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(54) Title: SYSTEMS AND METHODS FOR BARCODING NUCLEIC ACIDS

(57) Abstract: The present invention generally relates to microfluidics and labeled nucleic acids. For example, certain aspects are generally directed to systems and methods for labeling nucleic acids within microfluidic droplets. In one set of embodiments, the nucleic acids may include "barcodes" or unique sequences that can be used to distinguish nucleic acids in a droplet from those in another droplet, for instance, even after the nucleic acids are pooled together. In some cases, the unique sequences may be incorporated into individual droplets using particles and attached to nucleic acids contained within the droplets (for example, released from lysed cells). In some cases, the barcodes may be used to distinguish tens, hundreds, or even thousands of nucleic acids, e.g., arising from different cells or other sources.

FIG. 1

LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

SYSTEMS AND METHODS FOR BARCODING NUCLEIC ACIDS

RELATED APPLICATIONS

This application claims the benefit of U.S. Prov. Pat. Apl. Ser. No. No.

61/982,001, filed April 21, 2014; U.S. Prov. Pat. Apl. Ser. No. No. 62/065,348, filed

October 17, 2014; U.S. Prov. Pat. Apl. Ser. No. No. 62/066,188, filed October 20, 2014;

and U.S. Prov. Pat. Apl. Ser. No. No. 62/072,944, filed October 30, 2014. Each of these

is incorporated herein by reference.

GOVERNMENT FUNDING

FIELD

The present invention generally relates to microfluidics and labeled nucleic acids.

BACKGROUND

Much of the physiology of metazoans is reflected in the temporal and spatial

variation of gene expression among constituent cells. Some of this variation is stable and

has helped us to define adult cell types, as well as numerous intermediate cell types in

development. Other variation results from dynamic physiological events such as the cell

cycle, changes in cell microenvironment, development, aging, and infection. Still other

expression changes appear to be stochastic in nature, and may have important

consequences. To understand gene expression in development and physiology, it has

been a dream of biologists to map gene expression changes not only in RNA levels, but

also in protein levels, and even to monitor post-translational modifications in every cell.

The methods available today for RNA sequence analysis (RNA-Seq) have the

capacity to quantify the abundance of RNA molecules in a population of cells with great

sensitivity. With some considerable effort these methods have been harnessed to analyze

RNA content in single cells. What is limiting are effective ways isolate and process

large numbers of individual cells for in-depth RNA sequencing, and to do so

quantitatively. This requires the isolation of cells under uniform conditions, preferably

with minimal loss of cells, especially in the case of clinical samples. The requirements

for the number of cells, the depth of coverage, and the accuracy of the measurements of

RNA abundance will depend on experimental considerations, which will include factors

such as the difficulty of obtaining material, the uniqueness of the material, the complexity of the cell population, and the extent to which cells are diversified in gene expression space. Lacking today high capacity single cell transcriptome data, it is hard to know the depth of coverage needed, but the presence of rare cell types in populations

5   of interest, such as occult tumor cells or tissue stem cell sub-populations, combined with other independent drivers of heterogeneity such as cell cycle and stochastic effects, suggests a demand for analyzing large numbers of cells.

Although analysis of RNA abundance by RNA-seq is well-established, the accuracy of single cell RNA-Seq is much more sensitive than bulk assays to the

10   efficiency of its enzymatic steps; furthermore the need for PCR or linear amplification from single cells risks introducing considerable errors. There are also major obstacles to parallel processing of thousands or even tens of thousands of cells, and to handling small samples of cells efficiently so that nearly every cell is measured. Over the past decade, microfluidics has emerged as a promising technology for single-cell studies with the

15   potential to address these challenges. Yet the number of single cells that can be currently processed with microfluidic chips remains low at 70-90 cells per run, which sets a limit for analysis of large numbers of cells in terms of running costs and the limited time during which cells remain viable for analysis. Moreover, capture efficiencies of cells into microfluidic chambers are often low, a potential issue for rare or clinical samples

20   where the number of cells available is limited.

SUMMARY

The present invention generally relates to microfluidics and labeled nucleic acids. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or

25   more systems and/or articles.

In one aspect, the present invention is generally directed to an article. In one set of embodiments, the article comprises a plurality of at least 10 microfluidic droplets, each of the droplets containing cell lysate including nucleic acid fragments. In some cases, a plurality of the nucleic acid fragments within a droplet are each bound to an

30   oligonucleotide tag. In certain embodiments, the oligonucleotide tag within the droplet is distinguishable from oligonucleotide tags within the other droplets of the plurality of droplets.

The article, in another set of embodiments, includes a plurality of at least 10 microfluidic droplets, each of the droplets containing cell lysate. In some embodiments, at least about 90% of the droplets contains only one particle. In some cases, the particle comprises an oligonucleotide covalently bonded thereto.

According to yet another set of embodiments, the article comprises a plurality of particles, at least about 90% of the particles comprising an oligonucleotide covalently bonded thereto, the oligonucleotide comprising at least 2 primer sites and at least 2 barcode regions. In some embodiments, at least about 90% of the particles are distinguishable from the other particles of the plurality of particles on the basis of the barcode regions of the oligonucleotides.

In one set of embodiments, the article comprises a plurality of at least 10,000 microfluidic droplets. In some embodiments, at least some of the droplets containing cell lysate include nucleic acid fragments. In certain cases, a plurality of the nucleic acid fragments within a droplet are bound to an oligonucleotide tag. The oligonucleotide tag within the droplet, in one embodiment, is distinguishable from oligonucleotide tags within the other droplets of the plurality of 10,000 microfluidic droplets.

The article, in another set of embodiments, includes a plurality of at least 10,000 microfluidic droplets. In some embodiments, at least some of the droplets contain cell lysate. At least about 90% of the plurality of 10,000 microfluidic droplets may contain only one particle in certain cases. In some embodiments, the particle may comprise an oligonucleotide covalently bonded thereto. The oligonucleotide within a droplet may be distinguishable from oligonucleotides within the other droplets of the plurality of 10,000 microfluidic droplets in various instances.

In another aspect, the present invention is generally directed to a method. In one set of embodiments, the method includes acts of encapsulating a cell and a particle within a microfluidic droplet, the particle comprising an oligonucleotide tag covalently bonded thereto, lysing the cell within the droplet to release nucleic acid from the cell, and bonding the released nucleic acid and the oligonucleotide tag within the droplet.

The method, in another set of embodiments, includes acts of providing a plurality of microfluidic droplets containing cells, at least about 90% of the droplets containing one cell or no cell, lysing the cells within the plurality of microfluidic droplets to release nucleic acid from the cells, and bonding the nucleic acid to oligonucleotide tags, wherein for at least about 90% of the droplets. In some cases, the oligonucleotide tag within the

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droplet may be distinguishable from oligonucleotide tags within the other droplets of the plurality of droplets.

According to still another set of embodiments, the method includes acts of providing a plurality of particles, attaching first oligonucleotides to the plurality of particles such that at least about 90% of the particles has covalently bonded thereto only one first oligonucleotide, where the first oligonucleotides are taken from a pool of at least 10 unique first oligonucleotides; and attaching second oligonucleotides to the first oligonucleotides such that at least about 90% of the first oligonucleotides has covalently bonded thereto only one second oligonucleotide, where the second oligonucleotides are taken from a pool of at least 10 unique second oligonucleotides.

In accordance with one set of embodiments, the method includes acts of encapsulating a cell and a hydrogel microsphere or particle within a droplet, where the hydrogel microsphere or particle has attached thereto a barcoded nucleic acid, lysing the cell within the droplet to release RNA and/or DNA from the cell, and enzymatically reacting the RNA and/or DNA with the barcoded nucleic acid.

The method, in another set of embodiments, comprises providing droplets containing cells such that no more than 10% of the droplets contains two or more cells, lysing the cells within the plurality of droplets to release RNA and/or DNA from the cells, and uniquely labeling the RNA and/or DNA with a droplet-specific barcode.

According to still another set of embodiments, the method includes acts of providing droplets containing cells such that no more than 10% of the droplets contains two or more cells, lysing the cells within the plurality of droplets to release RNA and/or DNA from the cells, and uniquely labeling the RNA and/or DNA with a barcode selected from a pool of at least 10,000 barcodes.

In yet another set of embodiments, the method includes acts of providing a plurality of microspheres or particles carrying nucleic acid, covalently attaching an oligonucleotide to the microspheres or particles, enzymatically extending the oligonucleotides with a first barcode selected at random from a pre-defined pool of first barcodes, and enzymatically extending the oligonucleotides with a second barcode selected at random from a pre-defined pool of second barcodes.

In one set of embodiments, the method includes encapsulating a plurality of cells and a plurality of particles within a plurality of at least 10,000 microfluidic droplets, at least some of the particles comprising an oligonucleotide tag covalently bonded thereto,

such that the droplets of the plurality of the at least 10,000 droplets contain one or more oligonucleotide tags distinguishable from oligonucleotide tags contained in other droplets of the plurality of droplets, lysing at least some of the cells within the droplets to release nucleic acid from the cell, and bonding the released nucleic acids and the

5   oligonucleotide tags within at least some of the droplets.

In another set of embodiments, the method includes providing a plurality of at least 10,000 microfluidic droplets containing cells, at least about 90% of the plurality of droplets containing one cell or no cell, lysing the cells within the plurality of microfluidic droplets to release nucleic acid from the cells, and bonding the released nucleic acid to

10   oligonucleotide tags, wherein for at least about 90% of the droplets, the oligonucleotide tag within the droplet is distinguishable from oligonucleotide tags within other droplets of the plurality of droplets.

The method, according to yet another set of embodiments, includes encapsulating a cell and a hydrogel microsphere or particle within a droplet, where the hydrogel

15   microsphere or particle may have attached thereto a barcoded nucleic acid, lysing the cell within the droplet to release nucleic acid from the cell, and enzymatically reacting the released nucleic acid with the barcoded nucleic acid.

The method, in accordance with still another set of embodiments, is directed to providing a plurality of at least about 10,000 microfluidic droplets containing cells such

20   that no more than 10% of the droplets contains two or more cells, lysing the cells within the plurality of droplets to release nucleic acid from the cells, and uniquely labeling the released nucleic acid with a droplet-specific barcode.

In yet another set of embodiments, the method comprises providing droplets containing cells such that no more than 10% of the droplets contains two or more cells,

25   lysing the cells within the plurality of droplets to release nucleic acid from the cells, and uniquely labeling the released nucleic acid with a barcode selected from a pool of at least 10,000 distinguishable barcodes.

The method, in another set of embodiments, includes providing a plurality of at least about 10,000 microfluidic droplets containing cells such that no more than 10% of

30   the droplets contains two or more cells, lysing the cells within the plurality of droplets to release nucleic acid from the cells, and uniquely labeling the released nucleic acid with a droplet-specific barcode.

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In yet another set of embodiments, the method includes providing droplets containing cells such that no more than 10% of the droplets contains two or more cells, lysing the cells within the plurality of droplets to release nucleic acid from the cells, and uniquely labeling the released nucleic acid with a barcode selected from a pool of at least

5   10,000 distinguishable barcodes.

In another aspect, the present invention encompasses methods of making one or more of the embodiments described herein. In still another aspect, the present invention encompasses methods of using one or more of the embodiments described herein.

Other advantages and novel features of the present invention will become

10   apparent from the following detailed description of various non-limiting embodiments of the invention when considered in conjunction with the accompanying figures. In cases where the present specification and a document incorporated by reference include conflicting and/or inconsistent disclosure, the present specification shall control. If two or more documents incorporated by reference include conflicting and/or inconsistent

15   disclosure with respect to each other, then the document having the later effective date shall control.

BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying figures, which are schematic and are not

20   intended to be drawn to scale. In the figures, each identical or nearly identical component illustrated is typically represented by a single numeral. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. In the figures:

25   Fig. 1 illustrates a flowchart in accordance with one embodiment of the invention;

Figs. 2A-2B illustrate a microfluidic device in another embodiment of the invention;

Fig. 3 illustrates cells and particles within droplets in yet another embodiment of

30   the invention;

Fig. 4 illustrates a microfluidic channel containing cells and particles within droplets in still another embodiment of the invention;

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Fig. 5 illustrates sample count as a function of collection time, in one embodiment of the invention;

Fig. 6 illustrates a distribution of read sequences, in another embodiment of the invention;

Fig. 7 illustrates production of oligonucleotide tags, in yet another embodiment of the invention;

Fig. 8 illustrates a microfluidic device in another embodiment of the invention;

Figs. 9A-9B illustrates particles containing oligonucleotide tags, in yet another embodiment of the invention;

Figs. 10A-10C illustrates extended oligonucleotide tags contained within droplets, in still another embodiment of the invention;

Fig. 11 illustrates sequencing of DNA fragments, in another embodiment of the invention;

Fig. 12 illustrate reverse transcription efficiency as a function of droplet size, in accordance with another embodiment of the invention;

Figs. 13A-13H illustrate microfluidic droplets for DNA barcoding thousands of cells, in one embodiment of the invention;

Figs. 14A-14G illustrate droplet integrity and random barcoding, in accordance with another embodiment of the invention;

Figs. 15A-15G illustrate the heterogeneous structure of certain ES cell populations, in yet another embodiment of the invention;

Figs. 16A-16C illustrate a gene correlation network, produced in accordance with still another embodiment of the invention;

Figs. 17A-17H illustrate temporal heterogeneity and population structure in differentiating ES cells, in yet another embodiment of the invention;

Fig. 18 illustrates a microfluidic device in another embodiment of the invention;

Fig. 19A-19B illustrate certain microfluidic devices, in yet another embodiment of the invention;

Figs. 20A-20C illustrate synthesis of oligonucleotide tags for particles, in still another embodiment of the invention;

Figs. 21A-21H illustrate quantification of DNA, in one embodiment of the invention;

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Figs. 22A-22E illustrate random barcoding and unique molecular identifier (UMIs) filtering, in another embodiment of the invention;

Figs. 23A-23D illustrate single cell gene expression of mES cells, in one embodiment of the invention;

Figs. 24A-24G illustrate the structure of the mES cell population, in another embodiment of the invention;

Fig. 25 illustrates, in still another embodiment of the invention, a tSNE map of principal genes;

Fig. 26 shows Table 2; and

Fig. 27 illustrates a microfluidic device in another embodiment of the invention.

DETAILED DESCRIPTION

The present invention generally relates to microfluidics and labeled nucleic acids. For example, certain aspects are generally directed to systems and methods for labeling nucleic acids within microfluidic droplets. In one set of embodiments, the nucleic acids may include "barcodes" or unique sequences that can be used to distinguish nucleic acids in a droplet from those in another droplet, for instance, even after the nucleic acids are pooled together. In some cases, the unique sequences may be incorporated into individual droplets using particles and attached to nucleic acids contained within the droplets (for example, released from lysed cells). In some cases, the barcodes may be used to distinguish tens, hundreds, or even thousands of nucleic acids, e.g., arising from different cells or other sources.

Certain aspects of the present invention are generally directed to systems and methods for containing or encapsulating nucleic acids with oligonucleotide tags within microfluidic droplets or other suitable compartments, and covalently bonding them together. In some cases, the nucleic acids may arise from lysed cells or other material within the droplets. The oligonucleotide tags within a droplet may be distinguishable from oligonucleotide tags in other droplets, e.g., within a plurality or population of droplets. For instance, the oligonucleotide tags may contain one or more unique sequences or "barcodes" that are different between the various droplets; thus, the nucleic acid within each droplet can be uniquely identified by determining the barcodes associated with the nucleic acid. This may be important, for example, if the droplets are "broken" and the nucleic acids from different droplets are subsequently combined or merged together, e.g., for sequencing or other analysis.

In some embodiments, the oligonucleotide tags are introduced into the droplets by initially attaching the oligonucleotide tags to a particle (e.g., a hydrogel or a polymeric particle), then subsequently releasing them from the particle after the particle has been incorporated into a droplet. The particles may be prepared in some cases such that most or all of the particles have only one uniquely distinguishable oligonucleotide tag, relative to other particles having other distinguishable oligonucleotide tags). If the particles are present within the droplets at a density of 1 particle/droplet (or less), then once the oligonucleotide tags are released from the particle, then most or all of the droplets will contain one unique oligonucleotide tag (or no unique oligonucleotide), thus allowing each droplet (and the nucleic acids contained therein) to be uniquely identified.

Turning now to Fig. 1, an example of one aspect of the invention is now provided. However, it should be understood that this is by way of example only; other examples and embodiments of the invention are discussed in further detail below. In the non-limiting example of Fig. 1, a population of cells 10 is desired to be analyzed, e.g., by sequencing their DNA, by identifying certain proteins or genes that may be suspected of being present in at least some of the cells, by determining their mRNA or transcriptome, or the like. Although cells are used in this example as a source of nucleic acid material, this is by way of example, and in other embodiments, the nucleic acid may be introduced into the droplets from other sources, or using other techniques.

The cells may first be encapsulated in a series of microfluidic droplets 40. Those of ordinary skill in the art will be aware of techniques for encapsulating cells within microfluidic droplets; see, for example, U.S. Pat. Nos. 7,708,949, 8,337,778, 8,765,485, or Int. Pat. Apl. Pub. Nos. WO 2004/091763 and WO 2006/096571, each incorporated herein by reference. In some cases, the cells may be encapsulated at a density of less than 1 cell/droplet (and in some cases, much less than 1 cell/droplet) to ensure that most or all of the droplets have only zero or one cell present in them. Thus, as is shown in Fig. 1, each of droplets 41, 42, 43... have either zero or one cell present in them.

Also encapsulated in the droplets are oligonucleotide tags 20, present on particles 30. Particles 30 may be, for example, microparticles, and may be a hydrogel or a polymeric particle, or other types of particles such as those described herein. The particles and the cells may be encapsulated within the droplets simultaneously or sequentially, in any suitable order. In one set of embodiments, each particle contains a unique oligonucleotide tag, although there may be multiple copies of the tag present on a

particle. For instance, each of the oligonucleotide tags may have one or more unique sequences or "barcodes" that are present. Thus, for example, particle 31 contains only copies of oligonucleotide tag 21, particle 32 contains only copies of oligonucleotide tag 22, particle 33 contains only copies of oligonucleotide tag 33, etc. In some cases, the particles may be present in the droplets at a density of less than 1 particle/droplet (and in some cases, much less than 1 particle/droplet) to ensure that most or all of the droplets have only zero or one particle present in them. In addition, in certain embodiments, the oligonucleotide tags may be cleavable or otherwise releasable from the particles.

It should be noted that according to certain embodiments of the invention, the oligonucleotide tags are initially attached to particles to facilitate the introduction of only one unique oligonucleotide tag to each droplet, as is shown in Fig. 1. (In other embodiments, however, a plurality of oligonucleotide tags may be present, e.g., containing the same unique barcode.) For example, if the particles are present in the droplets at a density of less than 1 particle/droplet, then most or all of the droplets will each have only a single particle, and thus only a single type of oligonucleotide tag, that is present. Accordingly, as is shown in Fig. 1, the oligonucleotide tags may be cleaved or otherwise released from the particles, e.g., such that each droplet 41, 42, 43, ... contains a unique oligonucleotide tag 21, 22, 23, ... that is different than the other oligonucleotide tags that may be present in the other droplets. Thus, each oligonucleotide tag present within a droplet will be distinguishable from the oligonucleotide tags that are present in the other droplets. Although light (hv) is used in Fig. 1 to cleave the oligonucleotides from the particles, it should be understood that this is by way of example only, and that other methods of cleavage or release can also be used, e.g., as discussed herein. For example, in one set of embodiments, agarose particles containing oligonucleotides (e.g., physically) may be used, and the oligonucleotides may be released by heating the agarose, e.g., until the agarose at least partially liquefies or softens.

In some cases, the cells are lysed to release nucleic acid or other materials 51, 52, 53, ... from the cells. For example, the cells may be lysed using chemicals or ultrasound. The cells may release, for instance, DNA, RNA, mRNA, proteins, enzymes or the like. In some cases, the nucleic acids that are released may optionally undergo amplification, for example, by including suitable reagents specific to the amplification method. Examples of amplification methods known to those of ordinary skill in the art include, but are not limited to, polymerase chain reaction (PCR), reverse transcriptase (RT) PCR

amplification, in vitro transcription amplification (IVT), multiple displacement amplification (MDA), or quantitative real-time PCR (qPCR).

Some or all of the nucleic acid or other material 51, 52, 53, ... may be associated with the oligonucleotide tags present in the droplets, e.g., by covalently bonding. For example, the nucleic acid or other material 51, 52, 53 may be ligated or enzymatically attached to the oligonucleotide tags present in the droplets. Thus, as is shown in Fig. 1, droplet 41 exhibits nucleic acids 51 attached to oligonucleotide tags 21, droplet 42 exhibits nucleic acids 52 attached to oligonucleotide tags 22, droplet 43 exhibits nucleic acids 53 attached to oligonucleotide tags 23, etc. Thus, the nucleic acids within each droplet are distinguishable from the nucleic acids within the other droplets of the plurality of droplets 50 by way of the oligonucleotide tags, which are unique to each droplet in this example.

It should also be understood that although Fig. 1 depicts cleavage of the oligonucleotide tags from the particles followed by lysis of the cells, in other embodiments, these need not necessarily occur in this order. For example, cell lysis may occur after cleavage, or both may occur simultaneously.

Droplet 41, 42, 43, ... may then be "burst" or "broken" to release their contents, and in some cases, the nucleic acids present in each droplet may be combined or pooled together, as is shown in Fig. 1. However, since the nucleic acids are labeled by the different oligonucleotide tags, the nucleic acids from one droplet (i.e., from one cell) can still be distinguished from those from other droplets (or other cells) using the oligonucleotide tags. Accordingly, subsequent analysis (e.g., sequencing) of the combined pool of nucleic acids may be performed, and the source of each nucleic acid (e.g., individual cells) may be determined be determining the different oligonucleotide tags.

Thus, for example, a population of normal cells and cancer cells (e.g., arising from a tissue sample or biopsy) may be analyzed in such a fashion, and the cancer cells may be identified as having abnormal DNA, even if present in a large pool of normal cells. For example, due to the ability to track DNA on a cellular level using the oligonucleotide tags, the abnormal DNA can still be identified even if outnumbered by a large volume of normal DNA. As other non-limiting examples, stem cells may be isolated from normal cells, or the isolation of rare cell types in a population of interest may be performed.

In another aspect, the present invention provides systems and methods for the parallel capture and barcoding of DNA or RNA from large numbers of cells, e.g., for the purpose of profiling cell populations, or other purposes such as those described herein. In some embodiments, this relies on the encapsulation of barcoded nucleic acids or other

5   suitable oligonucleotide tags, e.g., attached to particles or microspheres (for example, hydrogel or polymer microspheres) together with cells and/or other reagents that may be used for RNA and/or DNA capture and/or amplification.

In one set of embodiments, the contents arising from substantially each individual cell may be labeled, e.g., with a unique barcode (which may be randomly determined, or

10   determined as discussed herein), which may allow in some cases for hundreds, thousands, tens of thousands, or even hundreds of thousands or more of different cells to be barcoded or otherwise labeled in a single experiment, e.g., to determine or define the heterogeneity between cells in a population or for screening cell populations, etc. Other purposes have been described herein.

15   In one set of embodiments, a microfluidic system is used to capture single cells into individual droplets (e.g., 50 pL to 10 nL volume), e.g., in a single reaction vessel. Each cell may be lysed and its RNA and/or DNA uniquely barcoded or labeled with a droplet-specific barcode, e.g., through an enzymatic reaction, through ligation, etc. Examples of microfluidic systems, including those with dimensions other than these, are

20   also provided herein. Some embodiments might also be used, in some embodiments, to quantify protein abundance in single cells in parallel to RNA or DNA, e.g., by first treating cells with DNA-tagged antibodies, in which case the DNA tags can be similarly barcoded with a droplet-specific barcode. Once the cell components in droplets have been barcoded, the droplets may be broken or burst and the sample can be processed,

25   e.g., in bulk, for high-throughput sequencing or other applications. After sequencing, the data can be split or otherwise analyzed according to the DNA barcodes.

To perform parallel barcoding of DNA, RNA and/or DNA-antibody tags in single cells, a single hydrogel or polymer particle or microsphere may be encapsulated into each droplet together with biological or chemical reagents and a cell, in accordance with

30   one set of embodiments. Particles or microspheres carrying a high concentration (e.g. 1 to 100 micromolar) of DNA fragments (hereafter "primers") may encode (a) a barcode sequence selected at random from a pool of, e.g., at least 10,000 barcodes (or at least 30,000 barcodes, at least 100,000 barcodes, at least 300,000 barcodes, or at least

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1,000,000 barcodes, etc.), with the same barcode found on all nucleic acid fragments on the particles or microspheres; and/or encode (b) one or more a primer sequences used for hybridization and capture of DNA or RNA. The number of distinct barcodes may be at least 10-fold, and in some cases at least 100-fold, larger than the number of cells to be

5   captured, in order to reduce the possibility of two or more cells occupying different droplets with particles or microspheres that carry the same barcode. For example, with 150,000 barcodes and 1,000 cells, on average just 3 cells will acquire a duplicate barcode (resulting in 997 detected barcodes).

In some embodiments, the encapsulation conditions are chosen such droplets

10   contain one particle (or microsphere) and one cell. The presence of empty droplets and/or droplets with single particles but without cells, and/or droplets with cells but without particles, may not substantially affect performance. However, the presence of two or more particles or two or more cells in one droplet may lead to errors that can be difficult to control for, so the incidence of such events is kept to minimum in some

15   instances, for example, less than about 10% or less than about 5%. Excepting the cells and particles, other biological and chemical reagents may be distributed equally among the droplets. The co-encapsulated cells and particles may be collected and processed according to the aim of the particular application. For example, in one particular embodiment, the DNA or RNA of single cells is captured by the primers introduced with

20   particle, and may then be converted into barcoded complimentary DNA upon reverse transcription or other DNA polymerization reaction.

After purification and optional DNA amplification, the base composition and barcode identity of cellular nucleic acids may be determined, for instance, by sequencing or other techniques. Alternatively, in some embodiments, primers introduced with

25   particles or microspheres can be used for amplification of specific nucleic acid sequences from a genome.

In some embodiments, the barcoded primers introduced using particles or microspheres can be cleaved therefrom by, e.g., light, chemical, enyzmatic or other techniques, e.g., to improve the efficiency of priming enzymatic reactions in droplets.

30   However, the cleavage of the primers can be performed at any step or point, and can be defined by the user in some cases. Such cleavage may be particularly important in certain circumstances and/or conditions; for example, some fraction of RNA and DNA molecules in single cells might be very large, or might be associated in complexes and

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therefore will not diffuse efficiently to the surface or interior of the particle or microsphere. However, in other embodiments, cleavage is not essential.

Techniques such as these can be used to analyze, for example, genomes, single nucleotide polymorphisms, specific gene expression levels, non-coding RNA, the whole transcriptome (or a portion thereof), entire genes or their sections, etc. However, the invention should not be limited to only these applications.

In one non-limiting embodiment, the 3' end of a barcoded primer is terminated with a poly-T sequences that may be used to capture cellular mRNA for whole-transcriptome profiling. The resulting library combining all cells can optionally be enriched using PCR-based methods or using hybridization capture-based methods (such as Agilent SureSelect), e.g., to allow sequencing of only a sub-set of genes of interest. In another embodiment, the 3' end of the barcoded primers may terminate with a random DNA sequence that can be used to capture the RNA in the cell. In another embodiment, the 3' end of the barcoded primers may terminate with a specific DNA sequence, e.g., that can be used to capture DNA or RNA species ("genes") of interest, or to hybridize to a DNA probe that is delivered into the droplets in addition to the particles or microspheres, for example, together with the enzyme reagents. In another embodiment, a particle or microsphere may carry a number of different primers to target several genes of interest. Yet another embodiment is directed to optimization of the size of droplets and the concentration of reaction components required for droplet barcoding.

Still another aspect of the present invention is generally directed to creating barcoded nucleic acids attached to the particles or microspheres. The nucleic acids may be attached to the surface of the particles or microspheres, or in some cases, attached or incorporated within the particle. For instance, the nucleic acids may be incorporated into the particle during formation of the particle, e.g., physically and/or chemically.

For example, one set of embodiments is generally directed to creating particles or microspheres carrying nucleic acid fragments (each encoding a barcode, a primer, and/or other sequences possibly used for capture, amplification and/or sequencing of nucleic acids). Microspheres may refer to a hydrogel particle (polyacrylamide, agarose, etc.), or a colloidal particle (polystyrene, magnetic or polymer particle, etc.) of 1 to 500 micrometer in size, or other dimensions such as those described herein. The microspheres may be porous in some embodiments. Other suitable particles or microspheres that can be used are discussed in more detail herein.

The preparation of DNA-carrying particles or microspheres, in some cases, may rely on the covalent attachment or other techniques of incorporation of an initial DNA oligonucleotide to the particles or microspheres, followed by enzymatic extension of each oligonucleotide by one or more barcodes selected, e.g., at random, from a pre-defined pool. The final number of possible unique barcodes may depend in some cases on the size of the pre-defined barcode pool and/or on the number of extension steps. For example, using a pool of 384 pre-defined barcodes and 2 extension steps, each particle or microsphere carries one of $384^2 = 147,456$ possible barcodes; using 3 extension steps, each particle or microsphere carries one of $384^3 = 56,623,104$ possible barcodes; and so on. Other numbers of steps may also be used in some cases; in addition, each pool may have various numbers of pre-defined barcodes (not just 384), and the pools may have the same or different numbers of pre-defined barcodes. The pools may include the same and/or different sequences.

Accordingly, in some embodiments, the possible barcodes that are used are formed from one or more separate "pools" of barcode elements that are then joined together to produce the final barcode, e.g., using a split-and-pool approach. A pool may contain, for example, at least about 300, at least about 500, at least about 1,000, at least about 3,000, at least about 5,000, or at least about 10,000 distinguishable barcodes. For example, a first pool may contain x_1 elements and a second pool may contain x_2 elements; forming a barcode containing an element from the first pool and an element from the second pool may yield, e.g., $x_1 x_2$ possible barcodes that could be used. It should be noted that x_1 and x_2 may or may not be equal. This process can be repeated any number of times; for example, the barcode may include elements from a first pool, a second pool, and a third pool (e.g., producing $x_1 x_2 x_3$ possible barcodes), or from a first pool, a second pool, a third pool, and a fourth pool (e.g., producing $x_1 x_2 x_3 x_4$ possible barcodes), etc. There may also be 5, 6, 7, 8, or any other suitable number of pools. Accordingly, due to the potential number of combinations, even a relatively small number of barcode elements can be used to produce a much larger number of distinguishable barcodes.

In some cases, such use of multiple pools, in combination, may be used to create substantially large numbers of useable barcodes, without having to separately prepare and synthesize large numbers of barcodes individually. For example, in many prior art systems, requiring 100 or 1,000 barcodes would require the individual synthesis of 100

or 1,000 barcodes. However, if larger numbers of barcodes are needed, e.g., for larger numbers of cells to be studied, then correspondingly larger numbers of barcodes would need to be synthesized. Such systems become impractical and unworkable at larger numbers, such as 10,000, 100,000, or 1,000,000 barcodes. However, by using separate "pools" of barcodes, larger numbers of barcodes can be achieved without necessarily requiring each barcode to be individually synthesized. As a non-limiting example, a first pool of 1,000 distinguishable barcodes (or any other suitable number) and a second pool of 1,000 distinguishable barcodes can be synthesized, requiring the synthesis of 2,000 barcodes (or only 1,000 if the barcodes are re-used in each pool), yet they may be combined to produce 1,000 x 1,000 = 1,000,000 distinguishable barcodes, e.g., where each distinguishable barcode comprises a first barcode taken from the first pool and a second barcode taken from the second pool. Using 3, 4, or more pools to assemble the barcode may result in even larger numbers of barcodes that may be prepared, without substantially increasing the total number of distinguishable barcodes that would need to be synthesized.

In some aspects, the DNA fragments or oligonucleotides can be released from the particles or microspheres using a variety of techniques including light, temperature, chemical, and/or enzymatic treatment. For example, with light, nucleic acid fragments may be released at a selected time and/or under desirable conditions, thus providing flexibility for their use.

In some embodiments, the particles or microspheres can be stored for long periods of time and used as a reagent for subsequent applications.

In yet another aspect, the present invention provides systems and methods for the parallel capture, barcoding and quantification of a panel of tens to hundreds, or more, of specific DNA and/or RNA sequences from large numbers of single cells, e.g., for the purpose of profiling cell populations or other purposes. Certain embodiments rely on encapsulation of barcoded nucleic acids, e.g., attached to particles such as hydrogel or polymer microspheres, together with cells and/or other reagents for, for example, RNA and/or DNA capture and amplification.

In some cases, systems and methods for labeling specific sets of genes (e.g., tens, or hundreds of genes, or more in some cases) arising from individual cells with a unique, random barcode, allowing hundreds, thousands, or even hundreds of thousands or more of different cells to be labeled or barcoded, e.g., in a single experiment, for the purpose

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of defining the heterogeneity between cells in a population or for screening cell populations, or for other purposes.

For example, in situations where a large number of cells are to be analyzed through multiplexed high-throughput sequencing, it may be desirable in some
5   embodiments to focus on a sub-set of genes of interest, for example between tens to hundreds of genes, rather than whole-transcriptome or whole-genome capture and sequencing.

Some embodiments are directed to the parallel barcoding of the contents of cells focusing on specific sequences of cellular DNA or RNA. These may include, for
10   example, the synthesis of DNA-barcoded microspheres (or other particles), and/or the use of such microspheres for the capture and barcoding of single cells in individual droplets (for example, 50 pL to 10 nL in volume, or other volumes described herein), e.g., in a single reaction vessel. In some cases, substantially each cell may be lysed and its RNA and/or DNA uniquely barcoded (tagged) with a droplet-specific nucleic acid
15   barcode, e.g., through an enzymatic reaction. In some embodiments, modifying the DNA-barcoded microspheres may be performed in such a way that they target only a specific panel of DNA sequences, rather than either using one sequence of interest or using random sequences. This may allow a high concentration of sequence-specific barcoded primers to be delivered into each droplet, which may, in some instances, allow
20   that the enzymatic barcoding and synthesis of complementary DNA occurs primarily for the sequences of interest. This may be used, for example, with any enzymatic approach in which a panel of sequence-specific primers can be used to capture genes of interest.

Some embodiments of the invention may be used to quantify protein abundance in single cells in parallel to RNA or DNA, for example, by first treating cells with DNA-
25   tagged antibodies, in which case one or more of the sequences or oligonucleotides on the particle or microsphere can be made complementary to the DNA tags delivered by the antibodies. In some cases, once the cell components in droplets have been barcoded, the droplets can be broken or burst and the sample can be processed, e.g., in bulk, for applications such as high-throughput sequencing. After sequencing, the data may be
30   split, in certain embodiments, according to the DNA barcodes thus providing information about the type, sequence, molecule count, origin of nucleic acids and/or proteins of interest, or the like.

In accordance with still another aspect, the present invention provides for optimizing reaction conditions for the enzymatic processing of cells within small volumes, for example, for cases where direct testing of the reactions would be extremely slow as it would require creating multiple microfluidic devices, or running microfluidic devices with large numbers of test samples. In some cases, this may also report specifically on the ideal volume required for enzymatic reverse transcription of mRNA into complementary DNA from single cells lysed in small volumes.

Certain embodiments of the invention provide for optimizing microfluidic reactions on single cells using reactions with a volume greater than 5 microliters, that can be performed using normal molecular biology reagents, e.g., without a microfluidic apparatus. This may be useful in certain applications, e.g., for testing parameters such as reaction volume over multiple orders of magnitude, which would otherwise require the design and synthesis of multiple test microfluidic devices, and the side-by-side comparison of the performance of such devices. It may also be useful for rapidly optimizing the conditions of microfluidic reactions, such as the optimal concentration of different reaction components.

In one set of embodiments, a bulk reaction is used to simulate the precise conditions present in a microfluidic volume. This is general and can be applied to optimize other aspects of microfluidic reactions, or other reactions. For example, this may be applied to test the ability of different additives to relieve inhibition of a reverse transcription (RT) reaction, and DNA primer concentrations necessary for performing RT reactions from lysed single cells in small volumes may be defined in certain embodiments.

The above discussions are non-limiting examples of various embodiments of the present invention. However, other embodiments are also possible. Accordingly, more generally, various aspects of the invention are directed to various systems and methods for systems and methods for labeling nucleic acids within microfluidic droplets, as discussed below.

In one aspect, the present invention is generally directed to systems and methods for labeling nucleic acids within a population of droplets, e.g., microfluidic droplets. In some cases, the microfluidic droplets may have an average diameter of the droplets of less than about 1 mm and/or the microfluidic droplets may be substantially monodisperse, e.g., as discussed herein.

In some cases, an oligonucleotide tag comprising DNA and/or other nucleic acids may be attached to particles and delivered to the droplets. In some cases, the oligonucleotide tags are attached to particles to control their delivery into droplets, e.g., such that a droplet will typically have at most one particle in it. In some cases, upon

5   delivery into a droplet, the oligonucleotide tags may be removed from the particle, e.g., by cleavage, by degrading the particle, etc. However, it should be understood that in other embodiments, a droplet may contain 2, 3, or any other number of particles, which may have oligonucleotide tags that are the same or different.

The oligonucleotide tags may be of any suitable length or comprise any suitable

10   number of nucelotides. The oligonucleotide tags may comprise DNA, RNA, and/or other nucleic acids such as PNA, and/or combinations of these and/or other nucleic acids. In some cases, the oligonucleotide tag is single stranded, although it may be double stranded in other cases. For example, the oligonucleotide tag may have a length of at least about 10 nt, at least about 30 nt, at least about 50 nt, at least about 100 nt, at least

15   about 300 nt, at least about 500 nt, at least about 1000 nt, at least about 3000 nt, at least about 5000 nt, at least about 10,000 nt, etc. In some cases, the oligonucleotide tag may have a length of no more than about 10,000 nt, no more than about 5000 nt, no more than about 3000 nt, no more than about 1000 nt, no more than about 500 nt, no more than about 300 nt, no more than about 100 nt, no more than about 50 nt, etc. Combinations of

20   any of these are also possible, e.g., the oligonucleotide tag may be between about 10 nt and about 100 nt. The length of the oligonucleotide tag is not critical, and a variety of lengths may be used in various embodiments.

The oligonucleotide tag may contain a variety of sequences. For example, the oligonucleotide tag may contain one or more primer sequences, one or more unique or

25   "barcode" sequences, one or more promoter sequences, one or more spacer sequences, or the like. The oligonucleotide tag may also contain, in some embodiments one or more cleavable spacers, e.g., photocleavable linker. The oligonucleotide tag may be attached to a particle chemically (e.g., via a linker) or physically (e.g., without necessarily requiring a linker), e.g., such that the oligonucleotide tags can be removed from the

30   particle via cleavage. Other examples include portions that may be used to increase the bulk of the oligonucleotide tag (e.g., using specific sequences or nonsense sequences), to facilitate handling (for example, a tag may include a poly-A tail), to increase selectivity of binding (e.g., as discussed below), to facilitate recognition by an enzyme (e.g., a

suitable ligase), to facilitate identification, or the like. Examples of these and/or other sequences are described in further detail herein.

As an example, in some embodiments, the oligonucleotide tags may comprise a "barcode" or a unique sequence. The sequence may be selected such that some or most of the oligonucleotide tags (e.g., present on a particle and/or in a droplet) have the unique sequence (or combination of sequences that is unique), but other oligonucleotide tags (e.g., on other particles or droplets) do not have the unique sequence or combination of sequences. Thus, for example, the sequences may be used to uniquely identify or distinguish a droplet, or nucleic acid contained arising from the droplet (e.g., from a lysed cell) from other droplets, or other nucleic acids (e.g., released from other cells) arising from other droplets.

The sequences may be of any suitable length. The length of the barcode sequence is not critical, and may be of any length sufficient to distinguish the barcode sequence from other barcode sequences. One, two, or more "barcode" sequence may be present in an oligonucleotide tag. A barcode sequence may have a length of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nt. More than 25 nucleotides may also be present in some cases.

In some cases, the unique or barcode sequences may be taken from a "pool" of potential barcode sequences. If more than one barcode sequence is present in an oligonucleotide tag, the barcode sequences may be taken from the same, or different pools of potential barcode sequences. The pool of sequences may be selected using any suitable technique, e.g., randomly, or such that the sequences allow for error detection and/or correction, for example, by being separated by a certain distance (e.g., Hamming distance) such that errors in reading of the barcode sequence can be detected, and in some cases, corrected. The pool may have any number of potential barcode sequences, e.g., at least 100, at least 300, at least 500, at least 1,000, at least 3,000, at least 5,000, at least 10,000, at least 30,000, at least 50,000, at least 100,000, at least 300,000, at least 500,000, or at least 1,000,000 barcode sequences.

In some cases, the oligonucleotide tag may contain one or more promoter sequences, e.g., to allow for production of the tags, to allow for enzymatic amplification, or the like. Those of ordinary skill in the art will be aware of primer sequences, e.g., P5 or P7. Many such primer sequences are available commercially. Examples of promoters include, but are not limited to, T7 promoters, T3 promoters, or SP6 promoters.

In some cases, the oligonucleotide tag may contain one or more primer sequences. Typically, a primer is a single-stranded or partially double-stranded nucleic acid (e.g., DNA) that serves as a starting point for nucleic acid synthesis, allowing polymerase enzymes such as nucleic acid polymerase to extend the primer and replicate the complementary strand. A primer may be complementary to and to hybridize to a target nucleic acid. In some embodiments, a primer is a synthetic primer. In some embodiments, a primer is a non-naturally-occurring primer. A primer typically has a length of 10 to 50 nucleotides. For example, a primer may have a length of 10 to 40, 10 to 30, 10 to 20, 25 to 50, 15 to 40, 15 to 30, 20 to 50, 20 to 40, or 20 to 30 nucleotides. In some embodiments, a primer has a length of 18 to 24 nucleotides. Examples of primers include, but are not limited to, P5 primer, P7 primer, PE1 primer, PE2 primer, A19 primer, or other primers discussed herein.

In some cases, the oligonucleotide tag may contain nonsense or random sequences, e.g., to increase the mass or size of the oligonucleotide tag. The random sequence can be of any suitable length, and there may be one or more than one present. As non-limiting examples, the random sequence may have a length of 10 to 40, 10 to 30, 10 to 20, 25 to 50, 15 to 40, 15 to 30, 20 to 50, 20 to 40, or 20 to 30 nucleotides.

In some cases, the oligonucleotide tag may comprise one or more sequences able to specifically bind a gene or other entity. For example, in one set of embodiments, the oligonucleotide tag may comprise a sequence able to recognize mRNA, e.g., one containing a poly-T sequence (e.g., having several T's in a row, e.g., 4, 5, 6, 7, 8, or more T's).

In one set of embodiments, the oligonucleotide tag may contain one or more cleavable linkers, e.g., that can be cleaved upon application of a suitable stimulus. For example, the cleavable sequence may be a photocleavable linker that can be cleaved by applying light or a suitable chemical or enzyme. A non-limiting example of a photocleavable linker can be seen in Fig. 20A. In some cases, for example, a plurality of particles (for instance, containing oligonucleotide tags on their surfaces) may be prepared and added to droplets, e.g., such that, on average, each droplet contains one particle, or less (or more) in some cases. After being added to the droplet, the oligonucleotide tags may be cleaved from the particles, e.g., using light or other suitable cleavage techniques, to allow the oligonucleotide tags to become present in solution, i.e., within the interior of the droplet. In such fashion, oligonucleotide tags can be easily

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loaded into droplets by loading of the particles into the droplets in some embodiments, then cleaved off to allow the oligonucleotide tags to be in solution, e.g., to interact with nucleotides or other species, such as is discussed herein.

In addition, in one set of embodiments, the oligonucleotide tag may comprise an

5   antibody, e.g., that can specifically bind to a protein suspected of being present in the cell (or droplet). For example, the droplet may contain one or more antibodies tagged with an oligonucleotide tag as described herein.

The oligonucleotide tag may be attached to a particle, e.g., as discussed herein. In some embodiments, a particle may comprise only one oligonucleotide tag, although

10   multiple copies of the oligonucleotide tag may be present on the particle; other particles may comprise different oligonucleotide tags that are distinguishable, e.g., using the barcode sequences described herein.

Any suitable method may be used to attach the oligonucleotide tag to the particle. The exact method of attachment is not critical, and may be, for instance, chemical or

15   physical. For example, the oligonucleotide tag may be covalently bonded to the particle via a biotin-steptavidin linkage, an amino linkage, or an acrylic phosphoramidite linkage. See, e.g., Fig. 20A for an example of an acrylic phosphoramidite linkage. In another set of embodiments, the oligonucleotide may be incorporated into the particle, e.g., physically, where the oligonucleotide may be released by altering the particle. Thus, in

20   some cases, the oligonucleotide need not have a cleavable linkage. For instance, in one set of embodiments, an oligonucleotide may be incorporated into particle, such as an agarose particle, upon formation of the particle. Upon degradation of the particle (for example, by heating the particle until it begins to soften, degrade, or liquefy), the oligonucleotide may be released from the particle.

25   The particle is a microparticle in certain aspects of the invention. The particle may be of any of a wide variety of types; as discussed, the particle may be used to introduce a particular oligonucleotide tag into a droplet, and any suitable particle to which oligonucleotide tags can associate with (e.g., physically or chemically) may be used. The exact form of the particle is not critical. The particle may be spherical or non-

30   spherical, and may be formed of any suitable material. In some cases, a plurality of particles is used, which have substantially the same composition and/or substantially the same average diameter. The "average diameter" of a plurality or series of particles is the arithmetic average of the average diameters of each of the particles. Those of ordinary

skill in the art will be able to determine the average diameter (or other characteristic dimension) of a plurality or series of particles, for example, using laser light scattering, microscopic examination, or other known techniques. The average diameter of a single particle, in a non-spherical particle, is the diameter of a perfect sphere having the same

5   volume as the non-spherical particle. The average diameter of a particle (and/or of a plurality or series of particles) may be, for example, less than about 1 mm, less than about 500 micrometers, less than about 200 micrometers, less than about 100 micrometers, less than about 75 micrometers, less than about 50 micrometers, less than about 25 micrometers, less than about 10 micrometers, or less than about 5 micrometers

10   in some cases. The average diameter may also be at least about 1 micrometer, at least about 2 micrometers, at least about 3 micrometers, at least about 5 micrometers, at least about 10 micrometers, at least about 15 micrometers, or at least about 20 micrometers in certain cases.

       The particle may be, in one set of embodiments, a hydrogel particle. See, e.g.,

15   Int. Pat. Apl. Pub. No. WO 2008/109176, entitled "Assay and other reactions involving droplets" (incorporated herein by reference) for examples of hydrogel particles, including hydrogel particles containing DNA. Examples of hydrogels include, but are not limited to agarose or acrylamide-based gels, such as polyacrylamide, poly-N-isopropylacrylamide, or poly N-isopropylpolyacrylamide. For example, an aqueous

20   solution of a monomer may be dispersed in a droplet, and then polymerized, e.g., to form a gel. Another example is a hydrogel, such as alginic acid that can be gelled by the addition of calcium ions. In some cases, gelation initiators (ammonium persulfate and TEMED for acrylamide, or Ca^{2+} for alginate) can be added to a droplet, for example, by co-flow with the aqueous phase, by co-flow through the oil phase, or by coalescence of

25   two different drops, e.g., as discussed in U.S. Patent Application Serial No. 11/360,845, filed February 23, 2006, entitled "Electronic Control of Fluidic Species," by Link, *et al.*, published as U.S. Patent Application Publication No. 2007/000342 on January 4, 2007; or in U.S. Patent Application Serial No. 11/698,298, filed January 24, 2007, entitled "Fluidic Droplet Coalescence," by Ahn, *et al.*; each incorporated herein by reference in

30   their entireties.

       In another set of embodiments, the particles may comprise one or more polymers. Exemplary polymers include, but are not limited to, polystyrene (PS), polycaprolactone (PCL), polyisoprene (PIP), poly(lactic acid), polyethylene, polypropylene,

polyacrylonitrile, polyimide, polyamide, and/or mixtures and/or co-polymers of these and/or other polymers. In addition, in some cases, the particles may be magnetic, which could allow for the magnetic manipulation of the particles. For example, the particles may comprise iron or other magnetic materials. The particles could also be

5   functionalized so that they could have other molecules attached, such as proteins, nucleic acids or small molecules. Thus, some embodiments of the present invention are directed to a set of particles defining a library of, for example, nucleic acids, proteins, small molecules, or other species such as those described herein. In some embodiments, the particle may be fluorescent.

10   In one set of embodiments, droplets are formed containing a cell or other source of nucleic acid, and a particle, e.g., comprising an oligonucleotide tag as described above. Any suitable method may be chosen to create droplets, and a wide variety of different techniques for forming droplets will be known to those of ordinary skill in the art. For example, a junction of channels may be used to create the droplets. The

15   junction may be, for instance, a T-junction, a Y-junction, a channel-within-a-channel junction (e.g., in a coaxial arrangement, or comprising an inner channel and an outer channel surrounding at least a portion of the inner channel), a cross (or "X") junction, a flow-focusing junction, or any other suitable junction for creating droplets. See, for example, International Patent Application No. PCT/US2004/010903, filed April 9, 2004,

20   entitled "Formation and Control of Fluidic Species," by Link, *et al.*, published as WO 2004/091763 on October 28, 2004, or International Patent Application No. PCT/US2003/020542, filed June 30, 2003, entitled "Method and Apparatus for Fluid Dispersion," by Stone, *et al.*, published as WO 2004/002627 on January 8, 2004, each of which is incorporated herein by reference in its entirety. In some embodiments, the

25   junction may be configured and arranged to produce substantially monodisperse droplets. The droplets may also be created on the fluidic device, and/or the droplets may be created separately then brought to the device.

             If cells are used, the cells may arise from any suitable source. For instance, the cells may be any cells for which nucleic acid from the cells is desired to be studied or

30   sequenced, etc., and may include one, or more than one, cell type. The cells may be for example, from a specific population of cells, such as from a certain organ or tissue (e.g., cardiac cells, immune cells, muscle cells, cancer cells, etc.), cells from a specific individual or species (e.g., human cells, mouse cells, bacteria, etc.), cells from different

organisms, cells from a naturally-occurring sample (e.g., pond water, soil, etc.), or the like. In some cases, the cells may be dissociated from tissue.

In addition, certain embodiments of the invention involve the use of other discrete compartments, for example, microwells of a microwell plate, individual spots on a slide or other surface, or the like. In some cases, each of the compartments may be in a specific location that will not be accidentally mixed with other compartments. The compartments may be relatively small in some cases, for example, each compartment may have a volume of less than about 1 ml, less than about 300 microliters, less than about 100 microliters, less than about 30 microliters, less than about 10 microliters, less than about 3 microliters, less than about 1 microliter, less than about 500 nl, less than about 300 nl, less than about 100 nl, less than about 50 nl, less than about 30 nl, or less than about 10 nl.

In one set of embodiments, the droplets (or other compartments) are loaded such that, on the average, each droplet has less than 1 particle in it. For example, the average loading rate may be less than about 1 particle/droplet, less than about 0.9 particles/droplet, less than about 0.8 particles/droplet, less than about 0.7 particles/droplet, less than about 0.6 particles/droplet, less than about 0.5 particles/droplet, less than about 0.4 particles/droplet, less than about 0.3 particles/droplet, less than about 0.2 particles/droplet, less than about 0.1 particles/droplet, less than about 0.05 particles/droplet, less than about 0.03 particles/droplet, less than about 0.02 particles/droplet, or less than about 0.01 particles/droplet. In some cases, lower particle loading rates may be chosen to minimize the probability that a droplet will be produced having two or more particles in it. Thus, for example, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the droplets may contain either no particle or only one particle.

Similarly, in some embodiments, the droplets (or other compartments) are loaded such that, on the average, each droplet has less than 1 cell in it. For example, the average loading rate may be less than about 1 cell/droplet, less than about 0.9 cells/droplet, less than about 0.8 cells/droplet, less than about 0.7 cells/droplet, less than about 0.6 cells/droplet, less than about 0.5 cells/droplet, less than about 0.4 cells/droplet, less than about 0.3 cells/droplet, less than about 0.2 cells/droplet, less than about 0.1 cells/droplet, less than about 0.05 cells/droplet, less than about 0.03 cells/droplet, less than about 0.02

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cells/droplet, or less than about 0.01 cells/droplet. In some cases, lower cell loading rates may be chosen to minimize the probability that a droplet will be produced having two or more cells in it. Thus, for example, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the droplets may contain either no cell or only one cell. In addition, it should be noted that the average rate of particle loading and the average rate of cell loading within the droplets may the same or different.

In some cases, a relatively large number of droplets may be created, e.g., at least about 10, at least about 30, at least about 50, at least about 100, at least about 300, at least about 500, at least about 1,000, at least about 3,000, at least about 5,000, at least about 10,000, at least about 30,000, at least about 50,000, at least about 100,000 droplets, etc. In some cases, as previously discussed, some or all of the droplets may be distinguishable, e.g., on the basis of the oligonucleotide tags present in at least some of the droplets (e.g., which may comprise one or more unique sequences or barcodes). In some cases, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the droplets may be distinguishable.

After loading of the particles and cells into droplets, the oligonucleotide tags may be released or cleaved from the particles, in accordance with certain aspects of the invention. As noted above, any suitable technique may be used to release the oligonucleotide tags from the droplets, such as light (e.g., if the oligonucleotide tag includes a photocleavable linker), a chemical, or an enzyme, etc. If a chemical or an enzyme is used, the chemical or enzyme may be introduced into the droplet after formation of the droplet, e.g., through picoinjection or other methods such as those discussed in Int. Pat. Apl. Pub. No. WO 2010/151776, entitled "Fluid Injection" (incorporated herein by reference), through fusion of the droplets with droplets containing the chemical or enzyme, or through other techniques known to those of ordinary skill in the art.

As discussed, in certain aspects, the droplets may contain nucleic acid. The nucleic acid may arise from a cell, or from other suitable sources. In one set of embodiments, if cells are present, the cells may be lysed within the droplets, e.g., to release DNA and/or RNA from the cell, and/or to produce a cell lysate within the droplet. For instance, the cells may be lysed via exposure to a lysing chemical or a cell

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lysis reagent (e.g., a surfactant such as Triton-X or SDS, an enzyme such as lysozyme, lysostaphin, zymolase, cellulase, mutanolysin, glycanases, proteases, mannase, proteinase K, etc.), or a physical condition (e.g., ultrasound, ultraviolet light, mechanical agitation, etc.). If a lysing chemical is used, the lysing chemical may be introduced into the droplet after formation of the droplet, e.g., through picoinjection or other methods such as those discussed in U.S. Pat. Apl. Ser. No. 13/379,782, filed December 21, 2011, entitled "Fluid Injection," published as U.S. Pat. Apl. Pub. No. 2012/0132288 on May 31, 2012, incorporated herein by reference in its entirety, through fusion of the droplets with droplets containing the chemical or enzyme, or through other techniques known to those of ordinary skill in the art. Lysing of the cells may occur before, during, or after release of the oligonucleotide tags from the particles. In some cases, lysing a cell will cause the cell to release its contents, e.g., cellular nucleic acids, proteins, enzymes, sugars, etc. In some embodiments, some of the cellular nucleic acids may also be joined to one or more oligonucleotide tags contained within the droplet, e.g., as discussed herein. For example, in one set of embodiments, RNA transcripts typically produced within the cells may be released and then joined to the nucleic acid tags.

In some embodiments, once released, the released nucleic acids from the cell (e.g., DNA and/or RNA) may be bonded to the oligonucleotide tags, e.g., covalently, through primer extension, through ligation, or the like. Any of a wide variety of different techniques may be used, and those of ordinary skill in the art will be aware of many such techniques. The exact joining technique used is not necessarily critical, and can vary between embodiments.

For instance, in certain embodiments, the nucleic acids may be joined with the oligonucleotide tags using ligases. Non-limiting examples of ligases include DNA ligases such as DNA Ligase I, DNA Ligase II, DNA Ligase III, DNA Ligase IV, T4 DNA ligase, T7 DNA ligase, T3 DNA Ligase, *E. coli* DNA Ligase, Taq DNA Ligase, or the like. Many such ligases may be purchased commercially. As additional examples, in some embodiments, two or more nucleic acids may be ligated together using annealing or a primer extension method.

In yet another set of embodiments, the nucleic acids may be joined with the oligonucleotide tags and/or amplified using PCR (polymerase chain reaction) or other suitable amplification techniques, including any of those recited herein. Typically, in PCR reactions, the nucleic acids are heated to cause dissociation of the nucleic acids into

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single strands, and a heat-stable DNA polymerase (such as Taq polymerase) is used to amplify the nucleic acid. This process is often repeated multiple times to amplify the nucleic acids.

In one set of embodiments, PCR or nucleic acid amplification may be performed within the droplets. For example, the droplets may contain a polymerase (such as Taq polymerase), and DNA nucleotides, and the droplets may be processed (e.g., via repeated heated and cooling) to amplify the nucleic acid within the droplets. The polymerase and nucleotides may be added at any suitable point, e.g., before, during, or after various nucleic acids encoding various conditions are added to the droplets. For instance, a droplet may contain polymerase and DNA nucleotides, which is fused to the droplet to allow amplification to occur. Those of ordinary skill in the art will be aware of suitable PCR techniques and variations, such as assembly PCR or polymerase cycling assembly, which may be used in some embodiments to produce an amplified nucleic acid. Non-limiting examples of such procedures are also discussed below. In addition, in some cases, suitable primers may be used to initiate polymerization, e.g., P5 and P7, or other primers known to those of ordinary skill in the art. In some embodiments, primers may be added to the droplets, or the primers may be present on one or more of the nucleic acids within the droplets. Those of ordinary skill in the art will be aware of suitable primers, many of which can be readily obtained commercially.

In some cases, the droplets may be burst, broken, or otherwise disrupted. A wide variety of methods for "breaking" or "bursting" droplets are available to those of ordinary skill in the art, and the exact method chosen is not critical. For example, droplets contained in a carrying fluid may be disrupted using techniques such as mechanical disruption or ultrasound. Droplets may also be disrupted using chemical agents or surfactants, for example, 1H,1H,2H,2H-perfluorooctanol.

Nucleic acids (labeled with oligonucleotide tags) from different droplets may then be pooled or combined together or analyzed, e.g., sequenced, amplified, etc. The nucleic acids from different droplets, may however, remain distinguishable due to the presence of different oligonucleotide tags (e.g., containing different barcodes) that were present in each droplet prior to disruption.

For example, the nucleic acids may be amplified using PCR (polymerase chain reaction) or other amplification techniques. Typically, in PCR reactions, the nucleic acids are heated to cause dissociation of the nucleic acids into single strands, and a heat-

stable DNA polymerase (such as Taq polymerase) is used to amplify the nucleic acid. This process is often repeated multiple times to amplify the nucleic acids.

In one set of embodiments, the PCR may be used to amplify the nucleic acids. Those of ordinary skill in the art will be aware of suitable PCR techniques and variations, such as assembly PCR or polymerase cycling assembly, which may be used in some embodiments to produce an amplified nucleic acid. Non-limiting examples of such procedures are also discussed below. In addition, in some cases, suitable primers may be used to initiate polymerization, e.g., P5 and P7, or other primers known to those of ordinary skill in the art. Those of ordinary skill in the art will be aware of suitable primers, many of which can be readily obtained commercially.

Other non-limiting examples of amplification methods known to those of ordinary skill in the art that may be used include, but are not limited to, reverse transcriptase (RT) PCR amplification, in vitro transcription amplification (IVT), multiple displacement amplification (MDA), or quantitative real-time PCR (qPCR).

In some embodiments, the nucleic acids may be sequenced using a variety of techniques and instruments, many of which are readily available commercially. Examples of such techniques include, but are not limited to, chain-termination sequencing, sequencing-by-hybridization, Maxam–Gilbert sequencing, dye-terminator sequencing, chain-termination methods, Massively Parallel Signature Sequencing (Lynx Therapeutics), polony sequencing, pyrosequencing, sequencing by ligation, ion semiconductor sequencing, DNA nanoball sequencing, single-molecule real-time sequencing, nanopore sequencing, microfluidic Sanger sequencing, digital RNA sequencing ("digital RNA-seq"), etc. The exact sequencing method chosen is not critical.

In addition, in some cases, the droplets may also contain one or more DNA-tagged antibodies, e.g., to determine proteins in the cell, e.g., by suitable tagging with DNA. Thus, for example, a protein may be detected in a plurality of cells as discussed herein, using DNA-tagged antibodies specific for the protein.

Additional details regarding systems and methods for manipulating droplets in a microfluidic system follow, e.g., for determining droplets (or species within droplets), sorting droplets, etc. For example, various systems and methods for screening and/or sorting droplets are described in U.S. Patent Application Serial No. 11/360,845, filed February 23, 2006, entitled "Electronic Control of Fluidic Species," by Link, et al.,

published as U.S. Patent Application Publication No. 2007/000342 on January 4, 2007, incorporated herein by reference. As a non-limiting example, by applying (or removing) a first electric field (or a portion thereof), a droplet may be directed to a first region or channel; by applying (or removing) a second electric field to the device (or a portion thereof), the droplet may be directed to a second region or channel; by applying a third electric field to the device (or a portion thereof), the droplet may be directed to a third region or channel; etc., where the electric fields may differ in some way, for example, in intensity, direction, frequency, duration, etc.

In certain embodiments of the invention, sensors are provided that can sense and/or determine one or more characteristics of the fluidic droplets, and/or a characteristic of a portion of the fluidic system containing the fluidic droplet (e.g., the liquid surrounding the fluidic droplet) in such a manner as to allow the determination of one or more characteristics of the fluidic droplets. Characteristics determinable with respect to the droplet and usable in the invention can be identified by those of ordinary skill in the art. Non-limiting examples of such characteristics include fluorescence, spectroscopy (e.g., optical, infrared, ultraviolet, etc.), radioactivity, mass, volume, density, temperature, viscosity, pH, concentration of a substance, such as a biological substance (e.g., a protein, a nucleic acid, etc.), or the like.

In some cases, the sensor may be connected to a processor, which in turn, cause an operation to be performed on the fluidic droplet, for example, by sorting the droplet, adding or removing electric charge from the droplet, fusing the droplet with another droplet, splitting the droplet, causing mixing to occur within the droplet, etc., for example, as previously described. For instance, in response to a sensor measurement of a fluidic droplet, a processor may cause the fluidic droplet to be split, merged with a second fluidic droplet, etc.

One or more sensors and/or processors may be positioned to be in sensing communication with the fluidic droplet. "Sensing communication," as used herein, means that the sensor may be positioned anywhere such that the fluidic droplet within the fluidic system (e.g., within a channel), and/or a portion of the fluidic system containing the fluidic droplet may be sensed and/or determined in some fashion. For example, the sensor may be in sensing communication with the fluidic droplet and/or the portion of the fluidic system containing the fluidic droplet fluidly, optically or visually, thermally, pneumatically, electronically, or the like. The sensor can be positioned

proximate the fluidic system, for example, embedded within or integrally connected to a wall of a channel, or positioned separately from the fluidic system but with physical, electrical, and/or optical communication with the fluidic system so as to be able to sense and/or determine the fluidic droplet and/or a portion of the fluidic system containing the

5   fluidic droplet (e.g., a channel or a microchannel, a liquid containing the fluidic droplet, etc.). For example, a sensor may be free of any physical connection with a channel containing a droplet, but may be positioned so as to detect electromagnetic radiation arising from the droplet or the fluidic system, such as infrared, ultraviolet, or visible light. The electromagnetic radiation may be produced by the droplet, and/or may arise

10   from other portions of the fluidic system (or externally of the fluidic system) and interact with the fluidic droplet and/or the portion of the fluidic system containing the fluidic droplet in such as a manner as to indicate one or more characteristics of the fluidic droplet, for example, through absorption, reflection, diffraction, refraction, fluorescence, phosphorescence, changes in polarity, phase changes, changes with respect to time, etc.

15   As an example, a laser may be directed towards the fluidic droplet and/or the liquid surrounding the fluidic droplet, and the fluorescence of the fluidic droplet and/or the surrounding liquid may be determined. "Sensing communication," as used herein may also be direct or indirect. As an example, light from the fluidic droplet may be directed to a sensor, or directed first through a fiber optic system, a waveguide, etc., before being

20   directed to a sensor.

Non-limiting examples of sensors useful in the invention include optical or electromagnetically-based systems. For example, the sensor may be a fluorescence sensor (e.g., stimulated by a laser), a microscopy system (which may include a camera or other recording device), or the like. As another example, the sensor may be an electronic

25   sensor, e.g., a sensor able to determine an electric field or other electrical characteristic. For example, the sensor may detect capacitance, inductance, etc., of a fluidic droplet and/or the portion of the fluidic system containing the fluidic droplet.

As used herein, a "processor" or a "microprocessor" is any component or device able to receive a signal from one or more sensors, store the signal, and/or direct one or

30   more responses (e.g., as described above), for example, by using a mathematical formula or an electronic or computational circuit. The signal may be any suitable signal indicative of the environmental factor determined by the sensor, for example a pneumatic signal, an electronic signal, an optical signal, a mechanical signal, etc.

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In one set of embodiments, a fluidic droplet may be directed by creating an electric charge and/or an electric dipole on the droplet, and steering the droplet using an applied electric field, which may be an AC field, a DC field, etc. As an example, an electric field may be selectively applied and removed (or a different electric field may be

5   applied, e.g., a reversed electric field) as needed to direct the fluidic droplet to a particular region. The electric field may be selectively applied and removed as needed, in some embodiments, without substantially altering the flow of the liquid containing the fluidic droplet. For example, a liquid may flow on a substantially steady-state basis (i.e., the average flowrate of the liquid containing the fluidic droplet deviates by less than 20%

10   or less than 15% of the steady-state flow or the expected value of the flow of liquid with respect to time, and in some cases, the average flowrate may deviate less than 10% or less than 5%) or other predetermined basis through a fluidic system of the invention (e.g., through a channel or a microchannel), and fluidic droplets contained within the liquid may be directed to various regions, e.g., using an electric field, without

15   substantially altering the flow of the liquid through the fluidic system.

In some embodiments, the fluidic droplets may be screened or sorted within a fluidic system of the invention by altering the flow of the liquid containing the droplets. For instance, in one set of embodiments, a fluidic droplet may be steered or sorted by directing the liquid surrounding the fluidic droplet into a first channel, a second channel,

20   etc.

In another set of embodiments, pressure within a fluidic system, for example, within different channels or within different portions of a channel, can be controlled to direct the flow of fluidic droplets. For example, a droplet can be directed toward a channel junction including multiple options for further direction of flow (e.g., directed

25   toward a branch, or fork, in a channel defining optional downstream flow channels). Pressure within one or more of the optional downstream flow channels can be controlled to direct the droplet selectively into one of the channels, and changes in pressure can be effected on the order of the time required for successive droplets to reach the junction, such that the downstream flow path of each successive droplet can be independently

30   controlled. In one arrangement, the expansion and/or contraction of liquid reservoirs may be used to steer or sort a fluidic droplet into a channel, e.g., by causing directed movement of the liquid containing the fluidic droplet. The liquid reservoirs may be positioned such that, when activated, the movement of liquid caused by the activated

reservoirs causes the liquid to flow in a preferred direction, carrying the fluidic droplet in that preferred direction. For instance, the expansion of a liquid reservoir may cause a flow of liquid towards the reservoir, while the contraction of a liquid reservoir may cause a flow of liquid away from the reservoir. In some cases, the expansion and/or

5   contraction of the liquid reservoir may be combined with other flow-controlling devices and methods, e.g., as described herein. Non-limiting examples of devices able to cause the expansion and/or contraction of a liquid reservoir include pistons and piezoelectric components. In some cases, piezoelectric components may be particularly useful due to their relatively rapid response times, e.g., in response to an electrical signal. In some

10   embodiments, the fluidic droplets may be sorted into more than two channels.

As mentioned, certain embodiments are generally directed to systems and methods for sorting fluidic droplets in a liquid, and in some cases, at relatively high rates. For example, a property of a droplet may be sensed and/or determined in some fashion (e.g., as further described herein), then the droplet may be directed towards a

15   particular region of the device, such as a microfluidic channel, for example, for sorting purposes. In some cases, high sorting speeds may be achievable using certain systems and methods of the invention. For instance, at least about 10 droplets per second may be determined and/or sorted in some cases, and in other cases, at least about 20 droplets per second, at least about 30 droplets per second, at least about 100 droplets per second, at

20   least about 200 droplets per second, at least about 300 droplets per second, at least about 500 droplets per second, at least about 750 droplets per second, at least about 1,000 droplets per second, at least about 1,500 droplets per second, at least about 2,000 droplets per second, at least about 3,000 droplets per second, at least about 5,000 droplets per second, at least about 7,500 droplets per second, at least about 10,000

25   droplets per second, at least about 15,000 droplets per second, at least about 20,000 droplets per second, at least about 30,000 droplets per second, at least about 50,000 droplets per second, at least about 75,000 droplets per second, at least about 100,000 droplets per second, at least about 150,000 droplets per second, at least about 200,000 droplets per second, at least about 300,000 droplets per second, at least about 500,000

30   droplets per second, at least about 750,000 droplets per second, at least about 1,000,000 droplets per second, at least about 1,500,000 droplets per second, at least about 2,000,000 or more droplets per second, or at least about 3,000,000 or more droplets per second may be determined and/or sorted.

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In some aspects, a population of relatively small droplets may be used. In certain embodiments, as non-limiting examples, the average diameter of the droplets may be less than about 1 mm, less than about 500 micrometers, less than about 300 micrometers, less than about 200 micrometers, less than about 100 micrometers, less than about 75

5   micrometers, less than about 50 micrometers, less than about 30 micrometers, less than about 25 micrometers, less than about 20 micrometers, less than about 15 micrometers, less than about 10 micrometers, less than about 5 micrometers, less than about 3 micrometers, less than about 2 micrometers, less than about 1 micrometer, less than about 500 nm, less than about 300 nm, less than about 100 nm, or less than about 50 nm.

10   The average diameter of the droplets may also be at least about 30 nm, at least about 50 nm, at least about 100 nm, at least about 300 nm, at least about 500 nm, at least about 1 micrometer, at least about 2 micrometers, at least about 3 micrometers, at least about 5 micrometers, at least about 10 micrometers, at least about 15 micrometers, or at least about 20 micrometers in certain cases. The "average diameter" of a population of

15   droplets is the arithmetic average of the diameters of the droplets.

In some embodiments, the droplets may be of substantially the same shape and/or size (i.e., "monodisperse"), or of different shapes and/or sizes, depending on the particular application. In some cases, the droplets may have a homogenous distribution of cross-sectional diameters, i.e., the droplets may have a distribution of diameters such

20   that no more than about 5%, no more than about 2%, or no more than about 1% of the droplets have a diameter less than about 90% (or less than about 95%, or less than about 99%) and/or greater than about 110% (or greater than about 105%, or greater than about 101%) of the overall average diameter of the plurality of droplets. Some techniques for producing homogenous distributions of cross-sectional diameters of droplets are

25   disclosed in International Patent Application No. PCT/US2004/010903, filed April 9, 2004, entitled "Formation and Control of Fluidic Species," by Link et al., published as WO 2004/091763 on October 28, 2004, incorporated herein by reference.

Those of ordinary skill in the art will be able to determine the average diameter of a population of droplets, for example, using laser light scattering or other known

30   techniques. The droplets so formed can be spherical, or non-spherical in certain cases. The diameter of a droplet, in a non-spherical droplet, may be taken as the diameter of a perfect mathematical sphere having the same volume as the non-spherical droplet.

In some embodiments, one or more droplets may be created within a channel by creating an electric charge on a fluid surrounded by a liquid, which may cause the fluid to separate into individual droplets within the liquid. In some embodiments, an electric field may be applied to the fluid to cause droplet formation to occur. The fluid can be

5   present as a series of individual charged and/or electrically inducible droplets within the liquid. Electric charge may be created in the fluid within the liquid using any suitable technique, for example, by placing the fluid within an electric field (which may be AC, DC, etc.), and/or causing a reaction to occur that causes the fluid to have an electric charge.

10   The electric field, in some embodiments, is generated from an electric field generator, i.e., a device or system able to create an electric field that can be applied to the fluid. The electric field generator may produce an AC field (i.e., one that varies periodically with respect to time, for example, sinusoidally, sawtooth, square, etc.), a DC field (i.e., one that is constant with respect to time), a pulsed field, etc. Techniques for

15   producing a suitable electric field (which may be AC, DC, etc.) are known to those of ordinary skill in the art. For example, in one embodiment, an electric field is produced by applying voltage across a pair of electrodes, which may be positioned proximate a channel such that at least a portion of the electric field interacts with the channel. The electrodes can be fashioned from any suitable electrode material or materials known to

20   those of ordinary skill in the art, including, but not limited to, silver, gold, copper, carbon, platinum, copper, tungsten, tin, cadmium, nickel, indium tin oxide ("ITO"), etc., as well as combinations thereof.

In another set of embodiments, droplets of fluid can be created from a fluid surrounded by a liquid within a channel by altering the channel dimensions in a manner

25   that is able to induce the fluid to form individual droplets. The channel may, for example, be a channel that expands relative to the direction of flow, e.g., such that the fluid does not adhere to the channel walls and forms individual droplets instead, or a channel that narrows relative to the direction of flow, e.g., such that the fluid is forced to coalesce into individual droplets. In some cases, the channel dimensions may be altered

30   with respect to time (for example, mechanically or electromechanically, pneumatically, etc.) in such a manner as to cause the formation of individual droplets to occur. For example, the channel may be mechanically contracted ("squeezed") to cause droplet formation, or a fluid stream may be mechanically disrupted to cause droplet formation,

for example, through the use of moving baffles, rotating blades, or the like. Other techniques of creating droplets include, for example mixing or vortexing of a fluid.

Certain embodiments are generally directed to systems and methods for splitting a droplet into two or more droplets. For example, a droplet can be split using an applied electric field. The droplet may have a greater electrical conductivity than the surrounding liquid, and, in some cases, the droplet may be neutrally charged. In certain embodiments, in an applied electric field, electric charge may be urged to migrate from the interior of the droplet to the surface to be distributed thereon, which may thereby cancel the electric field experienced in the interior of the droplet. In some embodiments, the electric charge on the surface of the droplet may also experience a force due to the applied electric field, which causes charges having opposite polarities to migrate in opposite directions. The charge migration may, in some cases, cause the drop to be pulled apart into two separate droplets.

Some embodiments of the invention generally relate to systems and methods for fusing or coalescing two or more droplets into one droplet, e.g., where the two or more droplets ordinarily are unable to fuse or coalesce, for example, due to composition, surface tension, droplet size, the presence or absence of surfactants, etc. In certain cases, the surface tension of the droplets, relative to the size of the droplets, may also prevent fusion or coalescence of the droplets from occurring.

As a non-limiting example, two droplets can be given opposite electric charges (i.e., positive and negative charges, not necessarily of the same magnitude), which can increase the electrical interaction of the two droplets such that fusion or coalescence of the droplets can occur due to their opposite electric charges. For instance, an electric field may be applied to the droplets, the droplets may be passed through a capacitor, a chemical reaction may cause the droplets to become charged, etc. The droplets, in some cases, may not be able to fuse even if a surfactant is applied to lower the surface tension of the droplets. However, if the droplets are electrically charged with opposite charges (which can be, but are not necessarily of, the same magnitude), the droplets may be able to fuse or coalesce. As another example, the droplets may not necessarily be given opposite electric charges (and, in some cases, may not be given any electric charge), and are fused through the use of dipoles induced in the droplets that causes the droplets to coalesce. Also, the two or more droplets allowed to coalesce are not necessarily required to meet "head-on." Any angle of contact, so long as at least some fusion of the droplets

initially occurs, is sufficient. See also, e.g., U.S. Patent Application Serial No. 11/698,298, filed January 24, 2007, entitled "Fluidic Droplet Coalescence," by Ahn, *et al.*, published as U.S. Patent Application Publication No. 2007/0195127 on August 23, 2007, incorporated herein by reference in its entirety.

5   In one set of embodiments, a fluid may be injected into a droplet. The fluid may be microinjected into the droplet in some cases, e.g., using a microneedle or other such device. In other cases, the fluid may be injected directly into a droplet using a fluidic channel as the droplet comes into contact with the fluidic channel. Other techniques of fluid injection are disclosed in, e.g., International Patent Application No.

10   PCT/US2010/040006, filed June 25, 2010, entitled "Fluid Injection," by Weitz, *et al.*, published as WO 2010/151776 on December 29, 2010; or International Patent Application No. PCT/US2009/006649, filed December 18, 2009, entitled "Particle-Assisted Nucleic Acid Sequencing," by Weitz, *et al.*, published as WO 2010/080134 on July 15, 2010, each incorporated herein by reference in its entirety.

15   A variety of materials and methods, according to certain aspects of the invention, can be used to form articles or components such as those described herein, e.g., channels such as microfluidic channels, chambers, etc. For example, various articles or components can be formed from solid materials, in which the channels can be formed via micromachining, film deposition processes such as spin coating and chemical vapor

20   deposition, laser fabrication, photolithographic techniques, etching methods including wet chemical or plasma processes, and the like. See, for example, *Scientific American*, 248:44-55, 1983 (Angell, *et al*).

       In one set of embodiments, various structures or components of the articles described herein can be formed of a polymer, for example, an elastomeric polymer such

25   as polydimethylsiloxane ("PDMS"), polytetrafluoroethylene ("PTFE" or Teflon®), or the like. For instance, according to one embodiment, a microfluidic channel may be implemented by fabricating the fluidic system separately using PDMS or other soft lithography techniques (details of soft lithography techniques suitable for this embodiment are discussed in the references entitled "Soft Lithography," by Younan Xia

30   and George M. Whitesides, published in the *Annual Review of Material Science*, 1998, Vol. 28, pages 153-184, and "Soft Lithography in Biology and Biochemistry," by George M. Whitesides, Emanuele Ostuni, Shuichi Takayama, Xingyu Jiang and Donald

E. Ingber, published in the *Annual Review of Biomedical Engineering*, 2001, Vol. 3, pages 335-373; each of these references is incorporated herein by reference).

Other examples of potentially suitable polymers include, but are not limited to, polyethylene terephthalate (PET), polyacrylate, polymethacrylate, polycarbonate,
5   polystyrene, polyethylene, polypropylene, polyvinylchloride, cyclic olefin copolymer (COC), polytetrafluoroethylene, a fluorinated polymer, a silicone such as polydimethylsiloxane, polyvinylidene chloride, bis-benzocyclobutene ("BCB"), a polyimide, a fluorinated derivative of a polyimide, or the like. Combinations, copolymers, or blends involving polymers including those described above are also
10   envisioned. The device may also be formed from composite materials, for example, a composite of a polymer and a semiconductor material.

In some embodiments, various structures or components of the article are fabricated from polymeric and/or flexible and/or elastomeric materials, and can be conveniently formed of a hardenable fluid, facilitating fabrication via molding (e.g.
15   replica molding, injection molding, cast molding, etc.). The hardenable fluid can be essentially any fluid that can be induced to solidify, or that spontaneously solidifies, into a solid capable of containing and/or transporting fluids contemplated for use in and with the fluidic network. In one embodiment, the hardenable fluid comprises a polymeric liquid or a liquid polymeric precursor (i.e. a "prepolymer"). Suitable polymeric liquids
20   can include, for example, thermoplastic polymers, thermoset polymers, waxes, metals, or mixtures or composites thereof heated above their melting point. As another example, a suitable polymeric liquid may include a solution of one or more polymers in a suitable solvent, which solution forms a solid polymeric material upon removal of the solvent, for example, by evaporation. Such polymeric materials, which can be solidified from, for
25   example, a melt state or by solvent evaporation, are well known to those of ordinary skill in the art. A variety of polymeric materials, many of which are elastomeric, are suitable, and are also suitable for forming molds or mold masters, for embodiments where one or both of the mold masters is composed of an elastomeric material. A non-limiting list of examples of such polymers includes polymers of the general classes of silicone
30   polymers, epoxy polymers, and acrylate polymers. Epoxy polymers are characterized by the presence of a three-membered cyclic ether group commonly referred to as an epoxy group, 1,2-epoxide, or oxirane. For example, diglycidyl ethers of bisphenol A can be used, in addition to compounds based on aromatic amine, triazine, and cycloaliphatic

backbones. Another example includes the well-known Novolac polymers. Non-limiting examples of silicone elastomers suitable for use according to the invention include those formed from precursors including the chlorosilanes such as methylchlorosilanes, ethylchlorosilanes, phenylchlorosilanes, dodecyltrichlorosilanes, etc.

5   Silicone polymers are used in certain embodiments, for example, the silicone elastomer polydimethylsiloxane. Non-limiting examples of PDMS polymers include those sold under the trademark Sylgard by Dow Chemical Co., Midland, MI, and particularly Sylgard 182, Sylgard 184, and Sylgard 186. Silicone polymers including PDMS have several beneficial properties simplifying fabrication of various structures of

10   the invention. For instance, such materials are inexpensive, readily available, and can be solidified from a prepolymeric liquid via curing with heat. For example, PDMSs are typically curable by exposure of the prepolymeric liquid to temperatures of about, for example, about 65 °C to about 75 °C for exposure times of, for example, about an hour. Also, silicone polymers, such as PDMS, can be elastomeric and thus may be useful for

15   forming very small features with relatively high aspect ratios, necessary in certain embodiments of the invention. Flexible (e.g., elastomeric) molds or masters can be advantageous in this regard.

          One advantage of forming structures such as microfluidic structures or channels from silicone polymers, such as PDMS, is the ability of such polymers to be oxidized,

20   for example by exposure to an oxygen-containing plasma such as an air plasma, so that the oxidized structures contain, at their surface, chemical groups capable of cross-linking to other oxidized silicone polymer surfaces or to the oxidized surfaces of a variety of other polymeric and non-polymeric materials. Thus, structures can be fabricated and then oxidized and essentially irreversibly sealed to other silicone polymer surfaces, or to

25   the surfaces of other substrates reactive with the oxidized silicone polymer surfaces, without the need for separate adhesives or other sealing means. In most cases, sealing can be completed simply by contacting an oxidized silicone surface to another surface without the need to apply auxiliary pressure to form the seal. That is, the pre-oxidized silicone surface acts as a contact adhesive against suitable mating surfaces. Specifically,

30   in addition to being irreversibly sealable to itself, oxidized silicone such as oxidized PDMS can also be sealed irreversibly to a range of oxidized materials other than itself including, for example, glass, silicon, silicon oxide, quartz, silicon nitride, polyethylene, polystyrene, glassy carbon, and epoxy polymers, which have been oxidized in a similar

fashion to the PDMS surface (for example, via exposure to an oxygen-containing plasma). Oxidation and sealing methods useful in the context of the present invention, as well as overall molding techniques, are described in the art, for example, in an article entitled "Rapid Prototyping of Microfluidic Systems and Polydimethylsiloxane," *Anal.*

5   *Chem.*, 70:474-480, 1998 (Duffy *et al.*), incorporated herein by reference.

Thus, in certain embodiments, the design and/or fabrication of the article may be relatively simple, e.g., by using relatively well-known soft lithography and other techniques such as those described herein. In addition, in some embodiments, rapid and/or customized design of the article is possible, for example, in terms of geometry. In

10   one set of embodiments, the article may be produced to be disposable, for example, in embodiments where the article is used with substances that are radioactive, toxic, poisonous, reactive, biohazardous, etc., and/or where the profile of the substance (e.g., the toxicology profile, the radioactivity profile, etc.) is unknown. Another advantage to forming channels or other structures (or interior, fluid-contacting surfaces) from oxidized

15   silicone polymers is that these surfaces can be much more hydrophilic than the surfaces of typical elastomeric polymers (where a hydrophilic interior surface is desired). Such hydrophilic channel surfaces can thus be more easily filled and wetted with aqueous solutions than can structures comprised of typical, unoxidized elastomeric polymers or other hydrophobic materials.

20   The following documents are incorporated herein by reference in their entirety for all purposes: U.S. Pat. Apl. Ser. No. 61/980,541, entitled "Methods and Systems for Droplet Tagging and Amplification," by Weitz, *et al.*; U.S. Pat. Apl. Ser. No. 61/981,123, entitled "Systems and Methods for Droplet Tagging," by Bernstein, *et al.*; Int. Pat. Apl. Pub. No. WO 2004/091763, entitled "Formation and Control of Fluidic

25   Species," by Link *et al.*; Int. Pat. Apl. Pub. No. WO 2004/002627, entitled "Method and Apparatus for Fluid Dispersion," by Stone *et al.*; Int. Pat. Apl. Pub. No. WO 2006/096571, entitled "Method and Apparatus for Forming Multiple Emulsions," by Weitz *et al.*; Int. Pat. Apl. Pub. No. WO 2005/021151, entitled "Electronic Control of Fluidic Species," by Link *et al.*; Int. Pat. Apl. Pub. No. WO 2011/056546, entitled

30   "Droplet Creation Techniques," by Weitz, *et al.*; Int. Pat. Apl. Pub. No. WO 2010/033200, entitled "Creation of Libraries of Droplets and Related Species," by Weitz, *et al.*; U.S. Pat. Apl. Pub. No. 2012-0132288, entitled "Fluid Injection," by Weitz, *et al.*; Int. Pat. Apl. Pub. No. WO 2008/109176, entitled "Assay And Other Reactions

Involving Droplets," by Agresti, *et al.*; and Int. Pat. Apl. Pub. No. WO 2010/151776, entitled "Fluid Injection," by Weitz, *et al.*

Also incorporated herein by reference are U.S. Prov. Pat. Apl. Ser. No. No. 61/982,001, filed April 21, 2014; U.S. Prov. Pat. Apl. Ser. No. No. 62/065,348, filed

5   October 17, 2014; U.S. Prov. Pat. Apl. Ser. No. No. 62/066,188, filed October 20, 2014; and U.S. Prov. Pat. Apl. Ser. No. No. 62/072,944, filed October 30, 2014.

In addition, the following are incorporated herein by reference in their entireties: U.S. Pat. Apl. Ser. No. 61/981,123 filed April 17, 2014; a PCT application filed April 17, 2015, entitled "Systems and Methods for Droplet Tagging"; U.S. Pat. Apl. Ser. No.

10   61/981,108 filed April 17, 2014; a PCT application filed on April 17, 2015, entitled "Methods and Systems for Droplet Tagging and Amplification"; a U.S. patent application filed on April 17, 2015, entitled "Immobilization-Based Systems and Methods for Genetic Analysis and Other Applications"; a U.S. patent application filed on April 17, 2015, entitled "Barcoding Systems and Methods for Gene Sequencing and

15   Other Applications"; and U.S. Pat. Apl. Ser. No. 62/072,944, filed October 30, 2014.

The following examples are intended to illustrate certain embodiments of the present invention, but do not exemplify the full scope of the invention.

EXAMPLE 1

This example makes use of hydrogel or polymer microspheres, each carrying

20   DNA fragments (primers) at a concentration of 1-100 micromolar. These primers can be cleaved from the microspheres by chemicals or by light, with each DNA fragment encoding (a) a barcode sequence selected at random from a pool of at least 10,000 barcodes (but more from typically over 100,000 barcodes), with the same barcode found on all nucleic acid fragments on each microsphere; and (b) one or more a primer

25   sequences used for hybridization and capture of DNA or RNA; (c) optionally, additional DNA sequences, for example a random nucleotide sequence for barcoding each molecule, or sequences used for amplification or capture of the barcoded products. Synthesis of these microspheres is described in more detail below.

In this example for droplet production, a microfluidic device prepared by soft-

30   lithography is used. Its schematics are indicated in Fig. 2 but emulsification can be also performed using other tools such as capillaries or tubing, for example. Other microfluidic configurations can also be used. Using this microfluidic device, droplets of

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~4 nL volume were produced (Figs. 3 and 4), but the size of the droplets could be readily adjusted based on the requirements of the enzymatic barcoding reaction.

The microfluidic device used in this example has one inlet for droplet carrier oil, and additional inlets for components of the droplet aqueous phase (Fig. 14). For the carrier oil, fluorinated oil (e.g. HFE-7500) containing ~0.75% (v/v) surfactant (PFPE-PEG-PFPE tri-block copolymer containing two perfluoropolyether blocks (PFPE) and one poly(ethylene)glycol (PEG) block) was used. The surfactant was used to prevent droplets from coalescing, and the amount may be adjusted, for instance, based on its physicochemical properties. The carrier oil used for emulsification is not limited to fluorinated liquids and alternative fluids such as based on hydrocarbons (e.g. mineral oil, hexane, etc.), silicon oil and other type of oils can be employed successfully. The three inlets used in this example delivered the following components: (1) a suspension of dissociated cells; (2) a cell lysis reagent; (3) a suspension of barcoded primer-carrying hydrogel or polymer microspheres; and (4) a reaction mixture used to enzymatically generate barcoded DNA complementary to the captured DNA or RNA. It is possible to pre-combine some of these components in some cases, e.g. (2) and (4).

The cell suspension was prepared in this example with the following considerations. If cells were adherent or from tissue, the cells could be first dissociated and optionally filtered or centrifuged to remove clumps of two or more cells. The mass density of the cell suspension buffer (typically PBS) was adjusted to minimize precipitation of cells during injection, for example by adding Optiprep at ~16% (v/v). The cell number density (cells per unit volume) may be adjusted to minimize incidences of two or more cells becoming captured in the same droplet. The precise calculation of the correct number density depends, for example, on factors such as the amount of multi-cell events that can be tolerated, and on the droplet volume, and on the relative droplet volume contributed by the cell suspension. For example, for 4 nL droplets with 50% of the droplet volume contributed by the cell suspension, a number density of 50 cells/microliter could be used to lead to an average occupancy of 0.1 cells/droplet, leading to approximately 5% of cell-containing droplets having more than one cell. If necessary, a small magnetic stirrer bar could be introduced into the cell syringe to allow continuous or occasional mixing of the cell suspension. During injection into the microfluidic device, the cells can be kept cold using an ice pack or other suitable techniques of cooling.

The enzyme reaction mix and/or lysis reagent(s) were prepared in this example such that their final concentrations after mixing with the cell suspension and with the microspheres were suitable for cell lysis and performance of the enzymatic reaction (e.g. reverse transcription reaction).

5   If using hydrogel microspheres, these spheres can be packed (concentrated) such that their delivery into droplets becomes ordered and synchronized, ensuring that the majority of droplets host exactly one microsphere. When using rigid polymer microspheres, these may be ordered, for example, using flow. In some cases, the aim is to ensure that the number of droplets having a single microsphere is relatively high, and

10   the number of droplets of having 0 or 2 microspheres is rare or even negligible.

To further increase the co-encapsulation events of cells and microspheres, for example, one cell and one DNA-barcoded microsphere per droplet, the cells could be ordered prior encapsulation.

As one non-limiting example, the aqueous phase is delivered into the device with

15   flow rates of 100 microliters/hour, 100 microliters/hour and 10-15 microliters/hour respectively for the cell suspension, the lysis/reaction mix, and the concentrated hydrogel microsphere suspension. For example, the number density of the cell suspension may be adjusted to 50,000/mL such that 5,000 cells are captured for barcoding within one hour of emulsification. However, the flow rates of all phases can be adjusted independently

20   between 1 and 10,000 microliters/hr, depending on the particular application.

After (or during) the encapsulation step, cells may be lysed and DNA fragments attached to the microsphere surface may be released inside the droplets using, e.g., light, chemical, enzymatic or other techniques.

The released DNA fragments may be used as primers for cell-encoded nucleic

25   acid amplification. For example, mRNA from the cells can be converted to cDNA using reverse transcription, or in another example, genes encoding cellular proteins can be synthesized using DNA polymerase.

To release the synthesized nucleic acid (DNA or RNA) into a mixture, the droplets may be broken in some cases, e.g., by chemical or physical techniques. The

30   released DNA may be collected and if necessary, can be amplified or further processed. The number of cells to be analyzed can be adjusted, for example, by first transferring a fraction of the droplet emulsion into a new reaction tube before droplet breaking (Fig. 5). For example, after collection of 200 microliters of droplet emulsion containing 5,000

cells, the emulsion can be first split into five tubes of 40 microliters, each containing approximately 1,000 cells. If desirable these samples can be processed separately. In addition, other adjustments can be performed in other embodiments.

The base composition of nucleic acids, including the barcode and the captured
5   sequence, can be determined by DNA sequencing or other techniques (Fig. 6).

Diagnostic tests can be carried out, for example, using quantitative real-time PCR (qPCR) to compare the abundance of captured DNA or RNA barcoded in droplets, to the abundance achieved when the enzymatic reactions are performed under controlled conditions, such as outside of droplets in a pooled bulk reaction, or using purified DNA
10   or RNA from an equivalent number of cells. qPCR makes use of two primers, one hybridizing to the end of the barcoded DNA fragments delivered by the microspheres; the other hybridizing to a target DNA or RNA sequence to be captured.

The above can be used to analyze, as non-limiting examples, genomes, single nucleotide polymorphisms, specific gene expression levels, non-coding RNA, the whole
15   transcriptome, entire genes or their sections, etc.

Fig. 2 shows schematics and operation of a microfluidic device, in accordance with one example of an embodiment of the invention. Other microfluidic device designs are also possible, e.g., as discussed herein. Fig. 2A shows schematics indicating the operation of a system. Cells, barcoded microspheres (barcoded beads) and reagents are
20   encapsulated into droplets using a microfluidic device. Fig. 2B shows a device having three inlets and one outlet. The inlets are used to introduce i) cells, ii) DNA-barcoded microspheres, iii) biological and/or chemical reagents and iv) carrier oil. Gels, cells and reagents can be introduced into device through any of the three inlets I, II, III. Encapsulation occurs at the flow-focusing junction and encapsulated samples are then
25   collected at the outlet. The flow rate of each inlet can be adjusted in order to obtain optimal conditions for cell and DNA-barcoded microsphere co-encapsulation. Fig. 3 shows digital images of cells and DNA-barcoded microspheres co-encapsulated together. Upper arrows show cells, lower arrows show microspheres. Time from the first frame is indicated. Fig. 4 shows an example of a device outlet showing microsphere and cell co-
30   encapsulation. Fig. 5 shows the number of barcoded samples vs emulsion volume and encapsulation (collection) time, produced in accordance with one embodiment of the invention. Fig. 6 shows a distribution of sequencing reads per abundant barcode showing largely uniform barcoding, in one embodiment of the invention.

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EXAMPLE 2

This example illustrates certain techniques for creating barcoded nucleic acids attached to the microspheres. First, the microspheres are synthesized incorporating a DNA primer (P1) into the hydrogel (Fig. 7). Several techniques of producing microspheres or various types hydrogel particles may be used. The microspheres described in this example makes use of polyacrylamide (pAc) hydrogel but alternative hydrogel materials can also be used (e.g. agarose, poly-N-isopropylacrylamide (pNIPAM) and others).

In one embodiment, an aqueous solution containing acrylamide (Ac), N,N'-methylenebisacrylamide (bis-Ac) and acrylic phosphoramidite modified DNA (Ac-DNA) and/or ammonium persulfate (APS) is prepared mixing individual components together.

Based on the pore size of the hydrogel mesh and the concentration of primer needed for the subsequent applications the amount of Ac and bis-Ac components as well as Ac-DNA concentration can be adjusted accordingly. For example, in one case, a mixture of ~0.0258% acrylamide, ~0.036% (v/v) N,N'-methylenebisacrylamide, 1-50 micromolar Ac-DNA, and ~0.2% APS is emulsified by a carrier oil containing 0.1-0.6% (v/v) polymerization inducer (N, N, N', N'-tetramethylethylenediamine refered as TEMED) for the production of hydrogel microspheres. As a carrier oil, fluorinated oil (e.g. HFE-7500) may be used, containing ~1.5% (v/v) surfactant (PFPE-PEG-PFPE tri-block copolymer containing two perfluoropolyether blocks (PFPE) and one poly(ethylene)glycol (PEG) block). The surfactant may be used, for example, to prevent droplets against coalescence. In some embodiments, its amount should be adjusted based on its physicochemical properties. The carrier oil used for emulsification is not limited to fluorinated liquids, and alternative fluids based on hydrocarbons (e.g. mineral oil, silicone oil, hexane, etc.) can be employed in other embodiments.

In this example for droplet production, a microfluidic device prepared by soft-lithography was used. Its schematics are indicated in Fig. 8, but emulsification can be also performed using other tools such as capillaries or tubing, for example. In addition, microfluidic devices having different schematics may also be used.

Using this microfluidic device, droplets of approximately 62 micrometers in diameter were produced (Fig. 9), but the size of droplets can be adjusted based on the requirements of other applications.

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In this example, the droplets were collected into a tube and then incubated at ~65 °C for >2 hour to induce the polymerization of polyacrylamide. The incubation period and temperature needed for polymerization could be varied accordingly. Polymerization of droplets can also be induced by light or various chemical means.

5   After polymerization, the emulsion may be broken by techniques such as chemical (e.g. perfluoroctanol) or physical techniques (e.g. electric field), which may cause the contents of the emulsion (e.g., the microspheres) to be released into the bulk solution. The released microspheres were then washed in hexane and aqueous buffers. In a typical example procedure, the microspheres were treated with hexane containing

10   1% (v/v) Span 80 and then three times with aqueous buffer (e.g. 10 mM Tris-HCl (pH 7.0), 10 mM EDTA and 0.1 % (v/v) Tween-20), and were then suspended in a buffer of desirable composition (e.g. 10 mM Tris-HCl (pH 7.0), 0.1 mM EDTA and 0.1 % (v/v) Tween-20). The final volume of the microspheres could differ from that seen during synthesis, and varies with conditions of the hydrogel suspension buffer.

15   The microspheres could be stored for extended periods of time, for example at 4 °C in a solution containing 10 mM EDTA, or in a solution containing 5 mM EDTA and 50% glycerol at -20 °C.

The incorporation of nucleic acid or primer into the microspheres or on its surface depends, for example, on functional groups present on the primers and/or the

20   material from which the microspheres are composed of. As a non-limiting example, a nucleic acid containing acrylic phosphoramidite at its 5' end can be incorporated into a polyacrylamide mesh of certain microspheres during polymerization process. As another example, acrydite-modified oligonucleotides can react covalently with thiol groups and thus, microspheres having thiol groups would bind acrydite-modified oligonucleotides.

25   In another example, oligonucleotides having amino groups can be covalently bound to the carboxy group of certain microspheres. In yet another example, oligonucleotides with a biotin group can be attached to streptavidin-coated microspheres. In yet another example, the particle may include antibodies or antibody fragments able to recognize certain oligonucleotide sequences present on the tags. [MOVE TO MAIN BODY OF

30   TEXT] Therefore, different types of incorporation of nucleic acids into/onto the microspheres are possible.

In one embodiment, primers P1 containing sequence for capture of a target nucleic acid (e.g. RNA or DNA), amplification (such as carrying a T7 promoter

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sequences or hybridization site for PCR primer), and/or sequencing may be used. In another embodiment, the P1 primer has a photocleavable site.

In one embodiment, the structure of P1 primer (direction from 5' to 3') is the following: acrylic phosphoramidite – photo-cleavable spacer – nucleotide sequence of
5   T7 promoter – nucleotide sequence for sequencing (PE1).

In another embodiment, the structure of the DNA primer N_1 from the first pool of DNA primers (direction from 5' to 3') is the following: adapter sequence (P2) – barcode sequence – nucleotide sequence complimentary to PE1.

In another embodiment, the structure of the DNA primer N_2 from the second pool
10   of DNA primers (direction from 5' to 3') is the following: Sequence of interest (P3) – barcode sequence – nucleotide sequence complimentary to P2.

In some cases, the microspheres carrying P1 primers may be split equally into N_1 pools, and each pool may be hybridized to one of N_1 distinct DNA templates, which have (sequentially from the 3' end to the 5' end): a DNA sequence complimentary to part or
15   all of the P1 primer allowing to form duplex with DNA P1 primer; one of N_1 unique nucleic acid barcodes composed of more than 6 defined nucleotides that are identical for all molecules within the same pool; optionally, a random nucleic acid sequence composed of more than 5 random nucleotides that differ between molecules with the same pool; and a DNA sequence (P2) that can be used as a hybridization site for
20   subsequent barcoding. The P2 sequence may contain sequence used for priming a sequencing reaction in later steps.

An enzymatic reaction may be performed on each of the N_1 pools leading to the extension of the P1 nucleic acid fragment by a copy of the template DNA fragments in each pool. In some cases, a ligation reaction can be used, e.g., instead of a
25   polymerization reaction.

The enzymatic reaction may be halted by addition of inhibitors such as EDTA, vanadium, or by other means.

The microspheres may be pooled together and optionally washed to remove the enzymes, or any excess template molecules.
30   The DNA fragments on the microspheres may be converted into single stranded DNA, for example, by removing the template molecules through denaturation, for example by washing the microspheres repeatedly in 0.1 M sodium hydroxide, or by other techniques.

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The microspheres now ending with the P2 sequence may be split equally into N_2 pools (typically $N_2=N_1$, although this is not required), and each pool may be hybridized to one of N_2 distinct DNA templates (as in step (2)), which have (sequentially from the 3' end to the 5' end): a DNA sequence complimentary to part or all of the P2 primer

5   allowing to form duplex with DNA P2 primer; one of N_2 unique nucleic acid barcodes composed of more than 6 defined nucleotides that are identical for all molecules within the same pool; a random nucleic acid sequence composed of more than 5 random nucleotides that differ between molecules within the same pool; and a DNA sequence site (P3) that may be used, for example, as a hybridization site for subsequent elongation,

10   or as a primer sequence used for single cell analysis operations (such as capture of DNA or RNA molecules).

In some embodiments, some of these steps may be repeated.

In some cases, microspheres may be produced, carrying single-stranded DNA fragments encoding primer P1, followed by a first barcode, followed by a sequence P2,

15   followed by a second barcode, followed by sequence P3. The number of unique microsphere pools is $N_1 \times N_2$ (see also Figs. 10 and 11).

In some cases, the prepared microspheres can be stored for extended periods of time and used as a reagent in subsequent application.

If required, additional repeats can be carried out with additional pools of $N_{3,4,}$ ...

20   barcode templates, each adding a barcode and sequence P4, P5, etc. The number of unique microsphere pools may grow with each step to $N_1 \times N_2 \times N_3 \times$....

Optionally, all of the microspheres can be hybridized together to a mixture of M DNA templates which have (sequentially from the 3' end to the 5' end): a DNA sequence complimentary to part or all of the final P3 (or P4, P5 etc) primer allowing to

25   form duplex with DNA P3 (or P4, P5 etc.) primer, and one of M sequences $S_1, ..., S_M$ that will be used as specific primer sequences for single cell analysis operations (such as capture of specific DNA or RNA molecules). These steps may be repeated. This may yield the same number of $N_1 \times N_2 \times N_3 \times$... pools of microspheres each carrying the above sequences but now the DNA fragments belong to M species of molecules that are

30   identical excepting a final M possible sequences $S_1, ..., S_M$.

In some cases, this may produce result in microspheres coated with ssDNA fragments, each of which encodes in the following order (from 5' to 3'): the P1 primer, for example containing a T7 promoter site and primer site PE1 that could be used as a

site for but not limited to for nucleic acid amplification and sequencing; two or more

DNA barcodes (each composed of 6 or more nucleotides), which are identical for all

primers coating a single bead, but differs between beads; optionally, a molecule-specific

DNA barcode (composed of 5 or more random nucleotides); the P2 "primer site 2" that

5   could be used as a primer for sequencing or/and for hybridization to DNA or RNA in

single cells for reverse transcription or PCR amplification; the P3 "primer site 3" that

could be used as a primer for sequencing or/and for hybridization to DNA or RNA in

single cells for reverse transcription or PCR amplification. One P3 fragment can also

encode one out of multiple gene specific primers (GSP), thus each bead coated with

10   multiple ssDNA fragments will contain all of the GSPs.

The microspheres carrying barcoded-DNA primers can be used, for example, as

reagents for sequencing or/and for hybridization to DNA or RNA in single cells, for

reverse transcription or PCR amplification and other applications that involve DNA

capture, amplification and sequencing.

15   Fig. 7 illustrates microspheres carrying PE1 primers hybridized to a pool of

single stranded DNA (ssDNA) primers carrying barcode sequence and primer sites PE1*

and P2*. In this example, the primer is then extended using DNA polymerase. The

extended primer is then converted to ssDNA (e.g. using increased temperature or

alkaline solution). The obtained ssDNA primer may then be hybridized to a second pool

20   of primers carrying a second barcode sequence and primer sites P2* and P3*. After

primer extension and conversion to ssDNA the microspheres can be used for different

applications, for example, applications aimed at capturing and sequencing nucleic acids

in a sample.

Fig. 8 illustrates schematics and design of microfluidic device used to produce

25   DNA-carrying microspheres, in accordance with one embodiment of the invention. The

device in this example includes one inlet for carrier oil and one inlet for reagents. The

droplets are generated at the flow focusing junction where two phases meet. The

droplets are collected at the collection outlet.

Figs. 9A and 9B are bright field images of DNA-carrying microspheres, produced

30   in accordance with one embodiment of the invention. In this example, the microspheres

composed of polyacrylamide hydrogel and DNA primer attached to the polymer mesh.

Scale bar is 50 micrometers.

Fig. 10 illustrates the evaluation of DNA extension efficiency of microspheres carrying barcoded-DNA primers, in another embodiment. Fig. 10A shows microspheres with DNA hybridized to a PE1 site with a FAM fluorescent probe, Fig. 10B shows microspheres with DNA hybridized to a P2 site with a FAM fluorescent probe. Fig. 10C shows microspheres with DNA hybridized to a P3 site with a fluorescent probe. These results show that DNA extension can be performed in hydrogel microspheres.

Fig. 11 illustrates high-throughput sequencing of DNA fragments from 11 individual microspheres, in yet another embodiment. An average of 140,000 molecules were sequenced from each microsphere. The plot shows the fraction of these ("primers") carrying the same barcodes on each microsphere, out of 384^2 possible barcodes. The identity of the barcodes is different for each of the microspheres. Each line corresponds to one microsphere. Under ideal conditions, 100% of DNA fragments on each microsphere would carry the same barcode, and 0% would carry the 2nd, 3rd, or other barcodes. The average achieved in this sample is 92% of the DNA fragments carrying the same barcode.

EXAMPLE 3

This example uses DNA barcoded microspheres are synthesized as described above, resulting in microspheres carrying the following single stranded DNA fragments with the following sequence elements (5' to 3'): the P1 primer, for example containing a T7 promoter site and primer site PE1 that could be used as a site for but not limited to for nucleic acid amplification and sequencing; two or more DNA barcodes (each composed of 6 or more nucleotides), which are identical for all primers coating a single bead, but differs between beads; optionally, a molecule-specific DNA barcode (composed of 5 or more random nucleotides); the P2 "primer site 2" that could be used as a primer for sequencing or/and for hybridization to DNA or RNA in single cells for reverse transcription or PCR amplification; and the P3 "primer site 3" that could be used as a primer for sequencing or/and for hybridization to DNA or RNA in single cells for reverse transcription or PCR amplification.

In this example, after synthesis of the DNA barcoded microspheres, the microspheres are pooled and then hybridized to a single mixture of M DNA templates which have (sequentially from the 3' end to the 5' end): a DNA sequence complimentary to part or all of the final $P3$ primer allowing to form duplex with the DNA $P3$ primer, and one of M sequences S_1, ..., S_M that will be used as specific primer sequences for

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single cell analysis operations such as capture of specific DNA or RNA molecules. These steps may be repeated, yielding the same number of $N_1 \times N_2$ pools of microspheres each carrying the sequences specified in 1, but now the DNA fragments belong to M species of molecules that are identical excepting a final M possible sequences $S_1, ..., S_M$.

In some cases, the DNA microspheres may be synthesized according to other methods to produce resulting microspheres have the same sequences described above.

EXAMPLE 4

This example shows that the reverse transcription (RT) of mRNA into complementary DNA from lysed cells becomes strongly inhibited for reaction volumes smaller than 3 nL per cell, specifically with the reaction yield Y follows a first-order inhibition with the droplet volume V, i.e. $Y=1/(1+K_{50}/V)$, where $K_{50}=1\text{-}3.3\text{nL}$ is the volume at which 50% inhibition occurs for at least three different cell culture lines tested (MCF7, K562 and THP-1 cells). By contrast, much of the current work with droplet microfluidics has focused on encapsulating cells in droplets with a volume of 10-100 pL volume. At such volumes reverse transcription reactions would be heavily inhabited.

Fig. 6 illustrates bulk tests for optimal droplet volume, in accordance with one embodiment of the invention.

In this example, tests of reaction efficacy on single cells in microfluidic volumes can be carried out by simulating droplet conditions in reaction wells containing 5 microliters or more of reaction mix, which may be adjusted to simulate the conditions within a single droplet.

To mimic a microfluidic volume of size V, intact cells were added to the bulk reaction at a final concentration of 1 cell per volume V. Thus, a single cell within a 4 nL droplet corresponds to running a reaction with a cell lysate of a concentration of 250 cells/microliters.

In addition, to mimic a microfluidic volume of size V, any reagents that are administered discreetly into droplets, such as by the use of microspheres that each carry m moles of reagent, the reagents are added to the bulk reaction at a final concentration of m moles per volume V. For example, if 1 femtomole of DNA fragments are delivered on microspheres into 4 nL droplets, the same DNA fragments would be added at a concentration of 0.42 micromolar to the bulk reactions.

Bulk reactions can be carried out in parallel in a 12-well, 96-well, or 384-well format to identify optimal reaction conditions.

Such diagnostic tests may provide a rapid method for optimizing the droplet size and composition for barcoding. For example, with three cell lines tested, a strong inhibition of the barcoding reaction when the droplets were made smaller than 3 nL volume was observed. See Fig. 12.

5   **EXAMPLE 5**

To interpret the gene expression of healthy and diseased tissues, it has been a dream of biologists to map gene expression changes in every cell. With such data one might hope to identify and track heterogeneous cell sub-populations, and infer regulatory relationships between genes and pathways. "Omics" methods such as RNA sequencing

10   have been harnessed to analyze single cells, but what is limiting are effective ways to routinely isolate and process large numbers of individual cells for in-depth sequencing, and to do so quantitatively. This example illustrates a droplet-microfluidic approach for parallel barcoding thousands of individual cells for subsequent profiling by next-generation sequencing. This shows a low noise profile and is readily adaptable to other

15   sequencing-based assays. These examples apply the technique to mouse embryonic stem (ES) cells to define the ES cell population structure and the heterogeneous onset of ES cell differentiation by LIF withdrawal. These results demonstrate the applications of droplet barcoding for deconstructing cell populations and inferring gene expression relationships with high-throughput single cell data.

20   These examples took advantage of droplet microfluidics to develop a novel technique for parallel barcoding of thousands of individual cells for subsequent profiling by next-generation sequencing (drop-Seq). The implementation used in these examples has a theoretical capacity to barcode tens of thousands of cells in a single run, although in practice some of the experiments focused on hundreds to thousands of cells per run,

25   since sequencing depth becomes limiting at very high cell counts. These examples evaluated drop-SEQ by profiling mouse embryonic stem (ES) cells before and after LIF withdrawal. A total of over 10,000 barcoded cells and control droplets were profiled, with ~3,000 ES and differentiating cells sequenced at greater depth for subsequent analysis. The following analysis identified the presence of rare sub-populations

30   expressing markers of distinct lineages that would be difficult to classify from profiling a few hundred cells. It was also found that key pluripotency factors fluctuated in a correlated manner across the entire ES cell population, and the possibility that such fluctuations might be used to associate novel factors with the pluripotent state was

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explored. Upon differentiation, dramatic changes were observed in the correlation structure of gene expression fluctuations, resulting from asynchronous inactivation of pluripotency factors, and the emergence of novel cell states. Altogether, these results show the potential of drop-SEQ to deconstruct cell populations and to infer gene expression relationships within a single experiment.

Design and implementation of a microfluidic platform for droplet barcoding and analysis of single cells. A protocol for RNA sequencing (RNA-Seq) was used, where mRNA is barcoded during a reverse transcription reaction, and cells are subsequently pooled and processed further for sequencing (Fig. 13A). For this, the drop-SEQ platform (Figs. 13A-13E and Fig. 18) encapsulated cells into droplets with lysis buffer, reverse transcription (RT) reagents, and barcoded oligonucleotide primers. mRNA released from each lysed cell remained trapped in the same droplet and was barcoded during synthesis of complementary DNA (cDNA). After barcoding, the material from all cells was combined by breaking the droplets, and the cDNA library was processed for sequencing (Fig. 13A).

One challenge in implementing this strategy was to ensure that each droplet carried primers encoding the same unique barcode, which should be different from barcodes in other droplets. This challenge was overcome by synthesizing a library of barcoded hydrogel microspheres (BHMs) that were co-encapsulated with cells (Fig. 13B). Each hydrogel carried covalently coupled, photo-releasable primers encoding one of 384^2 (i.e. 147,456) pre-defined barcodes. This pool size allowed randomly labeling 3,000 cells with 99% unique labeling, and 10,000 cells with 97% unique labeling (see below). Figs. 19-21 describe a method used to synthesize BHMs using a split-pool approach; see below. This can be extended in a straightforward manner to yield larger numbers of barcodes for larger-scale cell capture, for example for targeted sequencing applications.

To co-encapsulate the BHMs and cells, a microfluidic device with four inlets for i) the BHMs, ii) cells, iii) RT/lysis reagents and iv) carrier oil; and one outlet port for droplet collection was used (Figs. 13C-13D). The device generated monodisperse droplets which varied in the range of 1-5 nL at a rate of ~10-50 drops per second, simultaneously mixing aliquots from the inlets (Fig. 13E). The flow of close-packed deformable hydrogels inside the chip could be efficiently synchronized, allowing nearly 100% hydrogel droplet occupancy. This feature ensured that randomly distributed cells

arriving into droplets would be nearly always exposed to a BHM. In typical conditions, the cell concentration was set to occupy only 10% of droplets to ensure a low probability of two-cell events (Fig. 13E). In these experiments, droplets contained at least one cell and one gel to produce a barcoded library for sequencing. Typically, over 90% of

5   productive droplets contained exactly one cell and one gel (Fig. 13F). The efficiency of the RT reaction was also tested with primers in solution or still bound to BHMs, and it was found that primer release was important for an efficient RT reaction from lysed cells in droplets (Fig. 13G). Therefore, prior to the RT reaction, the BHM-bound primers were photo-released into the droplets by exposure to UV light (Fig. 13A).

10   In these examples, the samples of a few hundred to a few thousand cells were sequenced to avoid extremely shallow sequencing depth, but this could also be used to readily capture and barcode higher cell numbers, e.g., with a throughput of 4,000-6,000 cells per hour. Indeed, after sequencing, it was found that the number of barcoded samples scaled generally linearly with emulsion volume collected (Fig. 13H), with

15   approximately 2,000-3,000 cells or control droplets barcoded for every 100 microliters of emulsion (~30 minutes collection time).

             Fig. 13 shows an example of a droplet microfluidic platform for DNA barcoding thousands of cells. Fig. 13A shows an overview of drop-SEQ workflow; on-chip operations occur in the first three boxes, off-chip operations occur in the next three

20   boxes, and sequencing/data analysis occur in the last two boxes. Fig. 13B is a schematic of the microfluidic device for combining DNA-barcoded hydrogel microspheres (BHMs) (big circles) with cells (small circles) and RT/lysis mix. BHMs primer legend: PC = photocleavable linker; T7 = T7 RNA polymerase promoter; PE1 = sequencing primer; BC = BHM-specific barcode; UMI = unique molecular identifier. Fig. 13C shows a

25   microfluidic device design. Fig. 13D shows snapshots of microfluidic modules for encapsulation (right) and collection (left). Cells and BHMs are annotated with lower and upper arrows, respectively. Other arrows indicate direction of the flow. Scale bars, 100 micrometers. Fig. 13E shows statistics of droplet occupancy over time. Fig. 13F shows statistics of cell and DNA-barcoding bead co-encapsulation events. Over 90% of cells

30   are encapsulated with a single DNA-barcoding bead. Fig. 13G shows BioAnalyzer traces of the prepared library with primers photo-released from the beads before (upper curve) or after (lower curve) reverse transcription. Fig. 13H shows the number of

barcodes detected for pure RNA and mES cells, with 2,159 cells collected per 100 microliter emulsion (30 minutes collection time).

Fig. 18 shows the design of droplet microfluidics device used in this example. The device included three inlets for RT and lysis reagent mix (1), cell suspension (2), DNA barcoding beads (3) and one inlet for the continuous phase (4). The fluid resistors incorporated into device damping fluctuations arising due to mechanical instabilities of syringe pumps. The aliquot samples were brought together via 60 micrometer wide channels into the main 70 micrometer wide channel where they flowed laminarly before being encapsulated into droplets at the flow-focusing junction (dashed box). Droplets are collected at the outlet (5) in form of an emulsion.

Fig. 19 shows the design of droplet microfluidics device for production of DNA-barcoding hydrogel beads. Fig. 19A shows an example design of the device. The device comprises one inlet for aqueous phase (reagents) and one inlet for continuous phase (carrier oil). Monodisperse hydrogel droplets were generated at the flow-focusing nozzle those dimensions are indicated in the dashed box on the right. Droplets were stabilized by surfactant in the 2000 micrometer long channel and collected in form of an emulsion at the outlet port. Fig. 19B shows digital images of hydrogel droplet production, stabilization and collection. Microfluidic channels are 50 micrometers deep. Scale bars denote 100 micrometers.

Fig. 20 shows synthesis of DNA-barcoding beads. Fig. 20A shows the structure DNA oligonucleotide containing acrylic phosphoroamidite moiety (left) and photo-cleavable spacer (right) attached to the 5' end of DNA primer carrying T7 RNA polymerase promoter (left on sequence) and PE1 primer site (right on sequence). Fig. 20B shows schematics of synthesis of barcoded hydrogel microspheres. In the first step the ssDNA primers, attached to polyacrylamide hydrogel, were hybridized to complimentary DNA oligonucleotides carrying PE1* and W1* primer sites and the first half of DNA barcode. The resulting DNA heteroduplex was converted to dsDNA by Bst 2.0 DNA polymerase (where the dashed lines indicate newly synthesized DNA strand) and denatured back to ssDNA form by alkaline treatment. In the second step, the process was repeated with a second DNA oligonucleotide carrying W1* sequence, the second-half of DNA barcode, unique molecular identifier (UMI) and polyA sequence. After primer extension and denaturation the DNA-barcoding beads contain T7 promoter, PE1 primer site, DNA barcode, W1 site, UMI and polyT sequence. Fig. 20C shows the

DNA sequence (SEQ ID NO: 6) of a fully assembled primer. The highlighted text

indicates different parts of oligonucleotide with T7 promoter

(TAATACGACTCACTATAGGG) (SEQ ID NO: 2), PE1 primer site

(CTCTTTCCCTACACGACGCTCTTC) (SEQ ID NO: 3), two DNA barcodes ([barcode1]

5   and [barcode2]), W1 adaptor site (AAGGCGTCACAAGCAATCACTC) (SEQ ID NO: 4),

UMI (NNNNNN) and poly-T tail (TTTTTTTTTTTTTTTTTTTTTTTV) (SEQ ID NO: 5). The

chemical moieties for acrylic phosporamidite and photo-cleavable spacer are denoted as

/5Acryd/ and /iSpPC/ respectively. The DNA sequences of [barcode1] and [barcode2] is

8 nucleotides long each.

10   Fig. 21 shows quantification of DNA primers incorporated into barcoded

hydrogel microspheres (BHMs). Figs. 21A-21D show iImaging of BHMs post-

synthesis, showing a bright field image of BHMs 63 micrometers in size (Fig. 21A), and

fluorescent confocal imaging after hybridization with complimentary DNA probes

targeting PE1 sequence (Fig. 21B), W1 sequence (Fig. 21C) and polyT sequence (Fig.

15   21D). Scale bars, 100 micrometers. Fig. 21E shows a BioAnalyzer electropherogram of

DNA primers after photo-cleavage from BHMs, showing the presence of full-length

barcodes (largest peaks), as well as synthesis intermediates (two smaller peaks). Peaks at

35 and 10380 base pairs are gel migration markers. Numbers above the peaks indicate

theoretical fragment size in base pairs, but these are not accurate for the single stranded

20   DNA products. Figs 21F-21H show results from deep sequencing the barcoded product

of 11 individual BHMs. Fig. 21F shows a rank plot of barcode abundances on each gel;

Figs. 21G and 21H show histograms of the fraction occupied on each BHM by the most-

abundant and second-most abundant barcodes detailed in Fig. 21G and Fig. 21H. Perfect

synthesis would result in 100% occupied by the top barcode, and 0% by all other

25   barcodes. It was observed that an average of ~92% of all primers attached to each BHM

carried the same dominant barcode.

EXAMPLE 6

        Validation of random barcoding and droplet integrity. The ability of the drop-

Seq platform to effectively compartmentalize and barcode cells was tested in this

30   example by applying this to a mixture of cells from mouse and human origin (mouse ES

cells and K562 erythroleukemia cells) at approximately equal proportions (Fig. 14A). In

this test each barcode should associate entirely with either mouse or human mapped

transcripts, with only a small fraction of 2-cell events leading to the appearance of

barcodes associating with both mouse and human. After sequencing, Fig. 14A shows that drop-SEQ provided unambiguous identification of cells in the composite cell mixture: 96% of barcodes tagged reads mapped to either the mouse or human transcriptome with more than 99% purity, and only 4% of barcodes showed a mixture of both organisms. This already low error rate could be reduced even further by diluting cell suspensions to reduce co-encapsulation events, or by sorting droplets on-chip prior to collection to eliminate multi-cell events.

Also explicitly tested was that cell barcodes were randomly sampled from the intended pool of 384^2 possible barcodes to ensure a very low probability of repeated barcodes. A comparison of barcode identities across eight independent runs covering a total of 11,085 control droplets and cells consistently showed excellent agreement with random sampling from the pool of 384^2 barcodes (Fig. 22A).

Fig. 14 shows tests of droplet integrity and random barcoding. Fig. 14A shows schematic and results of droplet integrity control experiment: mouse and human cells are co-encapsulated to allow unambiguous identification of barcodes shared across multiple cells; 4% of barcodes share mixed mouse/human reads.

Fig. 22 shows random barcoding and unique molecular identifier (UMIs) filtering. Fig. 21A shows pair-wise tests of random barcoding for eight drop-Seq runs covering between 140-2,930 cells or pure RNA control droplets. Upper triangle shows the observed (left) and expected (right) number of shared barcodes for each pair of runs with 3842 random barcoding. Lower triangle shows p-values assuming uniform random barcoding from a pool of 3842 barcodes, which predicts that the observed number of shared barcodes should be hypergeometrically distributed about the expected value. The p values have not been corrected for multiple hypothesis testing. Figs. 22B-22D show UMI filtering. Fig. 21B shows the expected number of observed UMIs as a function of the number of detected mRNA molecules (black curve) can be shown to have the form

$n_{obs}(m) = N_{UMI}\left(1 - e^{-\frac{m}{N_{UMI}}}\right)$, where m is the number of detected mRNA molecules, and N_{UMI} = 4,096 is the total size of the available UMI pool. This function is contrasted with the ideal linear relationship (approximately straight line), showing the point of saturation.

Fig. 22C is an example of the number of mapped reads vs. number of distinct UMIs per gene in the data from a single mES cell; data points correspond to unique gene symbols. The curve indicates no amplification bias, i.e. where each mapped read corresponds to a

distinct UMI. Most genes show some amplification bias. Figs. 22D and 22E show log-log plots of the inter-cell CV (standard deviation/mean) as a function of the mean transcript abundance for genes detected in the mES cell population, without UMI filtering (Fig. 22D), and following UMI filtering (Fig. 22E). Each data point

5   corresponds to a single gene symbol.

EXAMPLE 7

Baseline technical noise for the drop-SEQ platform. Two sources of technical noise in single cell RNA-Seq are (a) variability between cells in mRNA capture efficiency, (b) the intrinsic sampling noise resulting from capturing finite numbers of

10   mRNA transcripts in each cell. The CEL-Seq protocol has been reported to suffer from a low capture efficiency of ~4% or less, and from a variability in capture efficiency of ~25% for pure RNA controls and ~50% for cells (coefficients of variation between samples) when performed in microtitre plates. Less is known about the impact of bioinformatic analysis on single cell sequencing data, but a potential problem may arise

15   from attributing ambiguous reads to multiple genes leading to spurious gene pair correlations. Technical noise can also arise during library amplification, but this noise source is mostly eliminated through the use of random unique molecular identifier (UMI) sequences, which allow bioinformatic removal of duplicated reads. This example illustrates implementation of a UMI-based filtering using random hexamers in all

20   experiments leading to a significant reduction in method noise (Fig. 22).

To test how technical noise in this system compares to previous applications of CEL-Seq, a technical control sample was analyzed that included total RNA diluted to single cell concentration (10 pg per droplet), mixed with ERCC RNA spike-in controls of known concentration (Fig. 14B). 953 barcoded control droplets were sequenced in a

25   single run with an average of 30×10^3 (+/- 21%) UMI-filtered mapped (UMIFM) reads per droplet (Fig. 14B). Between five to fifteen thousand unique gene symbols were identified in each droplet (25,209 detected in total), with the number correlating strongly with UMIFM counts (Fig. 14C). This showed an excellent linear readout of UMIFM counts compared to ERCC spike-in input concentration (Fig. 14D) down to

30   concentrations of 0.5 molecules/droplet on average; below that limit, there was a slight tendency to over-count the number of observed transcripts.

Another important measure of method performance is its sensitivity, i.e. the likelihood of detecting an expressed gene. The sensitivity was almost entirely a function

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of the transcript abundance (Fig. 14E), and was predicted extremely well for all genes based on the global capture efficiency of mRNA molecules (see below), measured from the ERCC spike-ins to be 7.1% (Fig. 14D). With this capture efficiency, genes were detected in 50% of droplets when 10 transcripts were present, and in >95% of droplets when >45 transcripts were present (Fig. 14E). This sensitivity and capture efficiency were higher than previously measured for CEL-Seq (3.4%).

In accuracy, this showed very low levels of technical noise, which can be assessed by comparing the coefficient of variation (CV = standard deviation / mean) of each gene across the cell population to its mean expression level (Fig. 14F). In a system limited only by sampling noise, all genes should be narrowly distributed about the power law curve CV=(mean)$^{-1/2}$ (Fig. 14F). This was indeed observed. More formally, after normalization, 99.5% of detected genes (N=25,209) had a distribution consistent with a Poisson distribution with a baseline technical noise 5-10% (Fig. 14F, dashed curve).

Fig. 14B shows an experimental schematic and histogram of UMI-filtered mapped (UMIFM) reads for RNA-Seq technical control experiment. Fig. 14C shows the number unique gene symbols detected as a function of UMIFM reads per droplet. Fig. 14D shows the mean UMIFM reads for spike-in molecules linearly related to their input concentration, with a capture efficiency β (beta) = 7.1%. Fig. 14E shows method sensitivity as a function of input RNA abundance; curve shows theory prediction, $1 - e^{-x*(1-e^{-\beta})}$, derived assuming only intrinsic sampling noise. Fig. 14F shows the coefficient of variation (CV) of spike-in and pure RNA transcripts plotted against the mean UMIFM counts after normalization. Solid curve shows the sampling noise limit; dashed curve shows the sampling noise limit with residual droplet-to-droplet variability in capture efficiency of 5%.

EXAMPLE 8

Noise modeling of single cell data. In anticipation of the single ES cell data, this example shows a technical noise model to better understand the effects of the low sampling efficiency of transcripts when measured on a per cell basis as compared to bulk measurements. The low efficiency had effects both on the observed variability of gene expression between cells, and on the covariation of gene expression among the cells. Three characteristics contribute to the effects: the capture efficiency of transcripts averaged across all cells; the cell-to-cell variation in capture efficiency; and the choice of a normalization scheme. By refining previous noise models a relationship between

biological and observed quantities was derived for the CVs of gene abundances across cells, the gene Fano Factors (variance/mean), and for pairwise correlations between genes (Fig. 14G; see below). The Fano Factor is a metric commonly used to measure noisy gene expression, and yet it is very sensitive to capture efficiency. This analysis

5   revealed that technical noise introduces not just baseline noise as widely appreciated, but it also spuriously amplifies existing biological variation (Fig. 14G, Eq. 1). Fig. 14G shows a summary of relationships between observed and underlying biological quantities for the CV, Fano Factor and gene pairwise correlations.

Also showed is that low sampling efficiencies significantly dampen correlations

10   between gene pairs in a predictable manner, setting an expectation to find relatively weak but significant correlations in the data (Fig. 14G, Eqs. 2-3). Knowing that relatively weak correlations are real and are an expected consequence of the statistics of single cell measurements helps derive useful information from the data including tests for highly variable genes (see below). These results also provide a basis for developing a

15   process for formally de-convolving noise from biological measurements based on fundamental counting statistics.

In addition, unexpectedly encountered and eliminated was an important source of anomalous gene expression correlation arising from reads mapping to two or more gene transcripts. Sequence analysis pipelines intended for bulk (non-single cell) applications

20   map ambiguous read probabilistically in a manner that can spuriously couple otherwise independently expressed genes. This problem may be particularly acute for 3'-sequencing of single cells since UTR regions can be similar across multiple genes; and in relatively uniform cell populations such as ES cells, which are characterized by a wide network of weak gene expression couplings that become comparable to those generated

25   spuriously. The problem is, however, more general when sampling efficiencies are low, since these serve to weaken even strong biological correlations (Fig. 14G, Eq. 3). These examples show that the read-mapping problem was overcome using a novel bioinformatic pipeline (see below) that makes use of repeated UMI tags across different reads to minimize ambiguities in mapping.

30   **EXAMPLE 9**

Single cell profiling of mouse ES cells. Single cell profiling is capable of identifying differentiated cell types from distinct lineages even with very low sequencing depths. What is less clear is the type of information that can be gained from studying a

relatively uniform population that is subject to stochastic fluctuations or to a dynamic environment. To explore the kind of information obtainable from our new method, this example studies mouse ES cells maintained in serum, as these cells are well studied and exhibit well-characterized fluctuations, but they are still uniform compared to

5   differentiated cell types and could pose a challenge for high throughput single cell sequencing.

To explore the behavior of drop-SEQ, different numbers of cells were harvested at different sequencing depths for each of the ES cell runs by collecting different emulsion volumes. 935 ES cells were collected for deep sequencing; 145, 302 and 2,160

10   cells after 2 days after LIF withdrawal; 683 cells after 4 days; and 169 and 799 cells after 7 days. The average number of reads obtained per cell in these runs ranged up to 208×10^3, and the average UMIFM counts ranged up to 29×10^3. Run statistics are detailed in Table 1.

The structure of the ES cell population. For the 935 ES cells, 1,507 genes were

15   identified that were significantly more variable than expected from Poisson statistics (10% FDR, see below and Table 2), and that were also expressed at a level of at least 10 UMIFM counts in at least one cell (Figs. 15A, 15B). Of the 1,507 abundant and variable genes, pluripotency factors previously reported to fluctuate in ES cells were found (*Nanog, Rex1/Zfp42, Dppa5a, Sox2, Esrrb*). Notably, the most highly variable genes

20   included known markers of Primitive Endoderm fate (*Col4a1, Col4a2, Lamb1, Lama1, Sox17, Sparc*), markers of Epiblast fate (*Krt8, Krt18, S100a6*), and epigenetic regulators of the ES cell state (*Dnmt3b*), but also genes with unknown association to ES cell regulation such as the stem cell antigen *Sca-1/Ly6a*, which may plays a role in regulating adult stem cell fate. Other genes showed very low noise profiles, consistent with Poisson

25   statistics (e.g. *Ttn*, Fig. 15B). The above-Poisson noise, defined as η (eta) $=CV^2-1/\mu$ (μ or mu being the mean UMIFM count), was evaluated for a select panel of genes (Fig. 15C) and found to be in qualitative agreement with previous reports. Unlike the CV or the Fano Factor, η (eta) scales linearly with its true biological value even for low sampling efficiencies (Fig. 14G, Eq. (1)).

30   Fig. 15 shows that drop-SEQ profiling reveals the heterogeneous structure of ES cell populations. Fig. 15A shows CV plotted against mean UMIFM counts for the mES cell transcriptome (middle and upper points) and the pure RNA technical controls (lower points). Genes marked in black are identified as significantly more variable than the

technical control. Solid and dashed curves are as in Fig. 14F, but with residual method

noise of 20% in the cell experiment. A subset of variable genes are annotated. Fig. 15B

shows illustrative gene expression distributions showing low (*Ttn*), moderate (*Trim28,*

Ly6a, Dppa5a) and high (*Sparc, S100a6*) expression variability, with fits to Poisson and

5   Negative Binomial distributions. Fig. 15C shows the above-Poisson (a.p.) noise, CV^2-

1/mean, plotted for pluripotency factors and compared with other factors.

EXAMPLE 10

To test the idea that ES cells exhibit heterogeneity between a pluripotent ICM-

like state and a more differentiated epiblast-like state, this example studied contrasting

10   the expression of candidate pluripotency and differentiation markers in single ES cells.

Gene pair correlations (Fig. 15D) at first appear consistent with a discrete two-state view,

since both the epiblast marker *Krt8* and the primitive Endoderm marker *Col4a1* were

expressed only in cells low for *Pou5f1* (shown) and the other pluripotency markers (not

shown). The differentiation-prone state was rare compared to the pluripotent state. The

15   correlations also showed other known regulatory interactions in ES cells, for example

Sox2, a known negative target of BMP signaling, was anti-correlated with the BMP

target *Id1*. What was more surprising, however, was the finding that multiple

pluripotency factors (*Nanog, Trim28, Esrrb, Sox2, Klf4, Zfp42*) fluctuated in tandem

across the bulk of the cell population (Fig. 15D, 23, 24). These observations together

20   were not explained by a simple two-state model, since they indicate that pluripotency

factors remain correlated independently of epiblast gene expression; instead they suggest

a continuum of states characterized by varying pluripotency. Not all pluripotency factors

showed significant correlations, however: *Oct4/Pou5f1* was much more weakly

correlated to other core pluripotency factors and other factors and instead correlated

25   strongly with cyclin D3 (Figs. 15D and 24), but not other cyclins, suggesting fluctuations

that belie a specific regulatory origin.

What then is the structure of the ES cell population inferred from the data? A

principal component analysis (PCA) was conducted of the ES cell population for the

highly variable genes, and it was found that multiple non-trivial dimensions of

30   heterogeneity (12 dimensions with 95% confidence), corresponding to the number of

principal components (PCs) in the data that cannot be explained by intrinsic noise in

expression (see Fig. 15E). This observation confirmd the presence of additional sources

of heterogeneity beyond the ICM-epiblast axis. Inspection of the first four principal

components, and their loadings (Fig. 15F), revealed the presence of at least three small but distinct cell sub-populations: one rare population (6/935 cells) expressed very low levels of pluripotency markers and high levels of *Col4a1/2, Lama1/b1/c1, Sparc*, and *Cd63*, which unambiguously identify primitive endoderm (PrEn)-like cells; a second cell population (15/935 cells) expressed high levels of *Krt8, Krt18, S100a6, Sfn* and other markers of the epiblast lineage. The third population presented a seemingly uncharacterized state, marked by expression of heat shock proteins *Hsp90, Hspa5* and other ER components such as the disulphide isomerase *Pdia6*. This population may represent ES cells under stress from dissociation.

PCA analysis is a powerful tool for visualizing cell populations that can be fractionated with just two or three principal axes of gene expression. However, when more than three non-trivial principal components exist there are more appropriate techniques for dimensionality reduction that represent the local structure of high-dimensional data. This example applied a method for dimensionality reduction known as t-distributed Stochastic Neighbor Embedding (t-SNE) (Fig. 15G). The map revealed no large sub-populations of ES cells, as expected, but revealed a continuum from high pluripotency to low pluripotency, with outlier populations identified by PCA lying at the population fringes. The map also revealed three additional fringe sub-populations characterized respectively by high expression of *Prdm1/Blimp1, Lin41/Trim71* and *SSEA-1/Fut4*. As with the *Hsp90*-hi population, it remains to be seen whether these populations represent distinct cell states endowed with distinct functional behaviors, or whether these are merely cells accessing outlier, but normal, states of ES cell gene expression. Thus, while the well-studied epiblast-like state in the ES cell population was identified, and evidence for collective fluctuations between ICM to epiblast-like state was found, these fluctuations are not the only axes of transcriptional heterogeneity in the ES cell population.

Fig. 15D shows heatmaps illustrating pairwise gene correlations. Fig. 15E shows an eigenvalue distribution obtained from principal component analysis of the mES cell population, revealing non-trivial modes of cellular heterogeneity detectable in the data (arrows). The smooth curve shows a typical eigenvalue distribution of a random permutation of the gene expression profile; the jagged curve shows the predicted Marcenko-Pastur eigenvalue distribution for a random matrix. Only eigenvalues lying beyond the curves were significant. Fig. 15F shows mES cell principal components and

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their loadings, showing the dominant uncorrelated modes of heterogeneity and revealing three rare ES cell sub-populations. Fig. 15G is a tSNE map of the mES cell population revealing additional fringe sub-populations and a pluripotency-to-epiblast axis.

Fig. 23 shows single cell gene expression of mES cells. Gene expression for principally variable genes at 0,2,4 and 7 days (Figs. 23A-23D, respectively). Expression of each gene is z-score standardized.

Fig. 24 shows the structure of the mES cell population. Fig. 24A shows pairwise correlations of selected genes across 935 mES cells. As discussed herein, Oct4/Pou5f1 correlated more strongly with Cyclin D3 and more weakly with Sox2, Klf4 and other pluripotency factors. The correlations reported here are as observed with no correction for subsampling (cf. Fig. 14G, Eq. (3)). Figs. 24B-24G show different projections of 3-dimensional tSNE map of the mES cell population reveal distinct cell sub-populations; the cells in each panel are colored according to the aggregate expression of the specified markers.

EXAMPLE 11

Putative pluripotency factors from gene expression covariation. The observation that genes co-vary in a population raises the question of whether correlations might disclose commonalities in gene regulation or function. In complex mixtures of cells, attempts at such inference may be confounded because gene-gene correlations could primarily arise from trivial differences between cell types, which reflect large-scale epigenetic changes rather than a particular regulatory program. The situation is different in a population consisting of just a single cell type: here, one might be more optimistic that fluctuations in cell state could reveal functional dependencies. The mES cell population satisfies this requirement as it shows relatively little discrete structure, beyond the presence of the small sub-populations described above.

To test whether gene expression covariation might contain regulatory information, this example explored the covariation partners of known pluripotency factors using a custom network neighborhood analysis (NNA) scheme (Fig. 16, see below). This scheme defines the set of genes most closely correlated with a given gene (or genes) of interest, and which also most closely correlate with each other. Given the sensitivity of correlations to sampling efficiency (Fig. 14G, Eq. (3)), the NNA analysis—which is only sensitive to correlation network topology—would be more robust than simply associating highly correlated genes. Remarkably, applied to the pluripotency

factors *Nanog* and *Sox2*, the NNA scheme strongly enriched for other pluripotency factors: of the 20 nearest neighbors of *Nanog*, eleven are documented as pluripotency factors, three more are associated with pluripotency, and one (*Slc2a3*) is syntenic with *Nanog*. Only one gene (*Rbpj*) has been shown to be dispensable for pluripotency,

5   leaving four genes with no previous documented connection to ES cells. It is tempting to predict that these genes also play a functional role in maintaining the pluripotent state. Similarly, the entire neighborhood of *Sox2* included factors directly or indirectly associated with pluripotency—including core pluripotency factors (6/9 genes); the threonine catabolic enzyme *Tdh*, which was recently shown to be expressed at high

10   levels in the ICM and is required for maintaining the pluripotent state; *Pcbp1* shown to be a binding partner of the pluripotency factor *Ronin/Thap11*, and the translation initiation factor subunit *Eif2s2* shown to be upregulated in response to Stat3 overexpression. Interestingly the same analysis may provide insight into other biological pathways. The neighborhood of Cyclin B (*Ccnb1*), for example, was small but contained

15   other core cell cycle genes *Cdk1*, *Ube2c* and *Plk1*.

The scheme is not generally applicable however to all regulatory functions: it was found that many other pathways seemingly independent of mES cell biology appear to have no meaningful NNA associations. This suggests that single cell covariation may capture fluctuations most specific to the biology of the cells being studied, and could be

20   harnessed more generally to identify other biological pathway components by artificially generating fluctuations through weak pathway-specific perturbations.

EXAMPLE 12

Population dynamics of differentiating ES cells. Upon LIF withdrawal, mES cells differentiate by a heterogeneous but poorly characterized process, leading

25   eventually to the formation of predominantly somatic (epiblast) lineages. The fate of pre-existing PrEn cells is unclear, as is the question of whether other cell lineages might transiently emerge and then vanish. In the single cell analysis, following LIF withdrawal the differentiating ES cell population underwent significant changes in population structure, which can be qualitatively appreciated from hierarchically clustering cells

30   according to the expression of highly variable genes (Fig. 17A). These changes and the following analysis reflect an unguided differentiation protocol; it would be instructive to apply the same methods to guided differentiation protocols in the future to identify how the inherent heterogeneity and variation in intermediate cell types depends on signaling.

To dissect the changes occurring in the cell population and to validate the quality of the data, this example first inspected the gene expression dynamics of pluripotency factors and differentiation markers (Fig 17B, 17C). The average expression of pluripotency factors *Rex1/Zfp42* and *Esrrb* levels dropped rapidly; *Pou5f1* and *Sox2* dropped more gradually; the epiblast marker *Krt8* increased steadily; and *Otx2*, a transcription factor required for transiting from the ICM to the epiblast state, transiently increased by day 2 and then decreased. It was however evident that the average expression was not representative of the dynamics in each cell: some cells failed to express epiblast markers and a fraction of cells continued to express pluripotency factors even seven days after LIF withdrawal, (Fig 17C), indicating that the timing of ES cell differentiation is itself heterogeneous.

A PCA analysis was performed of cells aggregated from all time points to identify whether this heterogeneity reflects global trends (Fig. 17D), and it was found that even after 7 days post-LIF withdrawal a fraction (5%, N=799) of cells overlapped with the mES cell population. The greatest temporal heterogeneity was evident at four days post-LIF, with cells spread broadly along the first principal component between the mES cell and differentiating state. The PCA analysis also revealed enrichment at days 2 and 4 for a strong metabolic signature (top GO annotation: Cellular Metobolic Process, p=1.4x10^{-8}) consistent with the metabolic changes occurring upon emergence from the pluripotent state.

In addition to heterogeneity arising from asynchrony in differentiation, after four and seven days there was evidence of emerging sub-populations with distinct patterns of gene expression, not all of which could be immediately attributed to known cell types. The population structure was visualized at these time points by t-SNE (Fig. 17G and Fig. 25), and tabulated the distinct sub-population markers in Table 3. At two days and four days post-LIF withdrawal, a rare population of *Zscan4*+ cells was identified, previously identified as rare Trophectoderm-forming cells (REF); this population was no longer detected by day 7. At four and seven days, another, less rare population emerged expressing normally maternally imprinted genes *H19*, *Rhox6/9*, *Peg10*, *Cdkn1* and others, suggesting widespread demethylation possibly associated with early primordial germ cell differentiation.

In addition to these populations, the resident PrEn cells could be detected at all time points (Figs. 17F, 17G), with PrEn population appearing to expand at two and four

days after LIF withdrawal, but then stagnating by seven days post-LIF. Overall, the analysis exposes the temporal heterogeneity of ES cell differentiation and the dynamics of distinct and novel ES cell sub-populations.

5 Fig. 17 shows temporal heterogeneity and population structure in differentiating ES cells. Fig. 17A shows changes in global population structure after LIF withdrawal are seen qualitatively by hierarchically clustering heatmaps of cell-cell correlations over the highly variable genes at each time point. Fig. 17B shows average dynamics of gene expression after LIF withdrawal are consistent with known patterns of differentiation. Fig. 17C shows the dynamics for the genes in Fig. 17B shown through probability

10 density (violin) plots for the fraction of cells expressing a given number of counts. Data points show the top 5% of cells. Figs. 17D and 17E show the first two principal components (PCs) (Fig. 17D), and PC loadings (Fig. 17E), of 3,034 cells from multiple time points showing rapid transient changes (PC 2) and asynchrony in differentiation (PC 1). Fig. 17F shows the dynamics of the fraction of epiblast and PrEn cells as a

15 function of time post-LIF. Fig. 17G shows tSNE maps of the differentiating ES cells after 4,7 days post-LIF reveal transient and emerging population sub-structure, and a tSNE map of genes after 4 days post-LIF (right panel) reveal putative population markers.

Fig. 25 shows a tSNE map of principal genes at 4 days post-LIF withdrawal.

20 This figure reproduces Fig. 17G with full gene annotation.

EXAMPLE 13

A reduction in promiscuous gene expression fluctuations during mES cell differentiation. This example addresses the hypothesis that mES cells are characterized by promiscuous gene expression, involving weakly-coupled expression of a wide number

25 of genes, which becomes refined during the process of differentiation. In a case where gene expression is more promiscuous, one might expect cells to occupy a larger sub-space of gene expression, as measured by the number of independent dimensions in which cells are distributed. By contrast, a more controlled pattern gene expression—even of a mixture of multiple cell types—would confine cells to a lower-dimensional

30 manifold reflecting one or more coherent states of gene expression. This example evaluated the intrinsic dimensionality of the ES cells and differentiating cells. It was found that the intrinsic dimensionality of gene expression decreased after differentiation (Fig. 17H), while the dimensionality for pure RNA and randomized data was

significantly higher than that of ES cells. This analysis supports the hypothesis that ES cell heterogeneity is associated with promiscuous weakly-coupled gene expression, which contrasts with heterogeneity after Lif withdrawal that arises from asynchrony in differentiation and a divergence of cell types.

5   Fig. 17H shows an estimation of the intrinsic dimensionality of gene expression variability of mES cells and following 7 days post-LIF, showing a shrinking sub-space of fluctuations during differentiation. Results are contrasted with pure RNA, which should lack correlations and thus display a maximal fluctuation sub-space.

EXAMPLE 14

10   These examples show the establishment of a platform for single cell capture, barcoding and transcriptome profiling, without physical limitations on the number of cells that can be processed. These examples showed high capture efficiencies, rapid collection times, very low inter-droplet CVs and a technical noise approaching the limits imposed by sampling statistics. These were reproducible across different experiments,

15 devices, BHM batches and emulsion volumes (Table 1). These can be readily applied to single cell transcriptomics of small clinical samples including tumor samples and tissue micro-biopsies, giving a quantitative picture of tissue heterogeneity. Depending on the desired application, this allows trading off sequencing depth with the size of the cell population, by collecting different emulsion volumes. These examples allow for

20 routinely identifying cell types, even rare sub-populations, based on gene expression. Owing to the low measurement noise, these allow one to distinguish discrete cell types from continuous fluctuations in gene expression, as was the case in ES cells. In addition to categorizing the cells, this type of data is valuable for identifying putative regulatory links between genes based on covariance, e.g., by exploiting natural and possibly subtle

25 variation between individual cells in a population. These examples only highlighted a few simple examples of such inference (Fig. 16), but this type single cell data lends itself to more formal approaches of reverse engineering.

          These examples can provide accurate information for many biological problems. This is illustrated by the complex and challenging problem of ES cell heterogeneity and

30 its dynamics during early differentiation. The ES cells do not host large sub-populations of distinct cell types, and therefore, analysis of their heterogeneity requires a sensitive method. To interpret the data from these cells, a statistical model was developed of single cell noise that addresses the question of how biological gene-gene correlations are

affected by low capture efficiencies and by technical variability between droplets, and we drew upon tools from machine learning to visualize the higher-dimensional organization of cells in gene expression 'space', and the dynamics of this organization. This analysis provided evidence in support of the hypothesis that ES cells fluctuate

5   between a more pluripotent state and a more differentiated state when maintained in serum and LIF. However, in addition to an ICM-like population expressing the differentiation markers such as *Krt8/18*, *S100a6* and *Fgf5*, other ES cell sub-populations were also identified associated with Primitive Endoderm fate, another sub-population expressing the primordial germ cell marker *Blimp1/Prdm1*, and sub-populations with less

10   obvious fate associations marked by high levels of ER-related proteins or the E3 ligase *Lin-41/Trim71*. The unbiased identification of these small cell sub-populations requires the scale enabled by drop-Seq. This is illustrated by the Primitive Endoderm-like cells, which represented less than ~1% of the cell population at all time points, and were too rare to be confidently detected by us in smaller samples of just 100-200 cells.

15   On the technical front, the drop-SEQ platform was developed for whole-transcriptome RNA sequencing, but the technology is highly flexible and should be readily adaptable to other applications requiring barcoding of RNA/DNA molecules, such as other RNA-Seq protocols, targeted sequencing approaches focusing on small panels of genes, ChIP-Seq, genomic sequencing, or chromatin proximity analysis (Hi-C).

20   One implementation made use of a very simple droplet microfluidic chip, having just a single flow-focusing junction (Fig. 13C) to combine cells, barcodes and RT reagents. Other versions of the platform might take further advantage of droplet microfluidic functionalities to allow multi-step enzymatic reactions through reagent pico-injection into existing droplets, or to perform target cell enrichment prior to sequencing by sorting

25   droplets on-chip. Moreover, drop-SEQ should be able to readily incorporate biochemical innovations targeting the relatively low capture efficiencies.

        Fig. 16 shows a gene correlation network neighborhoods reveal pluripotency-associated factors. Connected correlation network neighborhoods of Nanog (Fig. 16A), Sox2 (Fig. 16B), and Cyclin B (Fig. 16C), generated by selecting network neighbors that

30   have at least three mutual neighbors (see below). In Figs. 16A and 16B, grey boxes indicate previously validated pluripotency factors; boxes Calcoco2, Eif2s2, and Igfbp2 indicate factors previously reported to be associated with a pluripotent state.

EXAMPLE 15

This example illustrates various methods and systems used in the above examples.

Microfluidic device design and operation. The design of the microfluidics device used in some of these examples is indicated in Fig. 18 and integrates several features. As described above, it contains four inlets for, i) barcoded hydrogel microspheres (BHMs), ii) cell suspension, iii) reverse transcription (RT) and lysis reagent mix and iv) carrier oil, and one outlet port for droplet collection. To reduce flow fluctuations potentially arising due to mechanics of syringe pumps, fluid resistors were incorporated in the form of serpentine channels, while passive filters at each inlet prevent channels from clogging. The device included two junctions, one for bringing the three aqueous inputs together, and a second junction for sample encapsulation, where aqueous and oil phases meet and droplet generation occurs. To stabilize drops against coalescence, 0.75% (w/w) EA-surfactant (RAN Biotechnologies Inc.,) dissolved in HFE-7500 (3M) fluorinated fluid, was used. The dimensions of the microfluidic channels were carefully chosen to maximize the number of BHM and cell co-encapsulation events. The width (60 micrometers) of the BHM reinjection channel was designed such as that the BHMs (63 micrometers in diameter) passing through this channel become slightly squeezed thus facilitating their close packing and arrangement into a single-file. The BHMs entering into the main channel (70 micrometers wide) could then move freely downstream the flow before being encapsulated into individual droplets. Because of their close packing, the arrival of BHMs became highly regular, allowing nearly 100% loading of single-bead per droplet. This ensured that i) almost each cell encapsulated into a droplet was exposed to one barcoded primer, and ii) there was a minimal loss of non-barcoded-cells.

Soft lithography. The microfluidic device with rectangular microfluidic channels 80 micrometers deep was manufactured following established protocols. Briefly, a 3 inch size silicon wafer was coated with SU-8 3050 photoresist (MicroChem) at uniform 80 micrometer film thickness, baked at 65 °C for 20 min, and exposed to 365 nm UV light for 40 s (at ~8 mW cm^2) through the mask having a corresponding design indicated in Fig. 18 and baked for 5 min at 95 °C. The un-polymerized photoresist was dissolved with propylene glycol monomethyl ether acetate, silicon wafer rinsed with isopropanol and dried on a 95 °C hot plate for 1 min. The PDMS base and cross-linker (Dow Corning) was mixed at a 10:1 ratio and ~30 mL poured into the Petri dish containing a

developed silicon wafer, degassed and incubated overnight at 65 °C. The PDMS layer
was then peeled-off and inlet-outlet ports were punched with a 1.2 mm biopsy punch
(Harris Uni Core). The patterned side of PDMS was then treated with oxygen plasma
and bounded to the clean glass slide. The micro-channels were treated with water

5   repellent Aquapel (PPG Industries) and the device was then used in the above-described
experiments.

Microfluidic device operation. During device operation, cell suspension and
RT/lysis mix were cooled with ice-cold jackets, and droplets were collected into a single
1.5 mL tube (DNA LoBind, Eppendorf) placed on an ice-cold rack (IsoTherm System,

10   Eppendorf). To prevent water loss from the droplets due to evaporation during RT
incubation, 200 microliters of mineral oil layer (Sigma) was placed on top of the
emulsion. Throughout the experiments, flow rates at 100 microliters/hr for cell
suspension, 100 microliters/hr for RT/lysis mix, 10-20 microliters/hr for BHMs and
80 microliters/hr for carrier oil were used to produce 4 nL drops at a frequency of 15

15   droplets per second. Each aqueous phase was injected into the microfluidic device via
polyethylene tubing (ID 0.38 x OD 1.09 mm, BB31695-PE/2) connected to a needle of a
sterile 1 mL syringe (Braun) placed on a syringe pump (Harvard Apparatus, PC2 70-
2226).

Loading barcoded hydrogel microspheres (BHMs) into the microfluidic device.

20   After synthesis, BHMs were stored in $T_{10}E_{10}T_{0.1}$ buffer containing 10 mM Tris-HCl
(pH 8.0), 10mM EDTA, 0.1% (v/v) Tween-20. Before loading onto the microfluidic
chip, BHMs were washed in $T_{10}E_{0.1}T_{0.1}$ buffer containing 10 mM Tris-HCl (pH 8.0), 0.1
mM EDTA and 0.1% (v/v) Tween-20, and then resuspended in 1X RT buffer (Invitrogen
Superscript III buffer) supplemented with 0.5% (v/v) IGEPAL CA-630 and concentrated

25   by centrifugation at 5000 rpm for 2 min. After removal of the supernatant BHMs were
concentrated for a second time to achieve a close packing and eventually loaded directly
into tubing connected to an oil-filled syringe for injection into the microfluidic device.
The composition of BHMs sample was 100 microliters concentrated BHMs,
20 microliters 10% (v/v) IGEPAL CA-630, 40 microliters 5X First-Strand buffer and 40

30   microliters nuclease-free water (total aliquot volume 200 microliters).

Cell preparation and injection. The cell encapsulation process relies on random
arrival of cells into the device. To minimize two or more cells from entering the same
drop, diluted cell suspensions were used (~50-100,000 cells/mL) to obtain an average

occupancy of 1 cell in 5-10 droplets. To prevent cell sedimentation in the syringe or
other parts of the system, the cells were suspended in 1X PBS buffer with 16% (v/v)
density gradient solution Optiprep (Sigma). 20,000 cells were typically used, suspended
in 160 microliters 5X PBS (Lonza 17-516F), 32 microliters Optiprep (Axis-Shield

5   1114542) and 8 microliters 1% (v/v) BSA (Thermo Scientific B14), in a total volume
200 microliters.

        Reverse transcription/lysis mix. The RT/lysis mix included 25 microliters 5X
First-Strand buffer (18080-044 Life Technologies), 9 microliters 10% (v/v) IGEPAL
CA-630 (#18896 Sigma), 6 microliters 25 mM dNTPs (Enzymatics N2050L), 10

10   microliters 0.1 M DTT (#18080-044, Life Technologies), 15 microliters 1 M Tris-HCl
(pH 8.0) (51238 Lonza), 10 microliters Murine RNase inhibitor (M0314, NEB),
15 microliters SuperScript III RT enzyme (200 U/microliters, #18080-044, Life
Technologies) and 60 microliters nuclease-free water (AM9937 Ambion), having a total
volume 150 microliters.

15   Surfactant and carrier oil used for production of droplets. The carrier oil was
HFE-7500 fluorinated fluid (3M) with 1% (w/w) EA surfactant (RAN Biotechnologies).
EA-surfactant is a tri-block copolymer having an average molecular weight of ~13.000 g
mol^{-1}. It has two perfluoropolyether tails (M_W ~6.000 g mol^{-1}) connected via
poly(ethylene)glycol (M_W ~600 g mol^{-1}) head group. The surfactant was highly soluble

20   in fluorinated fluids and nearly insoluble in the aqueous phase providing equilibrium
interfacial tension of ~2 mN/m.

        Barcoding inside droplets. After cell encapsulation primers were released from
the BHMs by exposing the tube containing the emulsion droplets to UV light (365 nm at
~10 mW/cm^2, BlackRay Xenon Lamp) while on ice. Next, the tube was heated to 50 °C

25   and incubated for 2 hours to allow cDNA synthesis to occur and then terminated by
heating for 15 min at 70 °C. The emulsion was then cooled on ice for 1 min and
demulsified by adding 1 volume of PFO solution (20% (v/v) perfluorooctanol and 80%
(v/v) HFE-7500). The aqueous phase from the broken droplets was transferred into a
separate DNA Lo-Bind tube (Eppendorf) and processed as per the CEL-SEQ protocol

30   with modifications described in the library preparation section.

        Synthesis and quality control of Barcoded Hydrogel Microspheres. BHM
synthesis relies on microfluidic emulsification of acrylamide:bis-acrylamide solution
supplemented with acrydate-modified DNA primer, which is incorporated into the

hydrogel mesh upon acrylamide polymerization. After polymerization, the BHMs are released from droplets, washed several times and processed by split-pool synthesis for combinatorial barcoding. Below is outlined a detailed protocol of performing such hydrogel bead synthesis followed by combinatorial barcoding.

5   BHM synthesis begins by emulsifying gel precursor solution into 62 micrometer size droplets using the microfludic chip indicated in Fig. 19. The composition of the dispersed phase was 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 15 mM NaCl containing 6.2% (v/v) acrylamide, 0.18% (v/v) bis-acrylamide, 0.3% (w/v) ammonium persulfate and 50 micromolar acrydate-modified DNA primer (IDT, see Fig. 20A for sequence).

10   As a continuous phase, fluorinated fluid HFE-7500 was used, carrying 0.4% (v/v) TEMED and 1.5% (w/w) EA-surfactant. The flow rates were 400 microliters/hr for the aqueous phase and 900 microliters/hr for the oil phase. Droplets were collected into a 1.5 mL tube under 200 microliters mineral oil and incubated at 65 °C for 12 hours to allow polymerization of beads to occur. The resulting solidified beads were washed

15   twice with 1 mL of 20% (v/v) 1H,1H,2H,2H-perfluorooctanol (B20156, Alfa Aesar) in HFE-7500 oil and twice with 1 mL of 1% (v/v) Span 80 (S6760, Sigma) in hexane (BDH1129-4LP, VWR) with 0.5-1 min incubation between each step and finally centrifuged at 5000 rcf for 30 s. After final centrifugation, the hexane phase was aspirated and the resulting BHM pellet was dissolved in 1 mL of TEBST buffer (10 mM

20   Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 10 mM EDTA and 0.1% (v/v) Triton X-100). To remove traces of hexane, the beads were washed three times with 1 mL TEBST buffer at 5000 rcf for 30 s and finally resuspended in 1 mL TEBST buffer and stored at 4 °C. These BHMs contained pores ~100 nm in size. In addition, the beads having elastic modulus of ~ 1 kPa were "squishy," which allows them to be packed into a concentrated

25   gel mass without losing their integrity.

          BHM split-pool combinatorial barcoding. To prepare barcoded primers on the hydrogel microspheres, the two-step enzymatic extension reaction summarized in Fig. 20B was used. To begin, a pre-loaded a 384-well plate was used with 9 microliters of 15 micromolar primer 5'-W1*-bc1-PE1* encoding the first-half of a barcode (where

30   'bc1' indicates a unique sequence for each well, see also Table 4 for nucleotide sequence information). 6 microliters of reaction mix was added, containing ~40,000 hydrogel beads (carrying 5'-Ac-PC-T7p-PE1 primer), 2.5x isothermal amplification buffer (NEB) and 0.85 mM dNTP (Enzymatics) into each well (accounting ~10^7 beads in total). After

denaturation at 85°C for 2 min and hybridization at 60 °C for 20 min, 5 microliters of *Bst*

enzyme mix was added (1.8U of *Bst* 2.0 and 0.3 mM dNTP in 1X isothermal

amplification buffer) giving a final volume in each well of 20 microliters. After

incubation at 60 °C for 60 min, the reaction was stopped by adding 20 microliters of stop

5   buffer into each well (100 mM KCl, 10 mM Tris-HCl (pH 8.0), 50 mM EDTA, 0.1%

(v/v) Tween-20) and incubated on ice for 30 min to ensure that EDTA chelates

magnesium ions and inactivates *Bst* enzyme. Next, the beads were collected into a

50 mL Falcon tube, centrifuged at 1000 rcf for 2 min and washed three times with 50 mL

of STOP buffer containing 10 mM EDTA. To remove the second strand the gels were

10   suspended in 20 mL of 150 mM NaOH, 0.5% (v/v) Brij 35P and washed twice with 10

mL of 100 mM NaOH, 0.5% (v/v) Brij 35P. The alkaline solution was then neutralized

with buffer 100 mM NaCl, 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.1% (v/v)

Tween-20 and washed once in 10 mL $T_{10}E_{10}T_{0.1}$ buffer (10 mM Tris-HCl (pH 8.0),

10 mM EDTA, 0.1% (v/v) Tween-20) and twice in 10 mL $T_{10}E_{0.1}T_{0.1}$ buffer (10 mM

15   Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.1% (v/v) Tween-20) and finally beads were

suspended in 1.3 mL of DST buffer.

For the second barcoding step, a second 384-microtiter plate was prepared, pre-

loaded with 9 microliters of 15 micromolar primer 5'-T19V*-UMI-bc2-W1* (where

'bc2' indicates a unique sequence for each well and UMI is a random hexanucleotide,

20   see also Table 4 for sequence information), and repeated the procedure as for the first

384-well plate.

Quantification of ssDNA primers on the beads. To quantify the amount of the

ssDNA primers per BHM, fluorescence *in situ* hybridization (FISH) was performed with

complimentary DNA probes targeting the un-extended DNA "stub" (PE1), the barcoded

25   primer after one extension step (W1) and the primer after two extension steps ($T_{19}V$) (see

Table 1 for sequence information). Hybridization was performed in a 40 microliters

volume at room temperature for 20 min by suspending ~4000 DNA-barcoding beads in

hybridization buffer (1 M KCl, 5 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.05% (v/v)

Tween-20) together with 10 micromolar FAM-labeled probe. The high salt

30   concentration was used to avoid melting of the probe targeting $T_{19}V$ (dA_{20}-FAM), which

has weak binding even at room temperature. The absence of background fluorescence

was validated in microspheres lacking DNA primers. After incubation, beads were

washed three times with 1.4 mL hybridization buffer, re-suspended in 40 microliters and

fluorescence intensity recorded under confocal microscope (Leica). The average fluorescence intensity of beads with PE1*-FAM, W1*-FAM and dA20-FAM was 2286 +/- 271, 1165 +/- 160 and 718 +/- 145, respectively (Figs. 21A-21D). This corresponds to incorporation efficiencies of ~50% for W1/PE1 and 60% for polyT/W1, which gives the final efficiency of 31% or ~15 micromolar of fully barcoded ssDNA primers per bead. Accounting of the BHM volume, this equals ~10^9 copies of fully extended ssDNA primers per single bead.

To validate the release of primers from the hydrogel mesh, ~4000 beads was suspended in 20 microliters DST buffer and exposed to UV light (365 nm at ~10 mW/cm^2) for 8 min. A gel electropherogram of 1 microliters of supernatant using a BioAnalyzer High Sensitivity DNA Analysis Kit (Agilent Technologies) confirmed the presence of three DNA bands (Fig. 21E), which is in agreement with FISH results from above.

Single-molecule sequencing of primers from single BHMs. To test the composition of BHMs after synthesis, 10 BHMs were randomly picked and sequenced using the Illumina MiSeq sequencing platform. For this purpose, the BHMs were first hybridized to a fluorescent FISH probe (PE1-FAM) as described above, and were manually picked using a dissection microscope (Nikon) under fluorescent illumination and transferred into 0.2 mL PCR tubes pre-filled with 5 microliters DNA Suspension (DS) buffer (10 mM Tris-HCl pH 8.0, 0.1mM EDTA). The tubes were then exposed to UV light (~10 mW/cm^2) for 15 min while keeping them on ice. After UV exposure, 0.5 microliters of 5 micromolar PE2-(barcode)$_n$-A19 primer (herein, *n* represents 10 different barcodes) was added to the tube and mixed with 4.5 microliters of *Bst* 2.0 ready-to-use reaction solution. The samples having 10 microliters final volume were then incubated at room temperature for 10 min, inactivated for 3 min at 95°C and cooled down on ice. Next, 20 microliters of master mix containing 50% (v/v) Kapa HiFi HotStart ready mix (2X, KK2601), 15% (v/v) PE1/PE2 primers, and 35% (v/v) nuclease-free water were added into each tube, and DNA was amplified with PCR (95 °C for 5 min, 30 cycles at 98 °C for 20 s, 60 °C for 15 s, 72 °C for 30 s and final step at 72 °C for 5 min). The size of the PCR products was assessed by gel-electrophoresis, purified with GenElute PCR CleanUp Kit (Na1020-1KT, Sigma) and all samples diluted down to 10 ng/microliters. In the final step all samples were pooled together and sequenced using MiSeq Illumina

platform by following manufacturer recommendations. Sequencing results of primers from 10 individual beads is presented in Fig. 21F-21H.

Limits on the number of cells per single sequencing run. For a large pool of barcoded hydrogel microspheres (BHMs), each carrying one of N barcodes, what is the maximum number of cells that can be captured before two or cells will carry the same barcode? This question is akin to the so-called birthday problem, with barcodes analogous to days of the year, and BHMs analogous to the people in a room. The expected number of observed barcodes from sampling n BHMs is $n_{obs} = N(1 - e^{-n/N})$. Thus, the expected multi-barcoding error, defined as the fraction of cells carrying the same barcode, is approximately $f_{err} = 1 - n_{obs}/n$. The error becomes large when n~N, so in practice the number of sampled cells must be much smaller than the number of barcodes, i.e. $n \ll N$, and therefore the limit of obtaining barcoded single-cells is $f_{err} \approx n/2N$. The number of barcoded single-cells n depends on the tolerated error, for example, allowing for an error of less than $f_{err}=1\%$ requires an upper limit $n=N/50$. Thus, for the value of $N=384^2$ which corresponds to two 384-well plates in our experiment, a 1% multiple-barcoding error arises at the limit $n=2,949$ cells. In practice, fewer cells can be used to produce negligible multi-barcoding errors.

Cell culture preparation. The mouse embryonic stem (mES) cells were maintained in ESC base media inside culture flasks pre-coated with gelatin at 37 °C in 5% CO_2 and 60-80% humidity at density ~3x10^5 cells ml^{-1}. The ESC media contained phenol red free DMEM (Gibco), supplemented with 15% (v/v) fetal bovine serum (Gibco), 2 mM L-glutamine, 1x MEM non-essential amino acids (Gibco), 1% (v/v) penicillin-streptomycin antibiotics, 110 micromolar beta-mercaptoethanol, 100 micromolar sodium pyruvate. For guided differentiation ESC base media was supplemented with Leukemia Inhibitory Factor (LIF) at final concentration 1000 U/mL and for unguided mES differentiation the media was without LIF. Within 2 days of LIF withdrawal the culture experienced significant morphological changes indicating the differentiation of mES cells.

Prior encapsulation the flask was washed with 1x PBS (without Mg^{2+} and Ca^{2+} ions) and treated with 1x trypsin/EDTA solution for 3 min at 37 °C. The trypsin was quenched by adding equal volume of ESC base media. Detached cells were centrifuged at 260g for 3 min and re-suspended in ~3 mL fresh ESC base media. After passing

through the 40 micrometer size strainer, cells were counted with hemocytometer and diluted in 0.5x PBS supplemented with 0.04% (v/v) BSA and 16% (v/v) OptiPrep solution to obtain desirable amount of cells (typically 20,000 cells in 200 microliters). The suspension was transferred into 1 mL syringe connected to microfluidics device and

5   injected at 100 microliter/hr flow rate. Following this procedure mES cells were prepared with LIF at Day 1 and without LIF at early Day 2, late Day 2, Day 4 and Day 7.

The K-562 cell line (ATCC, CCL-243) was maintained in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin at 37 $^\circ$C in 5% CO_2 and 60-80% humidity atmosphere, at density ~$3x10^5$ cells ml^{-1}. For encapsulation

10   experiments K-562 cells were prepared as outlined above but using DMEM media and mixed with mES cells at ratio 1:1.

DNA-library preparation. Library preparation was based on a modified CEL-Seq protocol. The workflow of DNA library preparation can summarized as follows: RT --> ExoI --> SPRI purification (SPRIP) --> SSS --> SPRIP --> T7 in vitro transcription

15   linear amplification --> SPRIP --> RNA Fragmentation --> SPRIP --> primer ligation --> RT --> library enrichment PCR.

Referring to the detailed protocol in Jaitin DA, *et al.* (2014) Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types. *Science* 343(6172):776-779, the following modifications were made to the protocol: the RT

20   primer included the P5/PE1 adaptor while the ligation primer includes the P7/PE2 adaptor, a flipped orientation to that in the protocol; prior to ExoI treatment, the aqueous phase from broken droplets was centrifuged at 4 $^\circ$C for 15 minutes at 14krcf to pellet cell debris and gels; during ExoI treatment, 10U HinFI were added to digest primer dimers that may have formed during the RT reaction; the original DNAse digestion step was

25   omitted after linear amplification; after linear amplification the resulting amplified RNA libraries were analyzed on an Agilent BioAnalyzer before proceeding; before primer ligation, the samples were treated with Shrimp Alkaline Phosphatase for 30 minutes. The number of final PCR cycles required for final library enrichment PCR ranged from 10-13 cycles. The remaining steps are otherwise unchanged.

30   Bioinformatic analysis. Paired-end sequencing was performed on Illumina MiSeq, HiSeq 2500 and NextSeq machines as detailed in Table 1. Read 1 was used to obtain the sample barcode and UMI sequences; read 2 was then mapped to a reference transcriptome as described below. The reads were first filtered based on presence in read

1 of two sample barcode components separated by the W1 adaptor sequence (see Fig. 20 and Table 4). Read 2 was then trimmed using Trimomatic (5) (version 0.30; parameters: LEADING:28 SLIDINGWINDOW:4:20 MINLEN:19). Barcodes for each read were matched against a list of the 384^2 pre-determined barcodes, and errors of up to two nucleotides mismatch were corrected. Reads with a barcode separated by more than two nucleotides from the reference list were discarded. The reads were then split into barcode-specific files for mapping and UMI filtering.

The trimmed reads were aligned using Bowtie (version 0.12.0, parameters: -n 1 –l 15 –e 300 –m 200 –best –strata -a) to the mouse transcriptome. The data sets were also reprocessed with different bowtie parameter sets without changing the qualitative results of the analysis. The reference transcriptome was built using all annotated transcripts (extended with a 125bp poly-A tail) from the UCSC mm10 genome assembly. A custom Python and PySAM script was used to process mapped reads into counts of UMI-filtered transcripts per gene. Alignments from bowtie were filtered in the following way: (1) for each read, we retained at most one alignment per gene, across all isoforms, by choosing the alignment closest to the end of the transcript. (2) If a read aligned to multiple genes, we excluded any alignments more than 400 bp away from the end of the transcript; this is motivated by the strong 3' bias of the CEL-SEQ method. (3) Reads mapping to more than 10 genes were excluded, and (4) a UMI filtering step described in the following paragraph was performed. Finally, (5) if a read still aligned to more than 2 genes after UMI filtering, the read was excluded altogether. In reporting the counts, for each gene, any other genes from which it could not be distinguished in at least one read was also reported; this allowed the exclusion of spurious correlations in our downstream analysis resulting from mapping ambiguities. The robustness of the pipeline to this final step was confirmed by re-processing the data with a maximum of 1-4 alignments per read. After steps (1-5) were carried out separately for each sample, the resulting gene expression tables were concatenated and loaded into MATLAB for analysis.

UMI filtering (step 4 above) was carried out as follows. Each distinct UMI was associated with a set of genes through the set of reads carrying the UMI. For each UMI, the minimal set of genes that can account for the full set of reads with this UMI was identified. This problem is known as the 'Hitting Set Problem' (or 'Set Cover Problem'). A greedy algorithm was applied to obtain the most parsimonious gene set for each UMI. Only one read per gene per UMI was kept. With this approach, some subsets

of genes may still be undistinguishable from each other because they are supported by the same set of ambiguously aligned reads. Step (5) in the previous paragraph was thus used to eliminate ambiguous reads beyond a predetermined threshold. To illustrate the UMI filtering step, consider a UMI with two reads, the first aligning to genes A and B and the second aligning to genes B and C. Although neither read is aligns unambiguously, gene B can explain the presence of both reads and thus the alignments to genes A and C are discarded, and just one of the two reads is kept for gene B.

UMI-filtered count normalization. Prior to normalization, the variation in the total UMI-filtered mapped (UMIFM) counts per sample barcode was 21% to 55% (coefficient of variation), see Table 1. The CV appeared to grow during differentiation, suggesting that some of the variation in total UMIFM counts arose from differences in cell size rather than in variation in RT efficiency. All counts were normalized by total-count normalization, i.e. the normalized counts for gene j in cell i is given in terms of the un-normalized counts, $m_{i,j}$, as $\widehat{m}_{i,j} = m_{i,j} \bar{M}/M_i$, where $M_i = \Sigma_j m_{i,j}$ and $\bar{M}$ is the average of M_i over all cells. Similar results are also obtained using sub-sample normalization.

Predicting method sensitivity. This section derives the form of the sensitivity curve (solid curve) in Fig. 14E, predicted for a case where the only limitation to detection is the capture efficiency, β or beta, which is assumed to be unbiased and uniform across all gene transcripts. All other biases, such as sequence-specific or length-specific bias, are assumed negligible. The excellent fit reinforces these assumptions. Let n be the number of transcripts for a given gene in a given droplet. The probability of detecting zero transcripts for the gene in this droplet is $p_0(n) = e^{-\beta n}$. The sensitivity S is then obtained by marginalizing $p_o(n)$ over the distribution of n, which, in the case of the pure RNA sample, is Poisson-distributed about a mean value $\bar{n}$. One obtains $S(\bar{n}) = 1 - \Sigma_{n=0}^{\infty} p_0(n) \, Poiss[n, \bar{n}]$, giving $S(\bar{n}) = e^{-\bar{n}(1-e^{-\beta})}$, which is the curve plotted in Fig. 14E, with the value of β (beta) measured from Fig. 14D. This curve can also be identified as the moment generating function (MGF) of the Poisson distribution evaluated at β (beta). The quality of the fit demands that variations in β (beta) between droplets be small, which is consistent with the low CV in the total counts between control droplets. For non-control samples, the input distribution for each gene is no longer a Poisson distribution, and the detection frequency $S(\bar{n})$ is instead different for

each gene and, under the assumptions given here, is equal to the MGF of the underlying gene expression distribution evaluated at β (beta).

Selection and filtering of principal gene sets for PCA and tSNE analysis. Since each gene carries intrinsic sampling noise that is uncorrelated to other genes, it is expected that for whole-transcriptome data, a large fraction of the variability observed across all genes will not be explained by the top principal components. For the same reasons, differences between cell sub-populations may appear weak if a large number of "bystander" genes (which vary little between populations) are included in evaluating cell-cell correlations. To overcome these sampling limitations, the ES cell population structure was analyzed using only a sub-set of genes chosen to reflect known ES cell biology while also reporting on the most variable genes at each time point. The general strategy for selecting an appropriate gene set was as follows: (1) for each time point, the top 200 most variable genes were included, as determined by the v-score (Table 2), which is closely related to the gene Fano Factors; these genes were complemented with a curated list of genes implicated in ES cell biology. (2) To reduce the gene set, a preliminary principal component analysis (PCA) was performed on the cell population, using the initial gene set, and used the results to select only "principal genes", i.e. genes contributing to non-random principal components (PCs) as determined by matrix randomization in Fig. 150E. The principal genes are those with the highest loading coefficients for each non-random PC, with the selection threshold set dynamically for each PC to reflect the structure of the loading coefficient distribution. (3) For each gene g in the set, the set was then re-expanded to include up to two additional genes that correlated most strongly with g. This final step allows inclusion of genes not present in the initial set, but which correlated strongly with the highly variable gene set. The final gene set derived at the end of step (3) was used for subsequent PCA and tSNE analysis at each time point.

Network neighborhood analysis. The distance metric $d=(1-(\text{Pearson correlation}))$ was used to define the distance between two genes, where the correlation is taken over all cells. An unweighted, directed network was constructed as follows: for a given gene G_0 of interest, a directed edge to its N nearest neighbor genes G_1 was defined (i.e. genes with the highest correlation to G_0). N additional directed edges were added from each member of the set G_1 to its N nearest neighbors, together forming a set G_2. The resulting preliminary network has $(N+1)*N$ directed edges in total, and up to $1+(N+1)*N$ vertices

representing G_0, G_1 and G_2. The network was then trimmed iteratively by removing any vertex that has fewer than X incoming edges. The final network is the "X-connected neighborhood of gene G_0." If it is not an empty set, it has: the gene G_0; some members of G_1 that are also nearest neighbors of at least X-1 other members of G_1; and some members of G_2 that are the nearest neighbors of at least X members of G_1. For the networks plotted in Fig. 16, the parameters N=50, X=3 were used.

Table 1. Sequencing run statistics

Sample (days post-LIF)	Emulsion volume (uL)	Platform	Total library reads (unfiltered)	Number cells / barcodes	Average filtered reads/cell	UMI Filtered Mapped (UMIFM) counts/cell	
						Average	Coeff. of variation (CV)
Pure RNA control	16	HiSeq 2500	166,031,332	953	89,116	24,191	21%
mES LIF+	40	NextSeq	413,138,104	935	199,193	29,239	36%
mES day 2, (early)	6	HiSeq 2500	119,859,024	145	119,386	20,524	35%
mES day 2, (late)	26	MiSeq	17,660,550	303	38,788	8,441	36%
mES day 4	40	MiSeq	11,557,428	683	10,237	4,661	43%
mES day 7	8	HiSeq 2500	92,805,168	169	153,035	27,065	38%
mES day 7	40	NextSeq	250,187,951	799	208,231	26,216	55%
mES day 2, early	95	HiSeq 2500	33,751,186	2,168	4,987	2,608	42%

Table 3. mES cell sub-population markers across time points

Data set	Cell group description	Cell group size (number / total)	Clustering index (-1<x<1)	High-expressed genes
mES day 0	Primitive endoderm-like	6/935	0.76	Gsn, Col4a1/2, Serpinh1, Lama1/b1/c1, Sparc, Srgn, P4ha2, Lrpap1, Podxl, Ctsl, S100a10, Pgk1, Slc2a3, Tfpi, Amn, Fbp2, Gpx3, Man2c1os, Lpar3, Cd63
	Epiblast-like	40/935 (all) 6/935 (Krt8-high)	0.20 (all) 0.55 (Krt8-high)	Actg1, Anxa2, Krt8/18/19, Plaur, Cnn1, Tagln, Plin2, Flnc, Tinagl1, Slc2a1, Fam160b2, Mmab, Sfn, Plec, S100a6, Flnb, Ngfrap1
	Hsp90-high	10/935	0.47	Atf5, Calr, Hsp90b1, Hspa5, Manf, Pdia6,

				Creld2, Hyou1, Derl3, Prph, Chchd10
	Prdm1-high	13/935	0.47	*Prdm1, Baat, Nsun6, Parp4, Srgn, Ssh1*
	Trim71-high	12/935	0.31	*Trim71, Cd44, Med10, Myo15, Bcl2/Cep83, Kdm1b, Sbk1, Csf1r Psg18/20, Prss44*
4,7 days post-LIF withdrawal	*H19/Rhox6/9+*	14/683, 10/899	0.36, 0.20	*H19, Igf2, Rhox6/9, Fabp3, Igfbp2, Sct, Vgf, Pmp22, Rhox5, Itm2a, Rhox5, 1700001F09Rik, Peg10*
	Pluripotency-high	21/683, 31/899	0.16, 0.15	*Trim28, Tex19.1, Tdh, Tdgf1, Spry4, Sox2, Psors1c2, Pou5f1, Phc1, Ogfod3, Mylpf, Mtl/2, Mkrn1, Mkm1, L1td1, Kcnj14, Gad1, G3bp2, Dnmt3l, Cdh16, Nlrp1a, 4930526L06Rik, 3110021A11Rik*
	Zscan4-hi	4/683, 0/899	0.45, N/A	*Zscan4a/c/d/f, Fbxo15, Tcstv1/3, Dazl, Calcoco2, Mylpf, Dcdc2c, Lmx1a, Ddit4l, Aqr, Clp1, Tmem92, Usp17la, 2310039L15Rik, B020031M17Rik, Gm4027, Gm20767, Gm7102, Gm8994*
	Primitive endoderm-like	7/683, 4/899	0.55, 0.65	*Gata6, Amn, Cd63, Ctsl, Col4a1/2, Lama1/b1/c1, Upp1, Sparc, P4ha2, Serpinh1, Fst, Lrpap1, P4hb, Ctsh, Clu, Epas1, Pga5*

Table 4. List of DNA oligonucleotides

1. BHM synthesis:	
Hydrogel-incorporated DNA primer	5'-/5Acryd/iSpPC/CGATGACG TAATACGACTCACTATAGGG ATACCACCATGG CTCTTTCCCTACACGACGCTCTTC-3' (SEQ ID NO: 7)
barcode 1 (W1*-bc1-PE1*)	5'-AAGGCGTCACAAGCAATCACTC 10987654321 AGATCGGAAGAGCGTCGTGTAGGGAAAGAG-3' (SEQ ID

                                            83

	NO: 8)
Barcode 2/UMI (T19V*-UMI-bc2-W1*):	5'-BAAAAAAAAAAAAAAAAAAAA NNNNNN 87654321 AAGGCGTCACAAGCAATCACTC-3' (SEQ ID NO: 9)
FAM-PE1*	/56-FAM/AGATCGGAAGAGCGTCGTGTAGGGAAAGAG (SEQ ID NO: 10)
FAM-W1*	/56-FAM/AAGGCGTCACAAGCAATCACTC (SEQ ID NO: 11)
FAM-A20	/56-FAM/AAAAAAAAAAAAAAAAAAAA (SEQ ID NO: 12)
Fully assembled DNA primers:	CGATGACG TAATACGACTCACTATAGGG ATACCACCATGG CTCTTTCCCTACACGACGCTCTTCCGATCT 1234567890 GAGTGATTGCTTGTGACGCCTT 12345678 NNNNN TTTTTTTTTTTTTTTTTTTV (SEQ ID NO: 13)
2. Library preparation:	
RNA ligation:	/5Phos/AGATCGGAAGAGCGGTTCAGCAGGAATGCC/3SpC3/ (SEQ ID NO: 14)
2nd RT primer:	GTCTCGGCATTCCTGCTGAAC (SEQ ID NO: 15)
PCR enrichment primers:	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTA CACGA (SEQ ID NO: 16) CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCT GCTGAAC (SEQ ID NO: 17)

EXAMPLE 16

This example illustrates a method of encapsulating cells into droplets. In this example, a droplet-barcoding-sequencing platform was used to encapsulate cells into droplets with lysis buffer, reverse transcription (RT) reagents, and barcoded

5   oligonucleotide primers. mRNA released from each lysed cell remains trapped in the same droplet and was barcoded during synthesis of complementary DNA (cDNA). After barcoding, the material from all cells is combined by breaking the droplets, and the cDNA library was processed for sequencing (Fig. 27).

In this example, a library of barcoded hydrogel microspheres (BHMs) were

10   synthesized that were coencapsulated with cells (Fig. 27). The BHMs carried covalently coupled, photoreleasable primers encoding one of 384^2 (i.e. 147,456) pre-defined barcodes. This pool size allowed randomly labeling 3,000 cells with 99% unique

labeling, and the number of cells that can be barcoded is far larger through the use of library barcodes to mark collection tubes of ~3k cells each. The method can be extended in a straightforward manner if larger-scale cell capture in a single library is desired.

5   Fig. 27 shows a droplet microfluidic platform for DNA barcoding thousands of cells. Schematic of single cell droplet barcoding. Cells are co-encapsulated with lysis buffer, reverse-transcription (RT) mix, and hydrogel microspheres carrying barcoded RT primers; after encapsulation primers are released from the hydrogels, and cDNA product in the droplets is tagged with a DNA barcode during reverse transcription. Droplets are then broken and material from all cells is linearly amplified before sequencing. UMI = a

10   random hexamer unique molecular identifier.

      While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or

15   one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific

20   application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and

25   equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not

30   mutually inconsistent, is included within the scope of the present invention.

      All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should
5   be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or
10 unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other
15 elements); etc.

As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and,
20 optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e. "one or the other but not both") when preceded by terms of exclusivity, such as
25 "either," "one of," "only one of," or "exactly one of." "Consisting essentially of," when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not
30 necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least

one" refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally

5   including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

10   When the word "about" is used herein in reference to a number, it should be understood that still another embodiment of the invention includes that number not modified by the presence of the word "about."

       It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or

15   acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

       In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, i.e., to mean

20   including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

       What is claimed is:

CLAIMS

1.   A method, comprising:

    encapsulating a plurality of cells and a plurality of particles within a plurality of at least 10,000 microfluidic droplets, at least some of the particles comprising an oligonucleotide tag covalently bonded thereto, such that the droplets of the plurality of the at least 10,000 droplets contain one or more oligonucleotide tags distinguishable from oligonucleotide tags contained in other droplets of the plurality of droplets;

    lysing at least some of the cells within the droplets to release nucleic acid from the cell; and

    bonding the released nucleic acids and the oligonucleotide tags within at least some of the droplets, without first amplifying the released nucleic acid.

2.   The method of claim 1, comprising encapsulating the plurality of cells in the plurality of microfluidic droplets such that at least about 90% of the microfluidic droplets contain one cell or no cell.

3.   The method of any one of claims 1 or 2, comprising encapsulating the plurality of particles in the plurality of microfluidic droplets such that at least about 90% of the microfluidic droplets contain one particle or no particle.

4.   The method of any one of claims 1-3, wherein the particles are encapsulated within the droplets at no more than about 1 particle/droplet.

5.   The method of any one of claims 1-4, wherein the particles are encapsulated within the droplets at no more than about 0.1 particles/droplet.

6.   The method of any one of claims 4-6, wherein the particles are encapsulated within the droplets at no more than about 0.01 particles/droplet.

7.   The method of any one of claims 4-6, wherein the cells are encapsulated within the droplets at no more than about 1 cell/droplet.

8.   The method of any one of claims 4-7, wherein the cells are encapsulated within the droplets at no more than about 0.1 cell/droplet.

9.   The method of any one of claims 1-8, wherein at least some of the particles are hydrogel particles.

10.   The method of any one of claims 1-8, wherein at least some of the particles are polymeric particles.

11.   The method of any one of claims 1-10, wherein at least some of the particles are microparticles.

12.   The method of any one of claims 1-11, wherein at least some of the particles comprise polyacrylamide.

13.   The method of any one of claims 1-12, wherein at least some of the particles comprise agarose.

14.   The method of any one of claims 1-13, wherein at least some of the particles comprise polystyrene.

15.   The method of any one of claims 1-14, wherein at least some of the particles comprise poly-N-isopropylacrylamide.

16.   The method of any one of claims 1-15, wherein at least some of the particles are magnetic.

17.   The method of any one of claims 1-16, wherein the plurality of particles have an average diameter of no more than about 500 micrometers.

18.   The method of any one of claims 1-17, wherein the plurality of particles have an average diameter of at least about 1 micrometer.

19. The method of any one of claims 1-18, wherein at least some of the oligonucleotide tags are covalently bonded to the particles via an acrylic phosphoramidite linkage.

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20. The method of any one of claims 1-18, wherein at least some of the oligonucleotide tags are covalently bonded to the particles via an amino linkage.

21. The method of any one of claims 1-18, wherein at least some of the oligonucleotide tags are covalently bonded to the particles via a biotin-steptavidin linkage.

10

22. The method of any one of claims 1-21, comprising covalently bonding the released nucleic acids and the oligonucleotide tags within at least some of the droplets.

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23. The method of any one of claims 1-22, comprising bonding the released nucleic acids and the oligonucleotide tags within at least some of the droplets using an enzyme.

20

24. The method of any one of claims 1-23, further comprising combining the released nucleic acids tagged with the oligonucleotide tags with nucleic acids released from other cells in other droplets.

25. The method of any one of claims 1-24, wherein the oligonucleotide tags uniquely identify the released nucleic acids from nucleic acids released from other cells.

26. The method of any one of claims 1-25, wherein at least some of the oligonucleotide tags comprise at least one barcode sequence.

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27. The method of claim 26, wherein the barcode sequence is selected from a pool of barcode sequences.

28.   The method of claim 27, wherein the pool of barcode sequences comprises at least 10,000 barcode sequences.

29.   The method of claim 28, wherein the pool of barcode sequences comprises at least 100,000 barcode sequences.

30.   The method of any one of claims 1-29, wherein at least some of the oligonucleotide tags comprise at least two barcode sequences.

31.   The method of claim 30, wherein the at least two barcode sequences is selected from a different pool of barcode sequences.

32.   The method of any one of claims 1-31, wherein at least some of the oligonucleotide tags comprise a primer sequence.

33.   The method of clam 32, wherein at least some of the oligonucleotide tags comprise more than one primer sequence.

34.   The method of any one of claims 1-33, further comprising releasing at least some of the oligonucleotide tags from the particles.

35.   The method of claim 34, comprising releasing at least some of the oligonucleotide tags from the particles by applying light.

36.   The method of any one of claims 1-35, wherein at least some of the oligonucleotide tags comprise a cleavable linker.

37.   The method of claim 36, wherein at least some of the cleavable linkers are photocleavable linkers.

38.   The method of claim 36, wherein at least some of the cleavable linkers are chemically cleavable linkers.

39.   The method of claim 36, wherein at least some of the cleavable linkers are enzymatically cleavable linkers.

40.   The method of any one of claims 1-39, comprising releasing at least some of the oligonucleotide tags from the particles prior to lysing the cells.

41.   The method of any one of claims 1-40, comprising releasing at least some of the oligonucleotide tags from the particles after lysing the cells.

42.   The method of any one of claims 1-41, wherein at least some of the oligonucleotide tags comprise a poly-T sequence.

43.   The method of any one of claims 1-42, wherein at least some of the oligonucleotide tags comprise a random DNA sequence.

44.   The method of any one of claims 1-43, wherein at least some of the oligonucleotide tags comprise a sequence able to specifically bind a gene.

45.   The method of any one of claims 1-44, wherein at least some of the oligonucleotide tags comprise a primer, and bonding the released nucleic acids and the oligonucleotide tags within at least some of the droplets comprises amplifying at least some of the nucleic acids using the primer to produce sequences wherein the released nucleic acids and the oligonucleotide tags are bonded together.

46.   The method of any one of claims 1-45, wherein lysing at least some of the cells comprises releasing DNA from the cells.

47.   The method of any one of claims 1-46, wherein lysing at least some of the cells comprises releasing RNA from the cells.

48.   The method of any one of claims 1-47, further comprising exposing at least some of the lysed cells to an antibody.

49.   The method of claim 48, wherein the antibody is a DNA-tagged antibody.

50.   The method of any one of claims 1-49, wherein at least some of the cells are lysed using a cell lysis reagent.

51.   The method of claim 50, comprising encapsulating a cell, a particle, and cell lysis reagent together within a microfluidic droplet.

52.   The method of any one of claims 1-51, further comprising breaking at least some of the droplets.

53.   The method of claim 52, comprising releasing at least some of the oligonucleotide tags from the particles prior to breaking the droplets.

54.   The method of claim 52, comprising releasing at least some of the oligonucleotide tags from the particles after breaking the droplets.

55.   The method of claims 52-54, further comprising sequencing at least some of the released nucleic acids.

56.   The method of claim 55, further comprising determining at least some of the released nucleic acids using the oligonucleotide tags.

57.   The method of any one of claims 1-56, wherein the plurality of microfluidic droplets have a volume of less than about 10 nl.

58.   The method of any one of claims 1-57, wherein the plurality of microfluidic droplets have an average diameter of less than about 1 mm.

59.   The method of any one of claims 1-58, wherein at least some of the cells arise from dissociated tissue.

60. An article, comprising:

a plurality of at least 10,000 microfluidic droplets, at least some of the droplets containing cell lysate including nucleic acid fragments, wherein a plurality of the nucleic acid fragments within a droplet are bound to an oligonucleotide tag, without first amplifying the released nucleic acid, wherein the oligonucleotide tags are covalently attached to particles, wherein the oligonucleotide tag within the droplet is distinguishable from oligonucleotide tags within the other droplets of the plurality of 10,000 microfluidic droplets.

61. The article of claim 60, comprising a plurality of at least 100,000 microfluidic droplets.

62. The article of any one of claims 60 or 61, comprising a plurality of at least 300,000 microfluidic droplets.

63. The article of any one of claims 60-62, comprising at least 1,000,000 microfluidic droplets.

64. The article of any one of claims 60-63, wherein at least about 90% of the droplets containing one particle or no particle.

65. The article of any one of claims 60-64, wherein at least some of the particles are hydrogel particles.

66. The article of any one of claims 60-65, wherein at least some of the particles are polymeric particles.

67. The article of any one of claims 60-66, wherein at least some of the particles are microparticles.

68. The article of any one of claims 60-67, wherein at least some of the particles comprise polyacrylamide.

69.   The article of any one of claims 60-68, wherein at least some of the particles comprise agarose.

70.   The article of any one of claims 60-69, wherein at least some of the particles comprise polystyrene.

71.   The article of any one of claims 60-70, wherein at least some of the particles comprise poly-N-isopropylacrylamide.

72.   The article of any one of claims 60-71, wherein at least some of the particles are magnetic.

73.   The article of any one of claims 60-72, wherein the particles have an average diameter of no more than about 500 micrometers.

74.   The article of any one of claims 60-73, wherein the particles have an average diameter of at least about 1 micrometer.

75.   The article of any one of claims 60-74, wherein at least some of the oligonucleotide tags are covalently bonded to at least some of the particles via an acrylic phosphoramidite linkage.

76.   The article of any one of claims 60-75, wherein at least some of the oligonucleotide tags are covalently bonded to at least some of the particles an amino linkage.

77.   The article of any one of claims 60-76, wherein at least some of the oligonucleotide tags are covalently bonded to at least some of the particles a biotin-steptavidin linkage.

78.   The article of any one of claims 60-77, wherein at least some of the oligonucleotide tags comprise at least one barcode sequence.

79. The article of claim 78, wherein the barcode sequence is selected from a pool of barcode sequences.

80. The article of claim 79, wherein the pool of barcode sequences comprises at least 100,000 barcode sequences.

81. The article of claim 80, wherein the pool of barcode sequences comprises at least 1,000,000 barcode sequences.

82. The article of any one of claims 60-81, wherein at least some the oligonucleotide tags comprise at least two barcode sequences.

83. The article of claim 82, wherein the at least two barcode sequences of the oligonucleotide tag is selected from a different pool of barcode sequences.

84. The article of any one of claims 60-83, wherein at least some of the oligonucleotide tags comprise a primer sequence.

85. The article of any one of claims 60-84, wherein at least some of the oligonucleotide tags comprise a cleavable linker.

86. The article of claim 85, wherein the cleavable linker is a photocleavable linker.

87. The article of claim 85, wherein the cleavable linker is a chemically cleavable linker.

88. The article of claim 85, wherein the cleavable linker is an enzymatically cleavable linker.

89. The article of any one of claims 60-88, wherein at least some of the oligonucleotide tags comprise a poly-T sequence.

90.   The article of any one of claims 60-89, wherein at least some of the oligonucleotide tags comprise a random DNA sequence.

91.   The article of any one of claims 60-90, wherein at least some of the oligonucleotide tags comprise a sequence able to specifically bind a gene.

92.   The article of any one of claims 60-91, wherein at least some of the oligonucleotide tags comprise primers.

93.   The article of any one of claims 60-92, wherein the microfluidic droplets have an average volume of less than about 10 nl.

94.   The article of any one of claims 60-93, wherein the microfluidic droplets have an average diameter of less than about 1 mm.

95.   The article of any one of claims 60-94, wherein the cells arise from dissociated tissue.

96.   A method, comprising:

providing a plurality of at least 10,000 microfluidic droplets containing cells, at least about 90% of the plurality of droplets containing one cell or no cell;

lysing the cells within the plurality of microfluidic droplets to release nucleic acid from the cells; and

bonding the released nucleic acid to oligonucleotide tags, without first amplifying the released nucleic acid, wherein the oligonucleotide tags are covalently attached to particles, wherein for at least about 90% of the droplets, the oligonucleotide tag within the droplet is distinguishable from oligonucleotide tags within other droplets of the plurality of droplets.

97.   The method of claim 96, wherein the particles are encapsulated within the droplets at no more than about 1 particle/droplet.

98.   The method of any one of claims 96 or 97, wherein the cells are encapsulated within the droplets at no more than about 1 cell/droplet.

99.   The method of any one of claims 96-98, further comprising combining the released nucleic acid tagged with the oligonucleotide with tag nucleic acids released from other cells in other droplets.

100   The method of claim 99, wherein the oligonucleotide tag uniquely identifies the released nucleic acids from nucleic acids released from other cells.

101.   The method of any one of claims 96-100, wherein the oligonucleotide tag comprises at least one barcode sequence.

102.   The method of any one of claims 96-101, wherein the oligonucleotide tag comprises a primer sequence.

103.   The method of any one of claims 96-102, further comprising breaking the droplets.

104.   The method of claims 96-103, further comprising sequencing the tagged nucleic acid.

105.   An article, comprising:

        a plurality of at least 10,000 microfluidic droplets, at least some of the droplets containing cell lysate, wherein at least about 90% of the plurality of 10,000 microfluidic droplets contain only one particle, the particle comprising an oligonucleotide covalently bonded thereto, wherein the oligonucleotide within a droplet is distinguishable from oligonucleotides within the other droplets of the plurality of 10,000 microfluidic droplets, without first amplifying the released nucleic acid from the cell lysate.

106.   The article of claim 105, comprising at least 30,000 microfluidic droplets.

107.   The article of claim 106, comprising at least 100,000 microfluidic droplets.

108.   The article of claim 107, comprising at least 1,000,000 microfluidic droplets.

5   109.   The article of any one of claims 105-108, wherein at least some of the particles are hydrogel particles.

110.   The article of any one of claims 105-109, wherein at least some of the particles are polymeric particles.

10   111.   The article of any one of claims 105-110, wherein at least some of the oligonucleotide tags comprise at least one barcode sequence.

112.   The article of any one of claims 105-111, wherein at least some of the
15   oligonucleotide tags comprise a primer sequence.

113.   The article of any one of claims 105-112, wherein at least some of the oligonucleotide tags comprise a cleavable linker.

20   114.   The article of any one of claims 105-113, wherein at least some of the oligonucleotide tags comprise a poly-T sequence.

115.   The article of any one of claims 105-114, wherein at least some of the oligonucleotide tags comprise a random DNA sequence.

25   116.   The article of any one of claims 105-115, wherein at least some of the oligonucleotide tags comprise a sequence able to specifically bind a gene.

117.   A method, comprising:
30   encapsulating a cell and hydrogel beads within a droplet, the hydrogel
       particle having attached thereto a barcoded nucleic acid;
               lysing the cell within the droplet to release nucleic acid from the cell; and

enzymatically reacting the released nucleic acid with the barcoded nucleic acid.

118.   The method of claim 117, further comprising cleaving the barcoded nucleic acid
from the hydrogel beads.

119.   A method, comprising:

providing a plurality of at least about 10,000 microfluidic droplets containing cells such that no more than 10% of the droplets contains two or more cells;

lysing the cells within the plurality of droplets to release nucleic acid from the cells; and

uniquely labeling the released nucleic acid with a droplet-specific barcode, without first amplifying the released nucleic acid.

120.   A method, comprising:

providing droplets containing cells such that no more than 10% of the droplets contains two or more cells;

lysing the cells within the plurality of droplets to release nucleic acid from the cells; and

uniquely labeling the released nucleic acid with a barcode selected from a pool of at least 10,000 distinguishable barcodes, without first amplifying the released nucleic acid.

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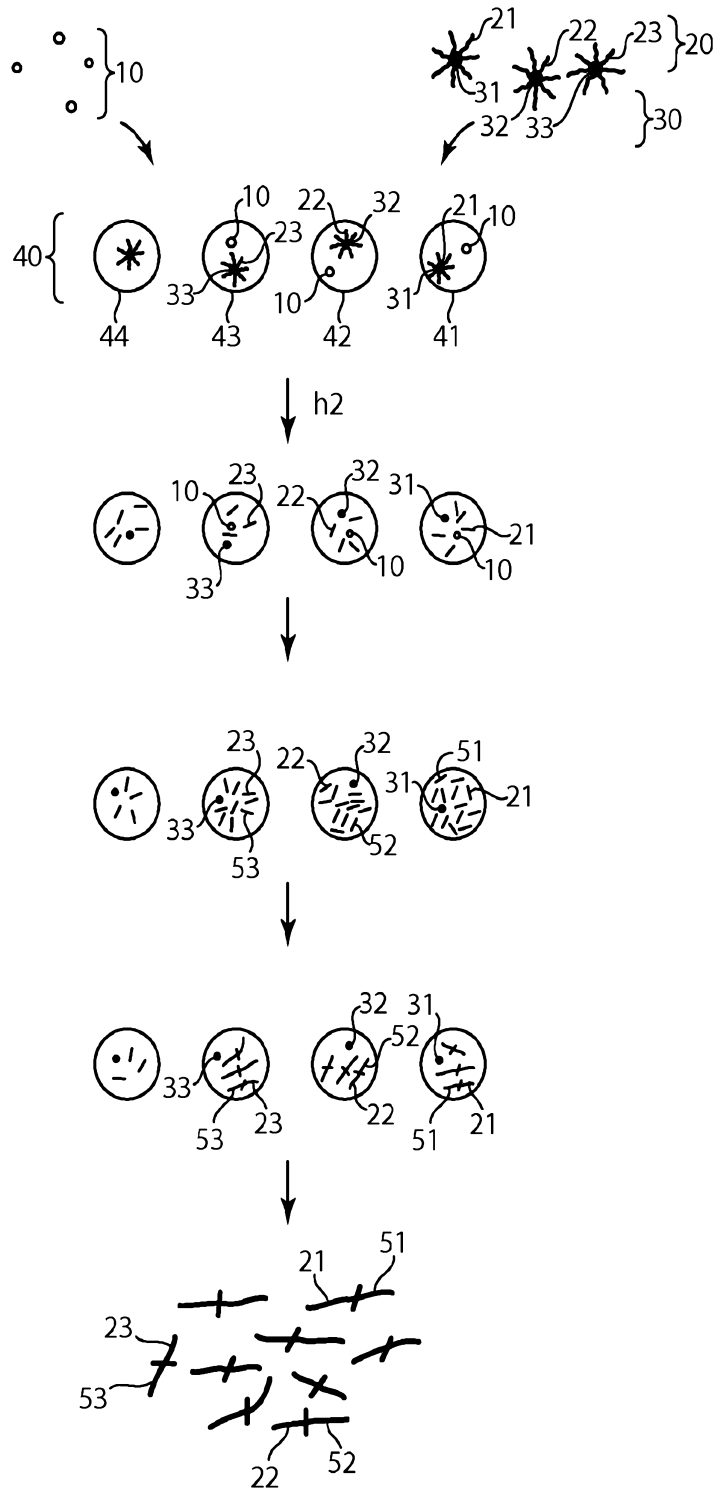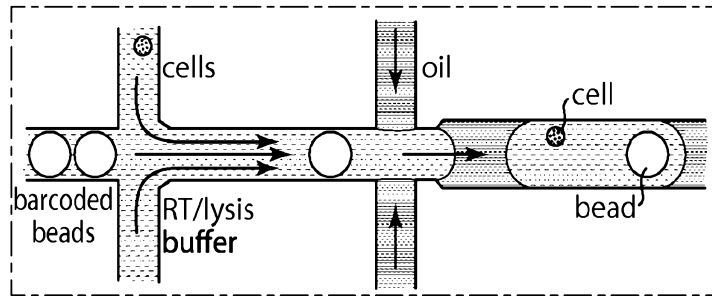
Fig. 1

Fig. 2A
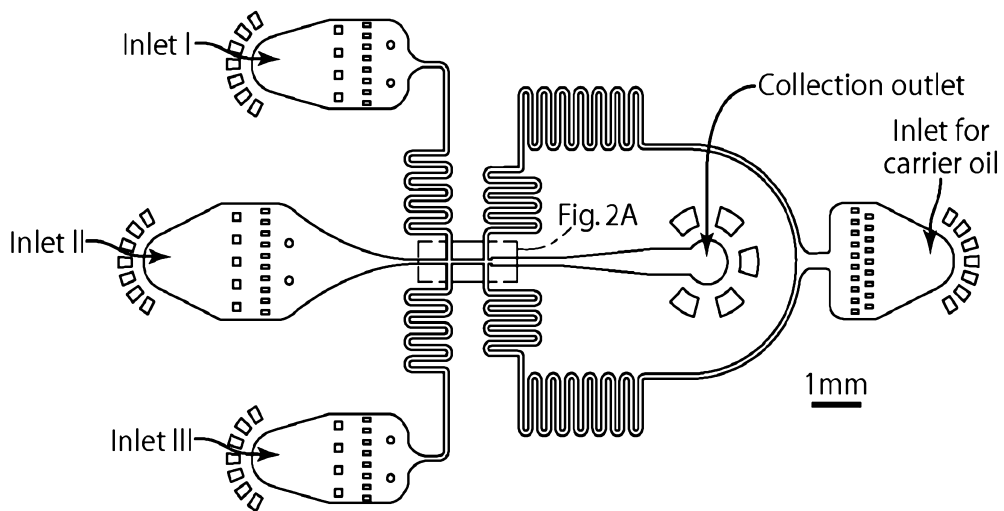
Fig. 2B

Fig. 3

Fig. 4

Fig. 5

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Fig. 6

Hydrogel
miscrosphere

ssDNA primers
attached to hydrogel mesh

Cleavable site   T7 promoter   Primer site PE1

Hybridization   Primer site PE1* barcode sequence Primer site P2*

Cleavable site   T7 promoter   Primer site PE1

Primer extension   Primer site PE1* barcode sequence Primer site P2*

Cleavable site   T7 promoter   Primer site PE1

Conversion to ss DNA

Cleavable site   T7 promoter   Primer site PE1   barcode sequence   Primer site P2

Primer site P2* barcode sequence Primer site P3*

Hybridization and Primer extension

Cleavable site   T7 promoter   Primer site PE1   barcode sequence Primer site P2

Conversion to ss DNA

Cleavable site   T7 promoter   Primer site PE1   barcode sequence Primer site P2 barcode sequence Primer site P3

Fig. 7

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Fig. 8

Fig. 9A

Fig. 9B

Fig. 10A   Fig. 10B   Fig. 10C
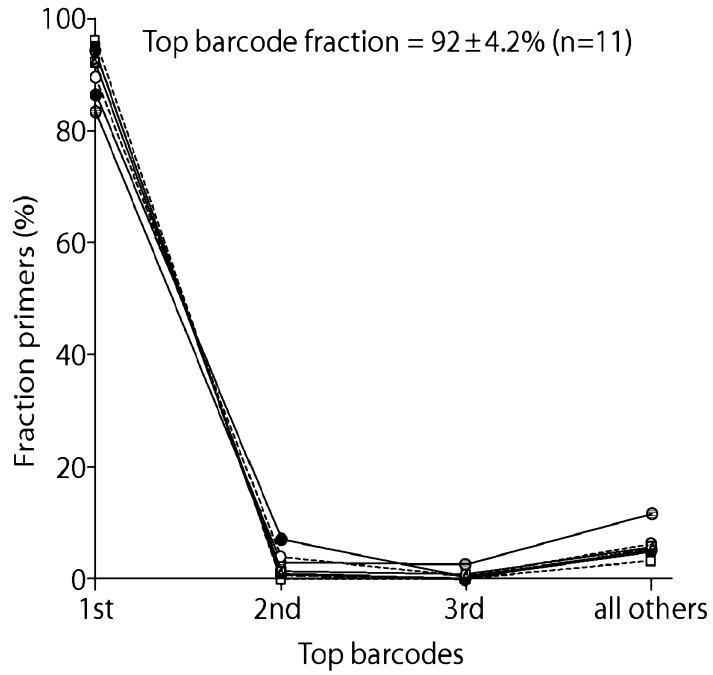
Fig. 11
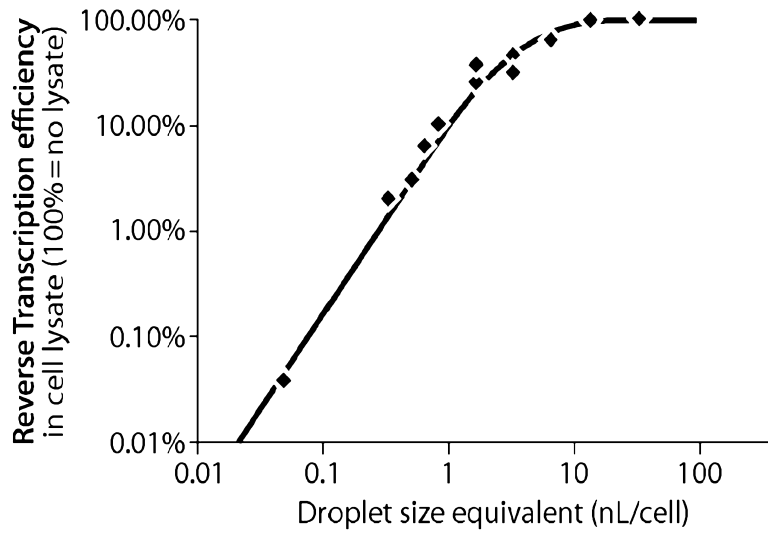
Fig. 12

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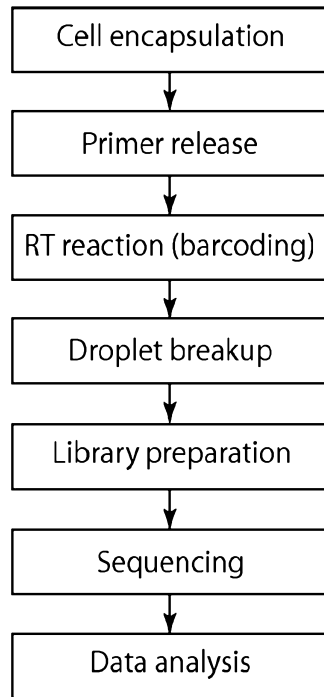
Fig. 13A
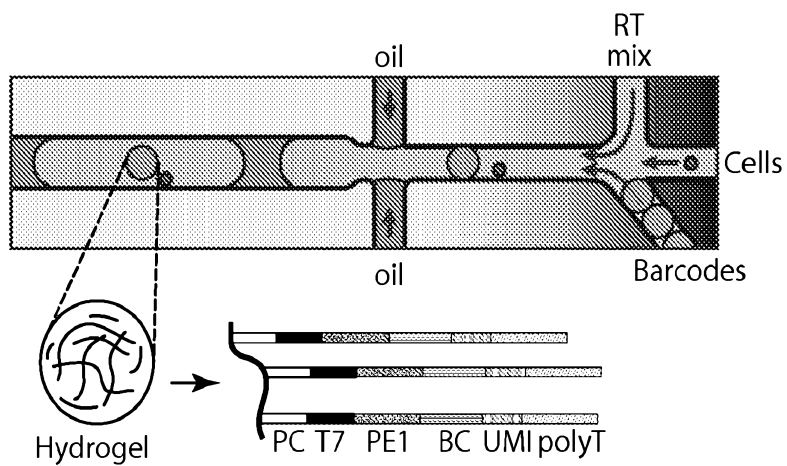
Fig. 13B
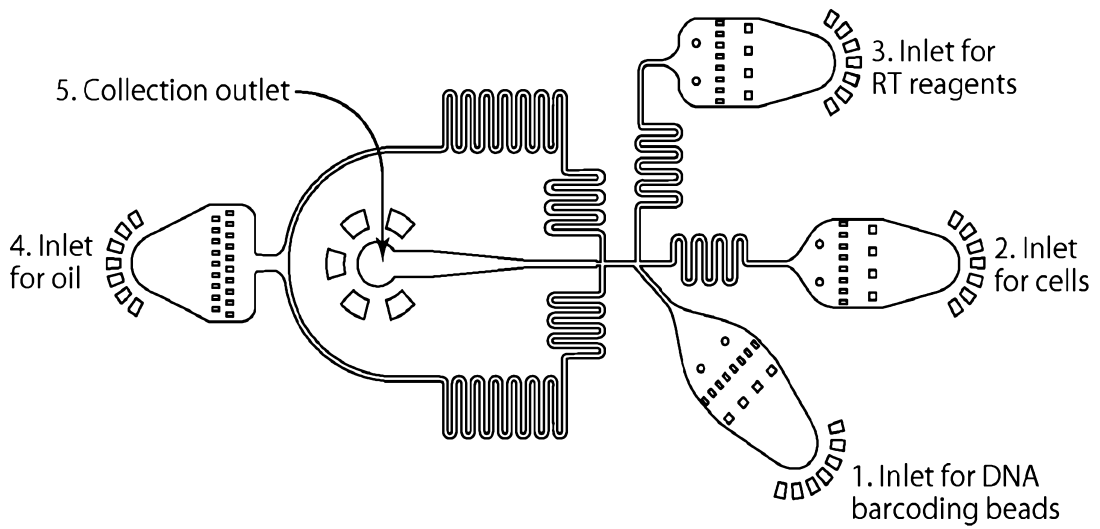
Fig. 13C
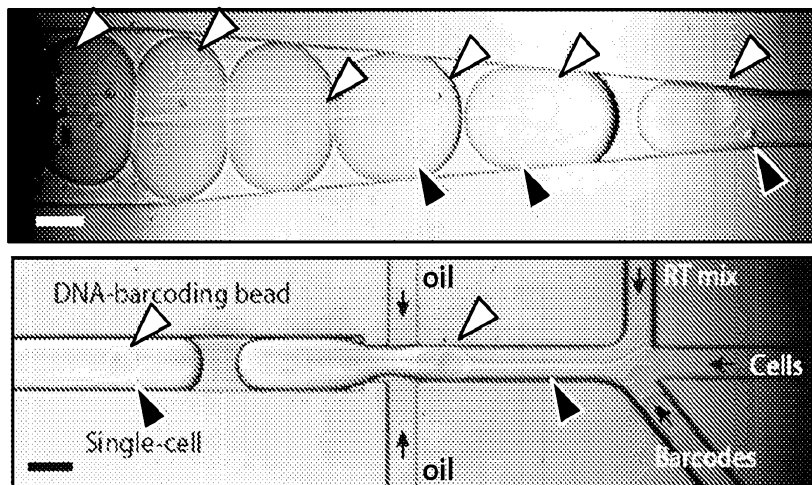
Fig. 13D
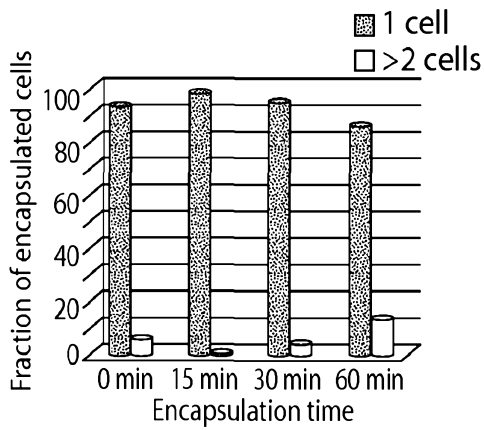
Fig. 13E
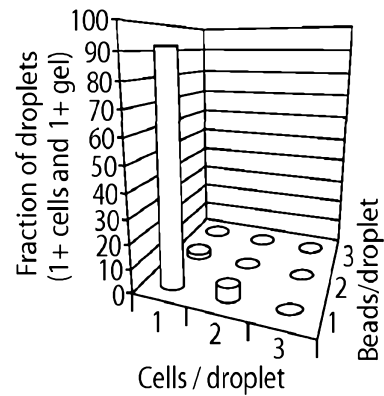
Fig. 13F
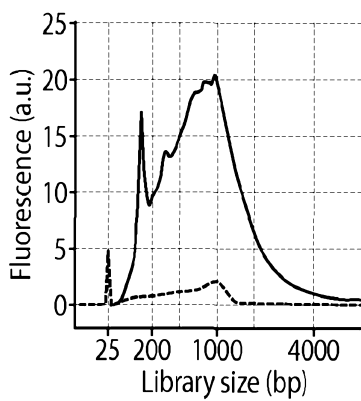
Fig. 13G
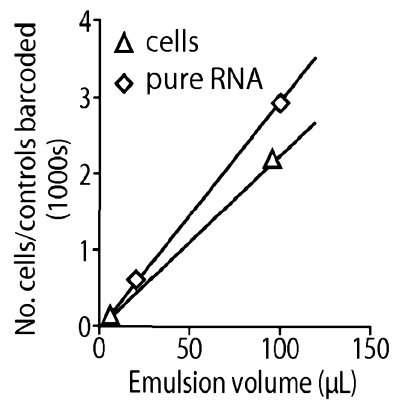
Fig. 13H
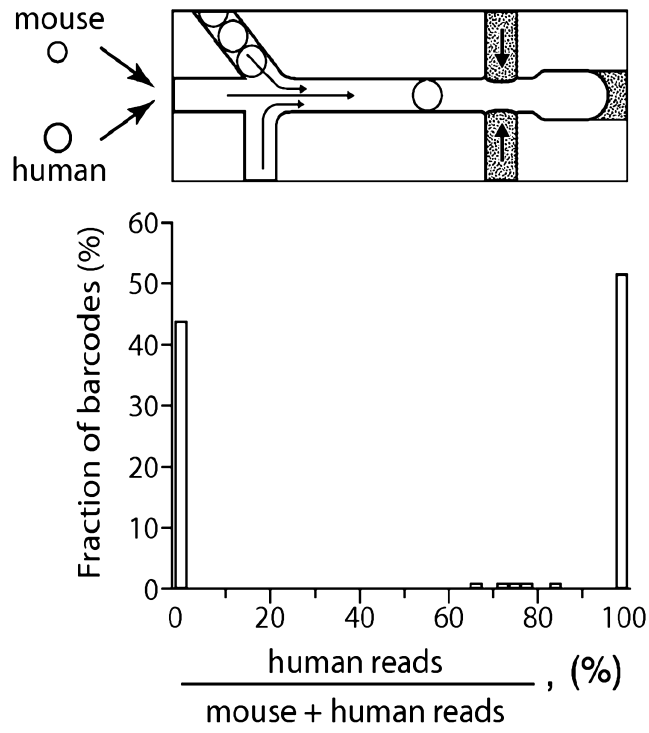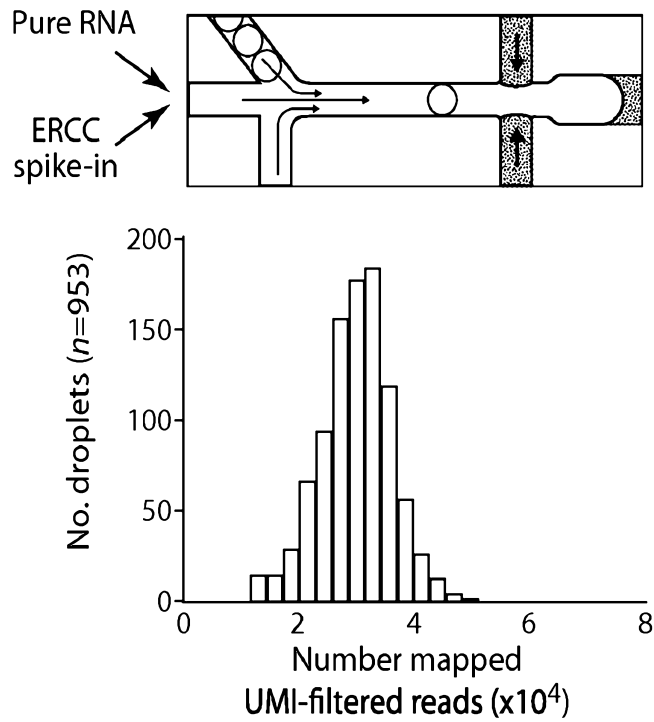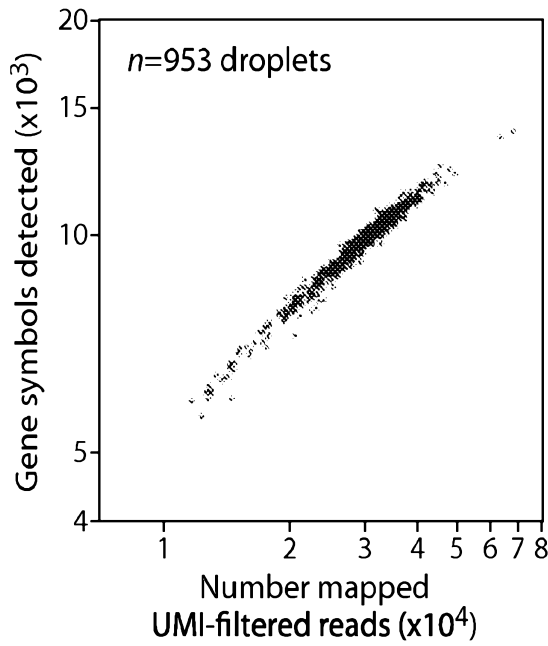
Fig. 14A

Fig. 14B

Fig. 14C
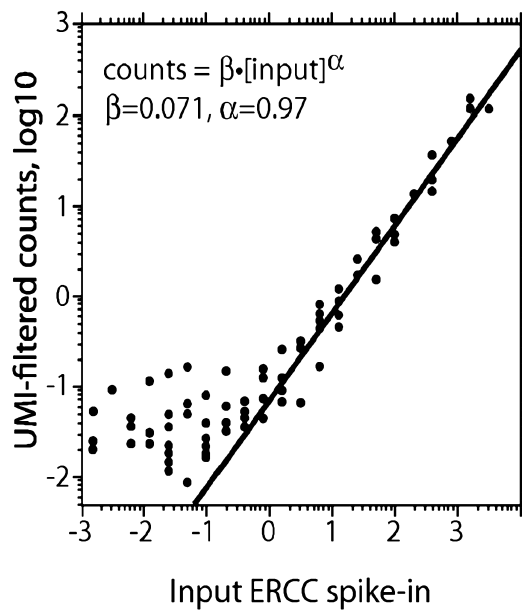
Fig. 14D

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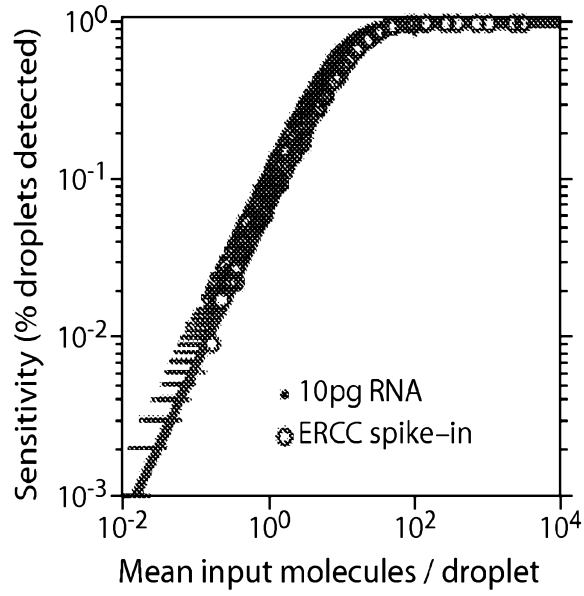
Fig. 14E
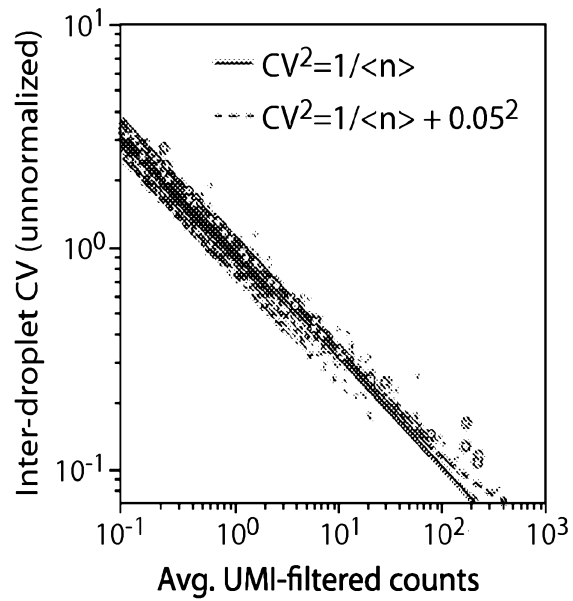
Fig. 14F

1) Above-Poisson noise:

$$\underbrace{CV^2 - \frac{1}{\mu}}_{\text{observed}} = \underbrace{\left(CV^2_{\text{bio}} - \frac{1}{\mu_{\text{bio}}}\right)}_{\text{biological variability}} \underbrace{\left(1 + CV^2_{\text{method}}\right)}_{\textbf{noise inflation}} + \underbrace{CV^2_{\text{method}}}_{\text{background}}$$

2) Fano factor:

$$F = \underbrace{\beta F_{\text{bio}} + (1-\beta)}_{\text{sampling diminution}} + \underbrace{CV^2_{\text{method}}}_{\text{background}}$$

3) Gene correlations:

$$\underbrace{\text{corr}_{i,j}}_{\text{observed}} \simeq \underbrace{\text{corr}^{(\text{bio})}_{i,j}}_{\text{biological}} \underbrace{\sqrt{\left(1 - F_i^{-1}\right)\left(1 - F_j^{-1}\right)}}_{\textbf{noise inflation}}$$

Fig. 14G

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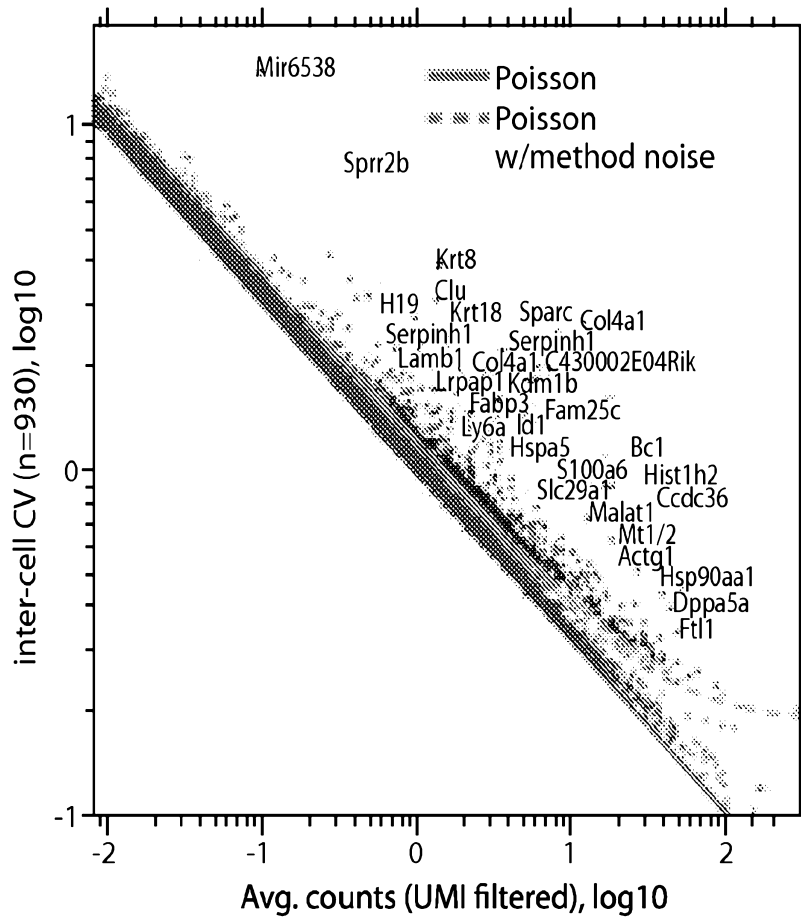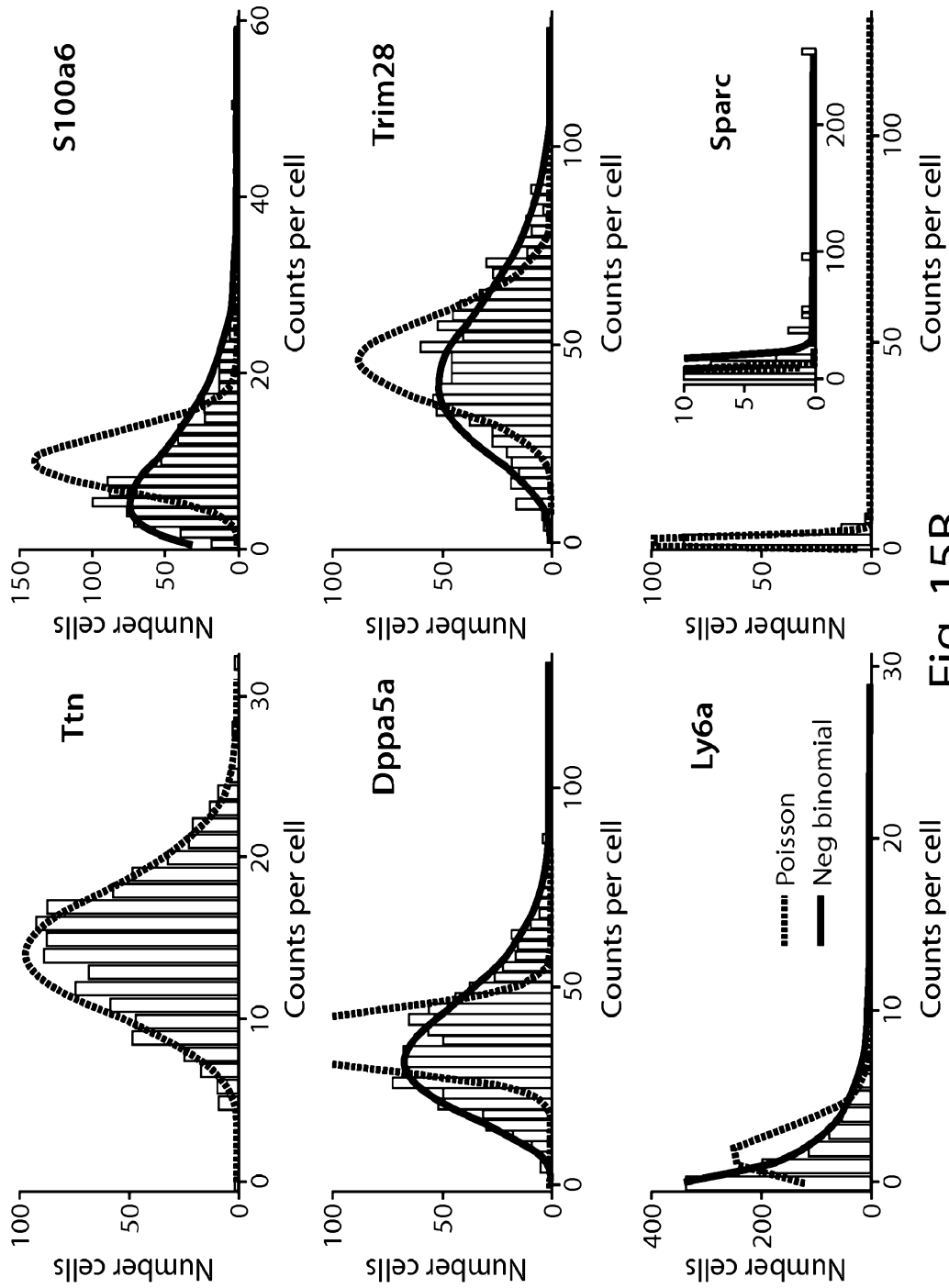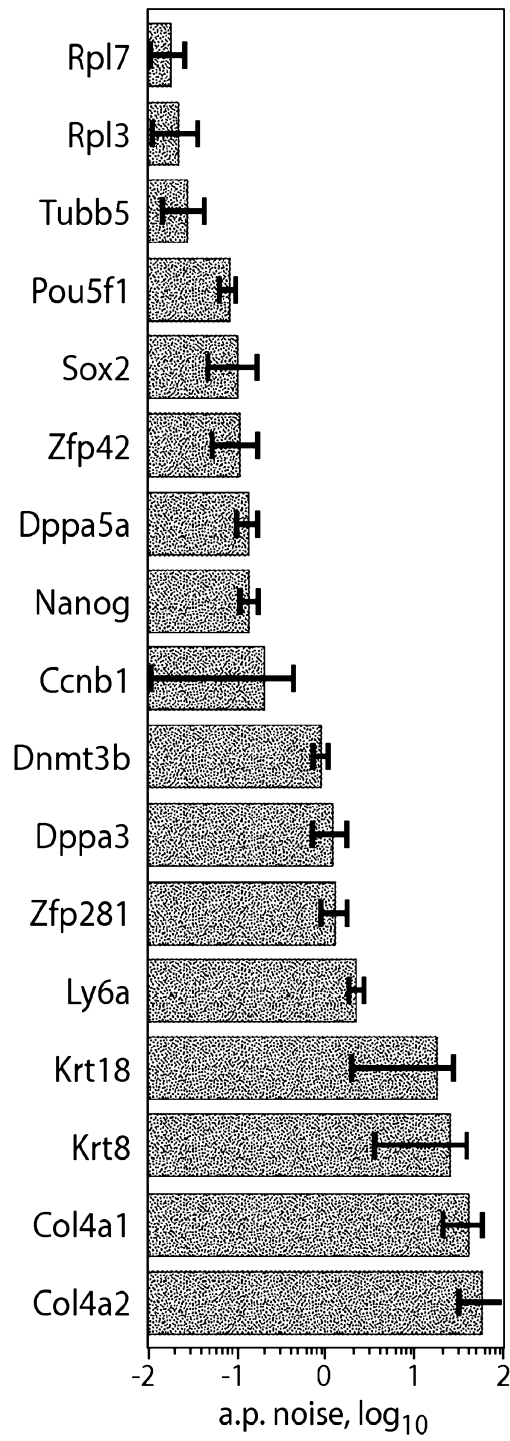
Fig. 15A

Fig. 15B
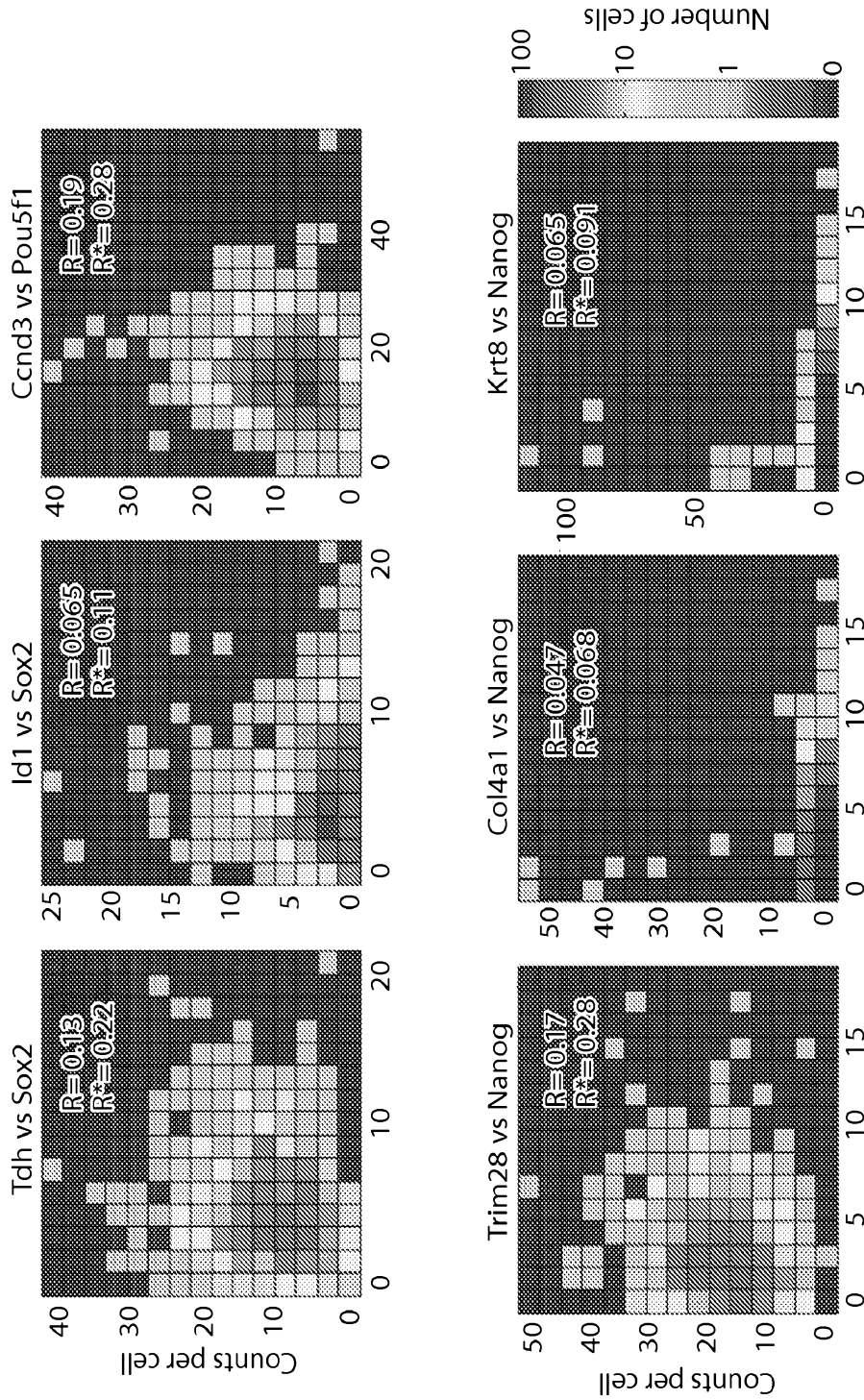
Fig. 15C
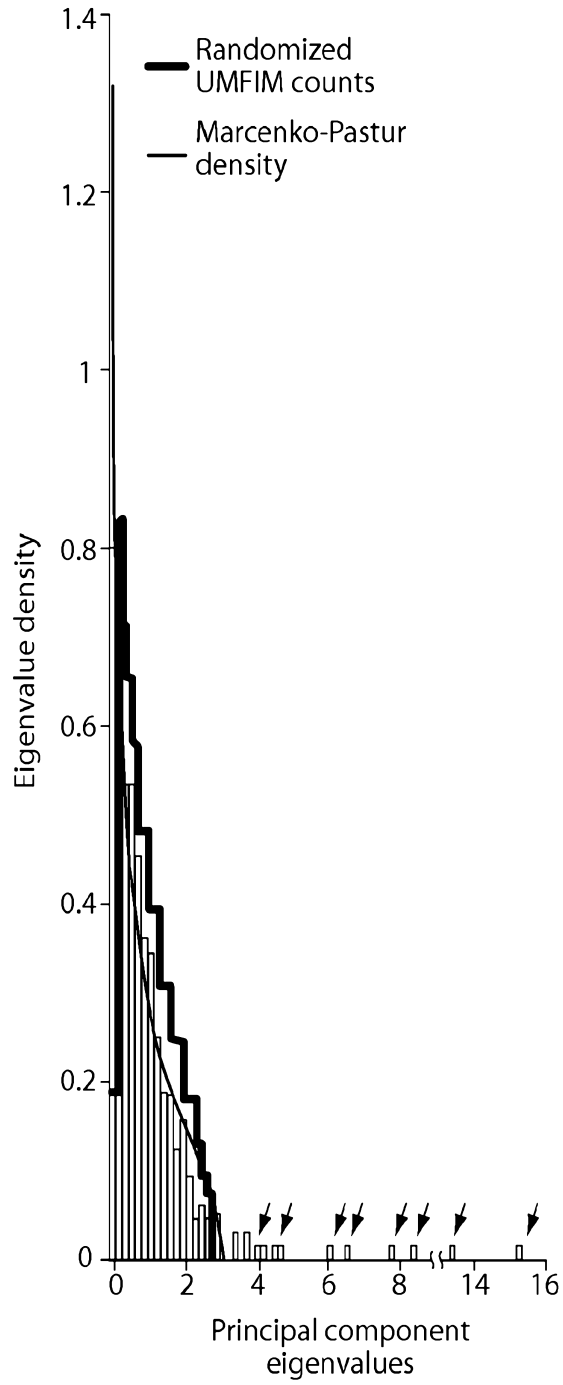
Fig. 15D

Fig. 15E
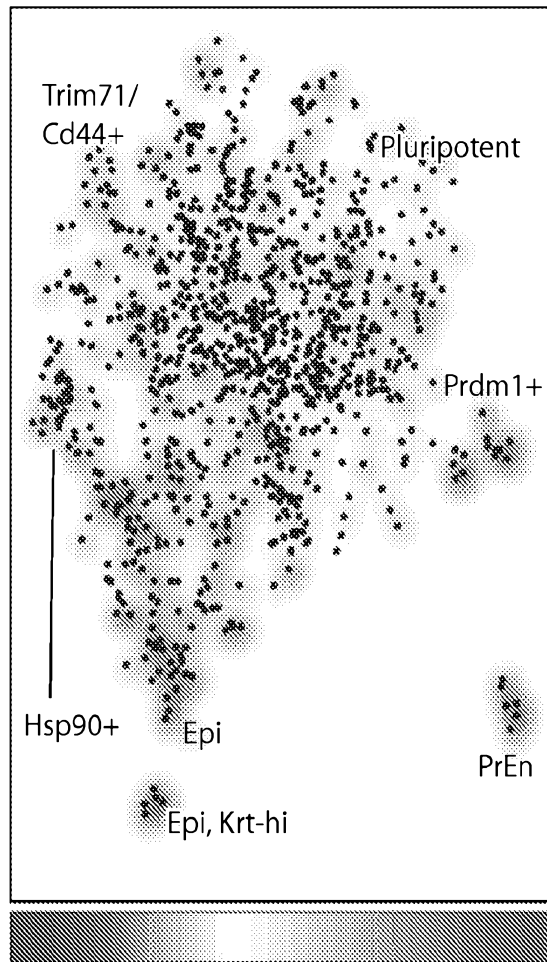
Fig. 15F

Pluripotency factor expression

Fig. 15G
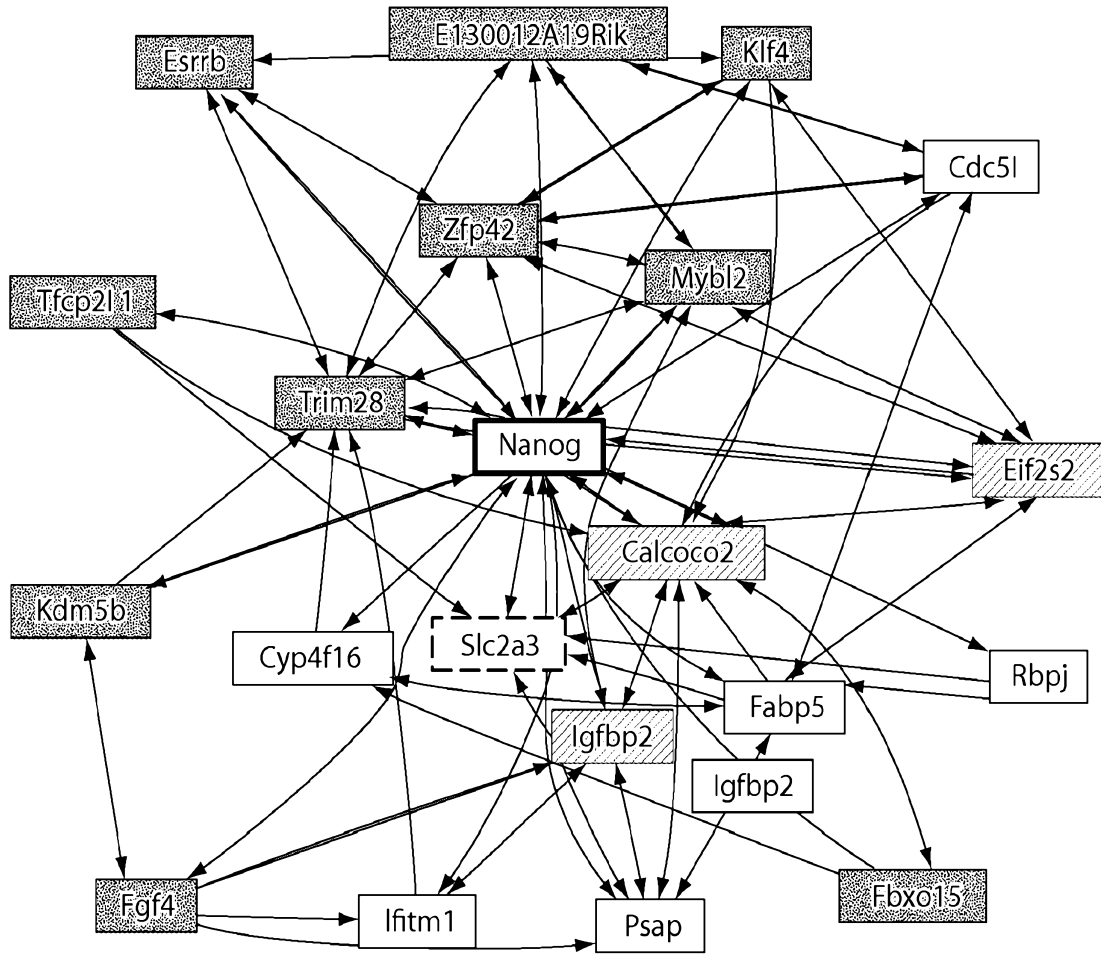
Fig. 16A
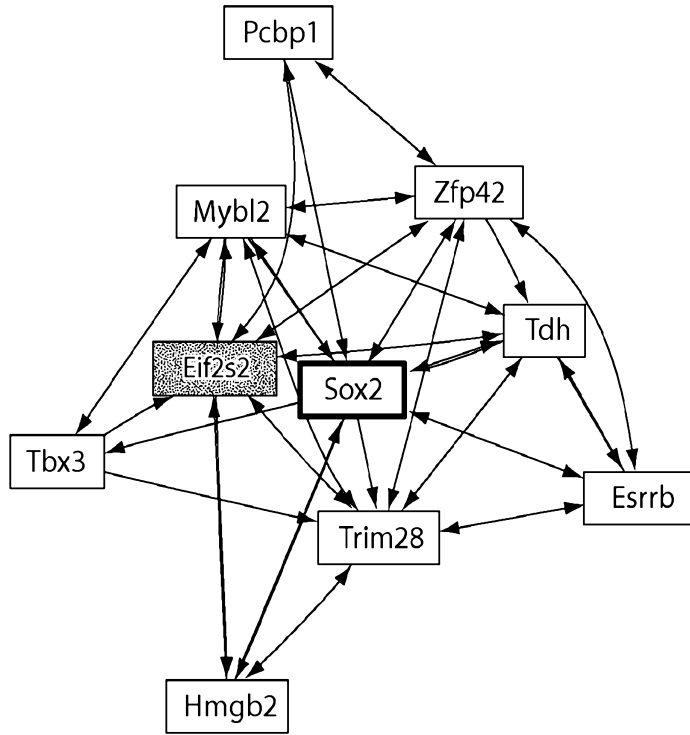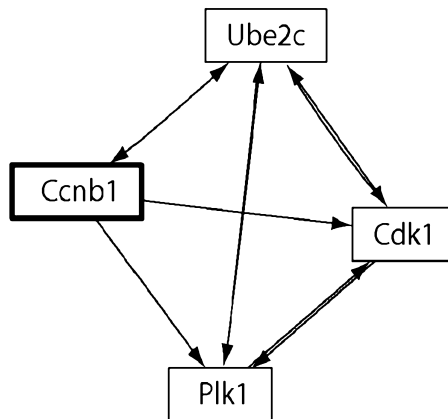
Fig. 16B

Fig. 16C

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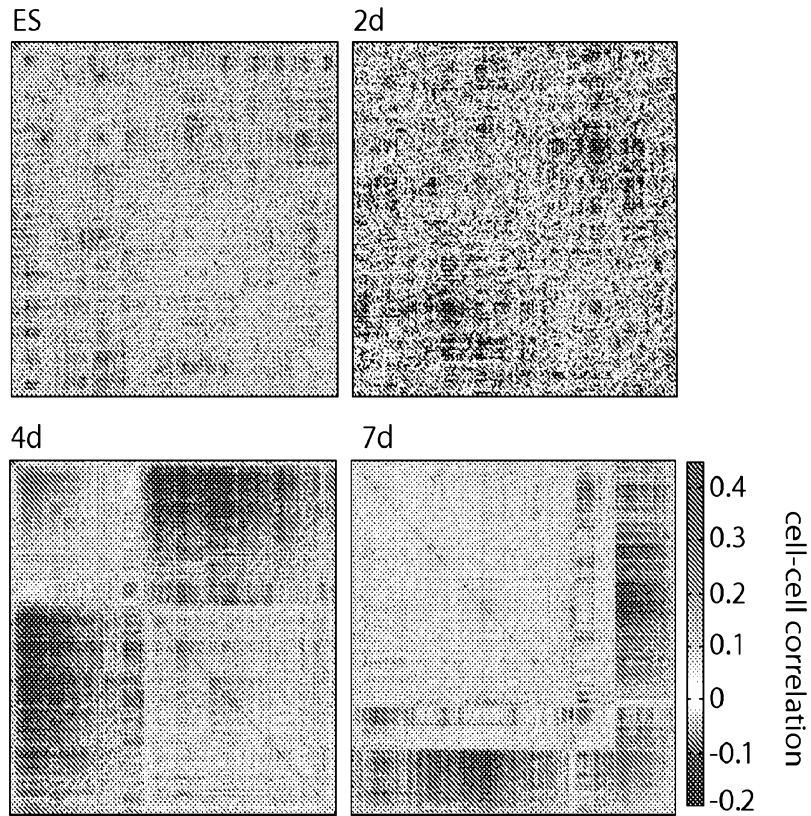
Fig. 17A
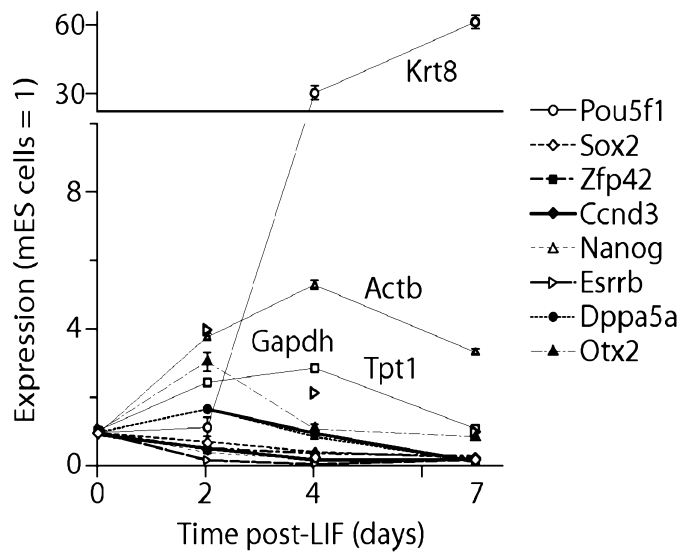
Fig. 17B
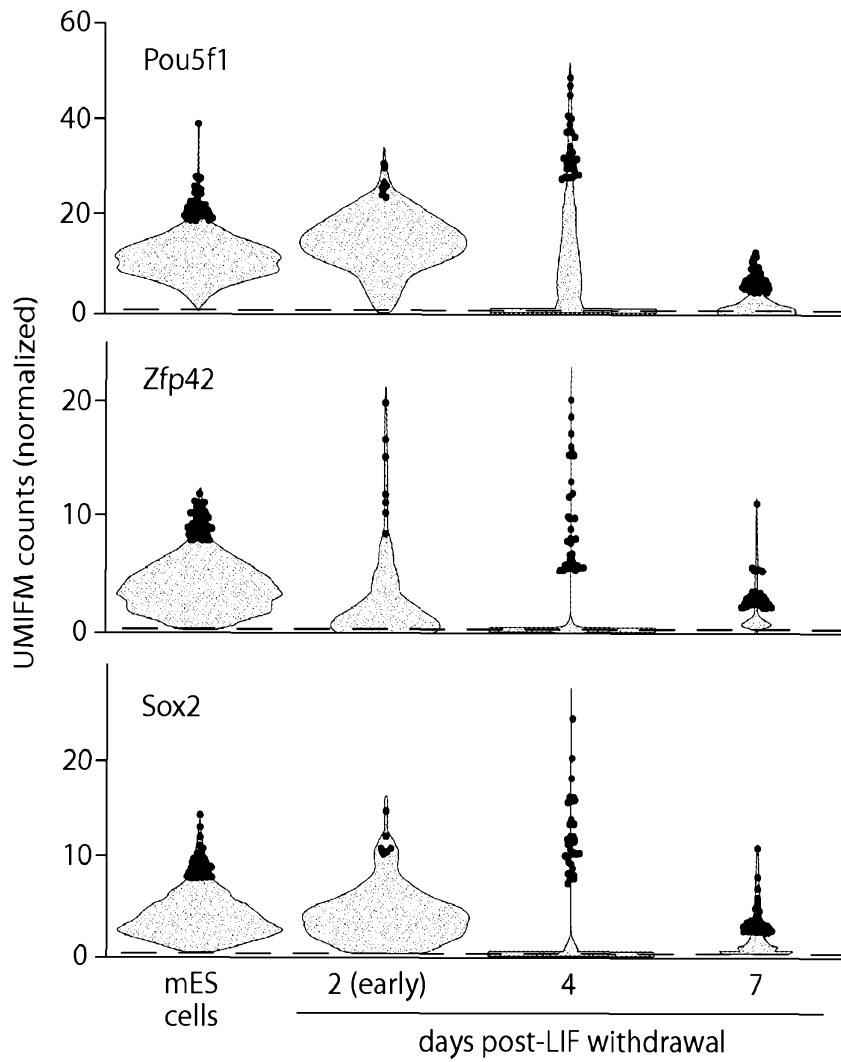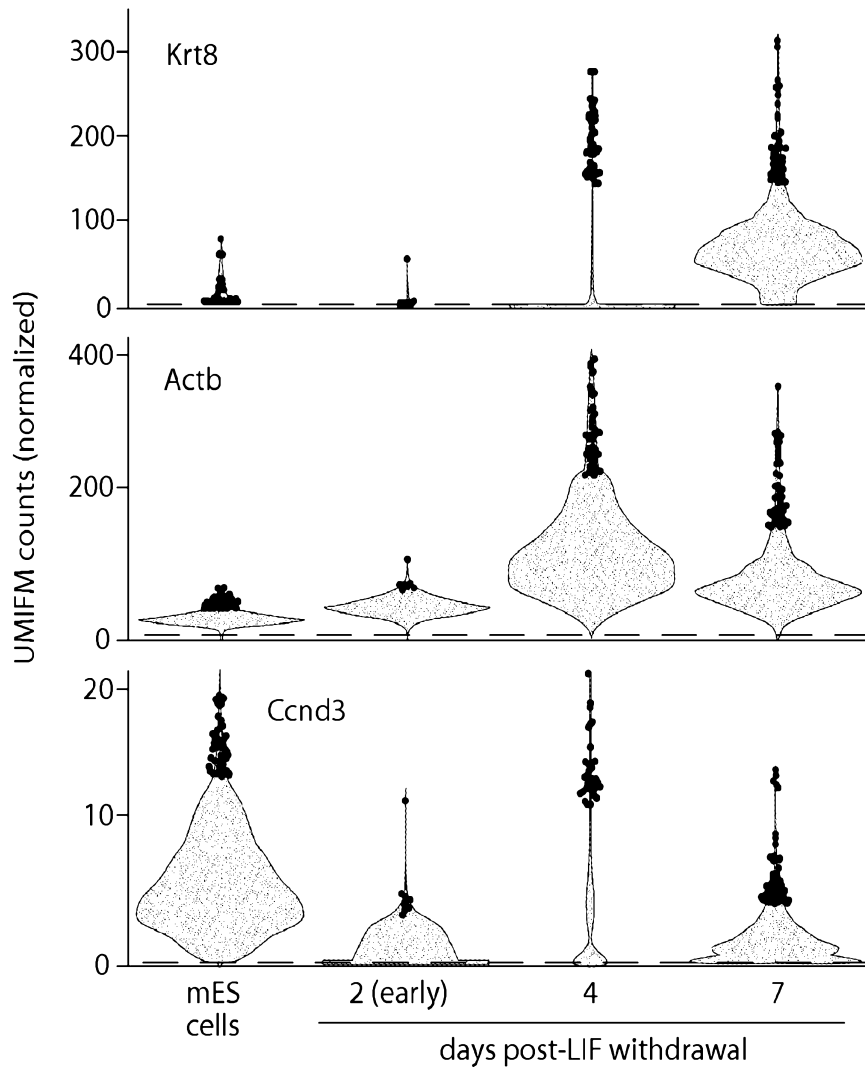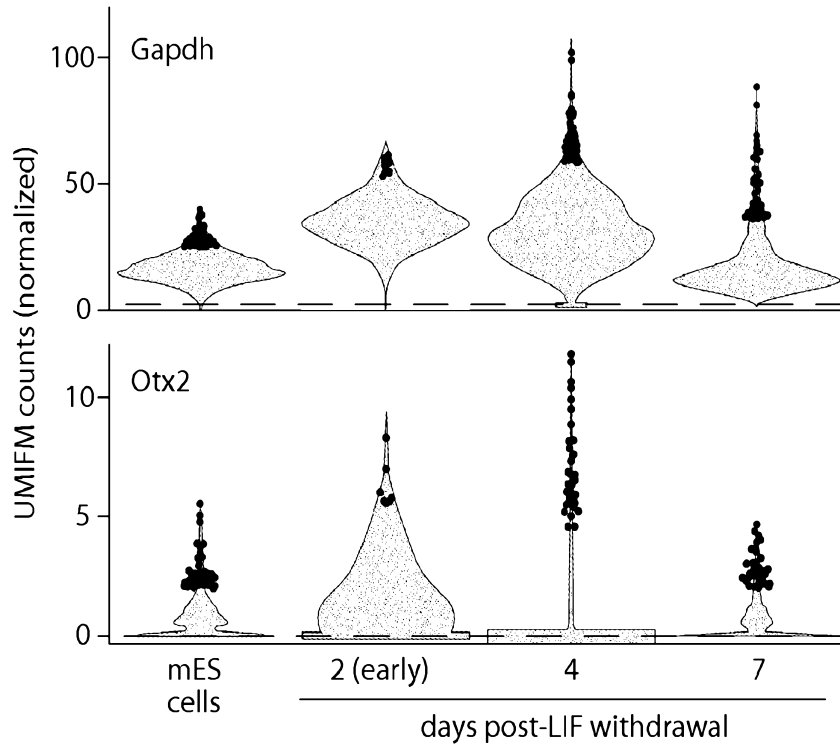
Fig. 17C-1

Fig. 17C-2

Fig. 17C-3
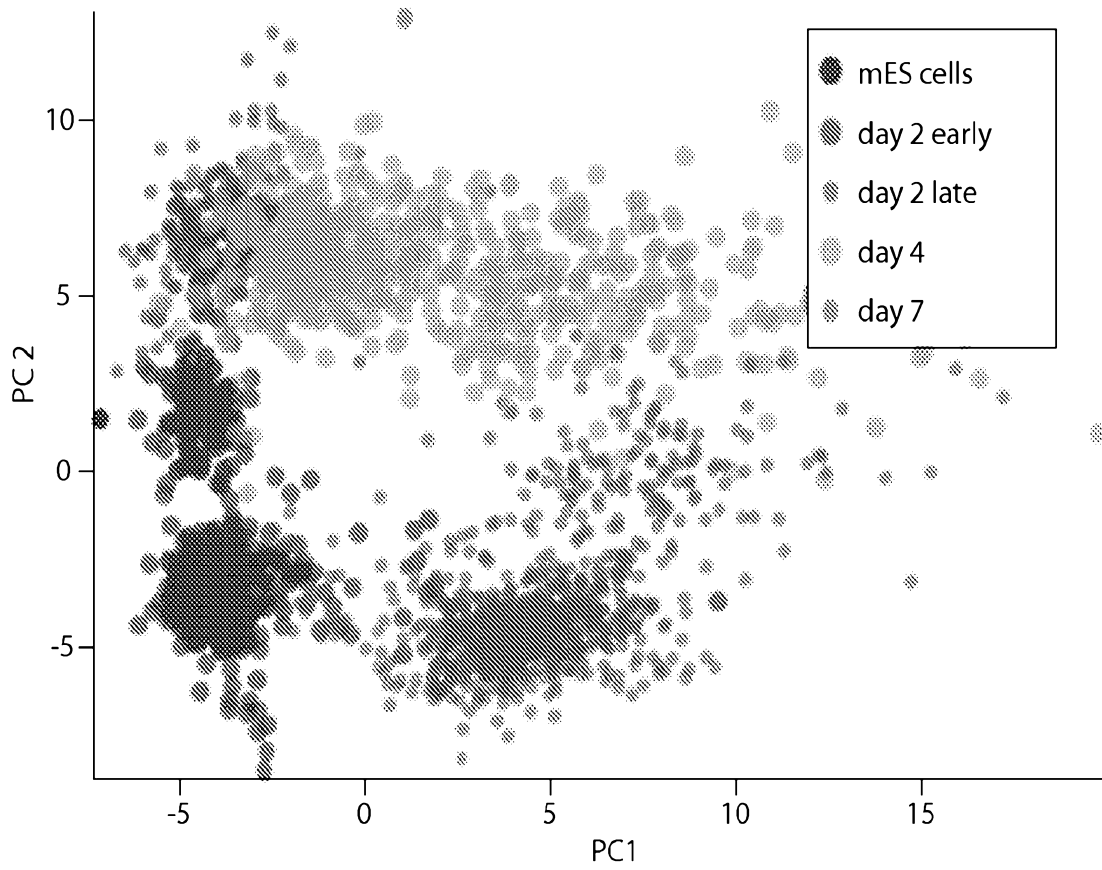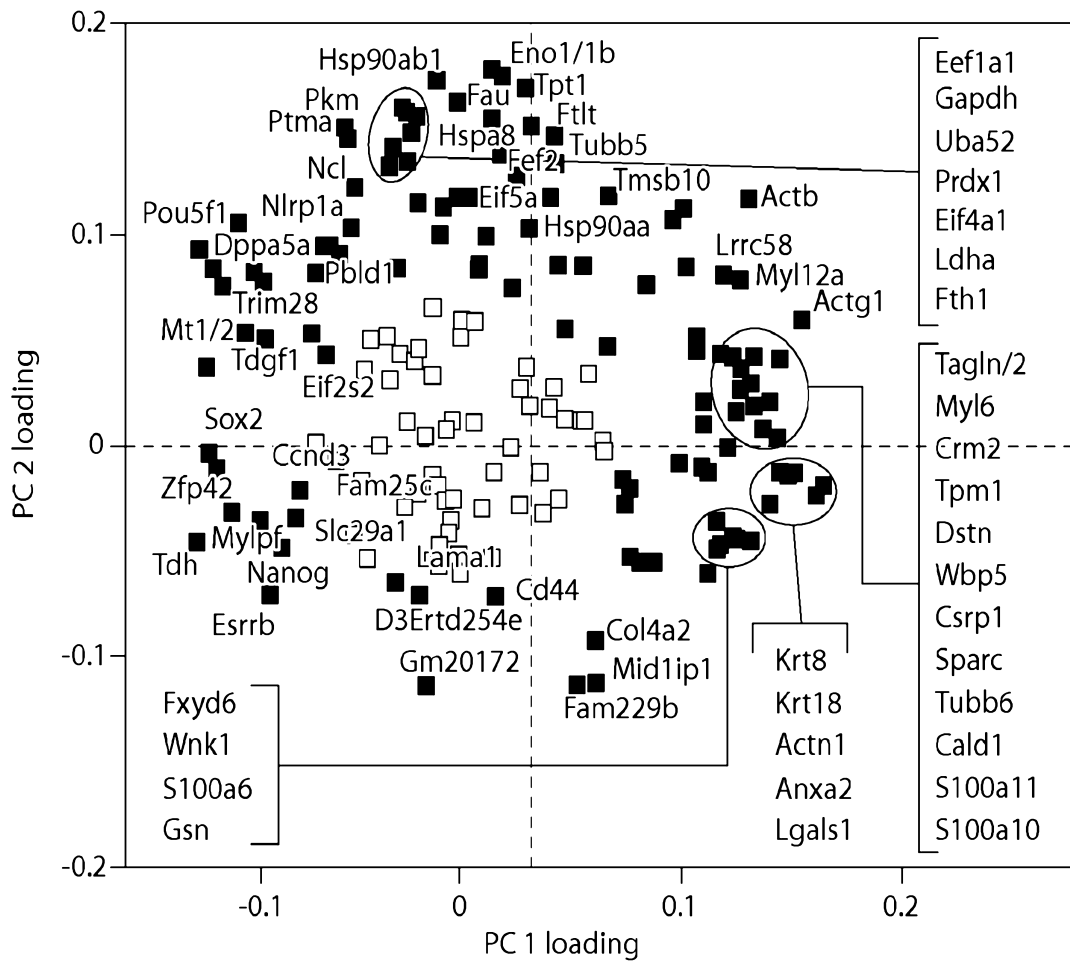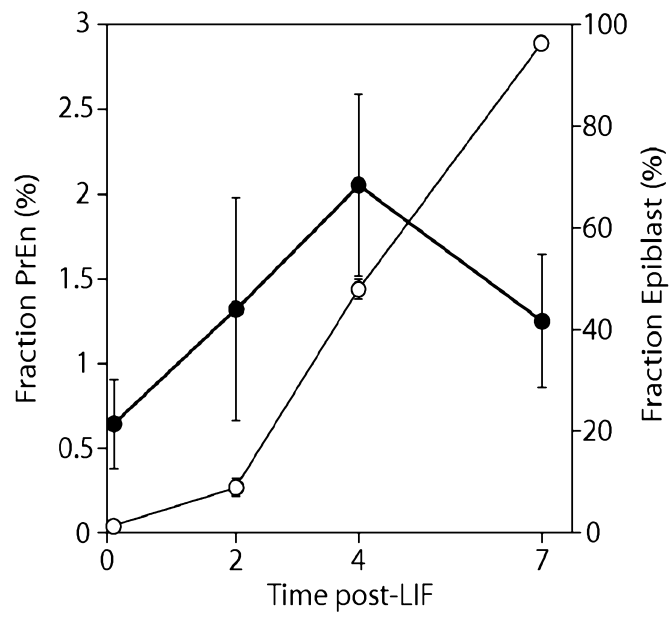
Fig. 17D

Fig. 17E

Fig. 17F
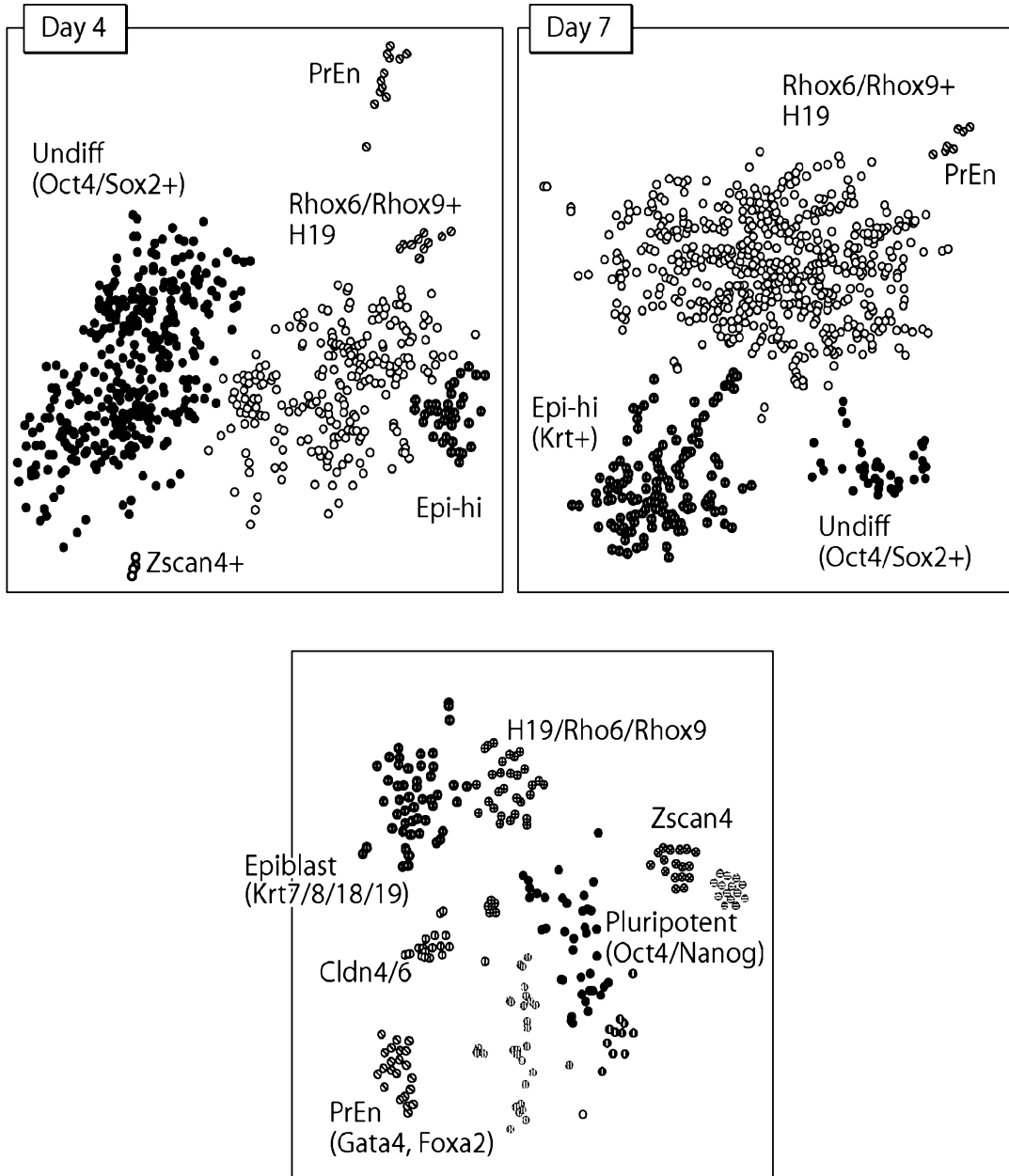
Fig. 17G
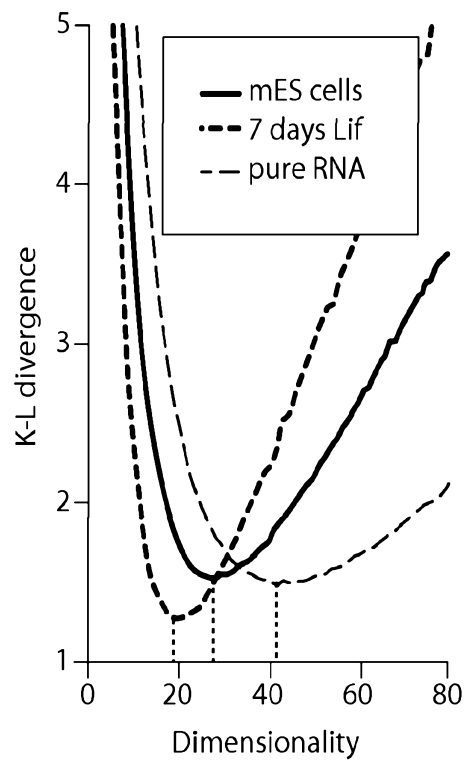
Fig. 17H

Fig. 18

Fig. 19A

Fig. 19B

CGATGACGTAATACCGACTCACYAYAGGGATACCACCATGGCTCTTTCCCTACACGACGCTCTTC-3'

Acrylic
phosphoroamidite   Photo-cleavable   TZ promoter   PE1 primer site
moiety   spacer

Fig. 20A

Hydrogel bead   ssDNA primers attached to hydrogel mesh

Photo-cleavable   T7 promoter   PE1 Primer site
spacer

Hybridization

PE1* Primer site   barcode 1   W1* site

Photo-cleavable   T7 promoter   PE1 Primer site
spacer

Primer extension

PE1* Primer site   barcode 1   W1* site

Photo-cleavable   T7 promoter   PE1 Primer site
spacer

Conversion to ss DNA

Photo-cleavable   T7 promoter   PE1 Primer site   barcode 1   W1 site
spacer

Hybridization and
Primer extension

W1* site   barcode 2   UMI*   poly-A

Photo-cleavable   T7 promoter   PE1 Primer site   barcode 1   W1 site
spacer

Conversion to ss DNA

Photo-cleavable   T7 promoter   PE1 Primer site   barcode 1   W1 site   barcode 2   UMI   poly-T
spacer

Fig. 20B

5'-/5Acryd//iSpPC/CGATGACG TAATACGACTCACTATAGGG ATACCACCATGG CTCTTTCCCTACACGACGCTCTTC
CGATCT barcode1 AAGGCGTCACAAGCAATCACTC barcode2 NNNNNN TTTTTTTTTTTTTTTTTTTTV -3'

Fig. 20C
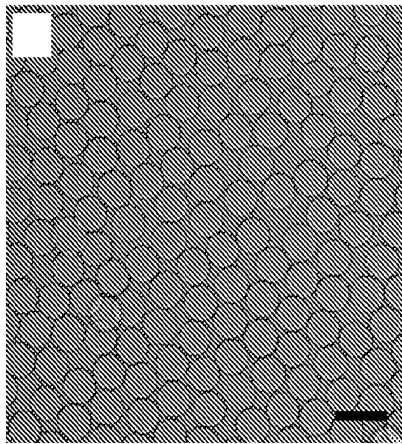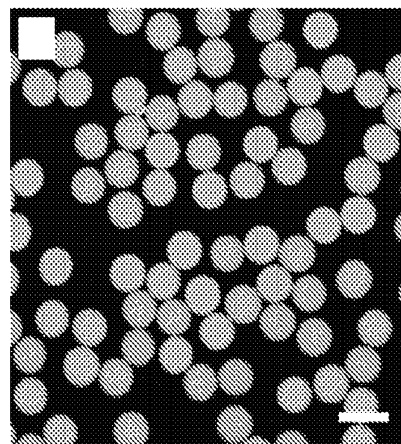
Fig. 21A

Fig. 21B
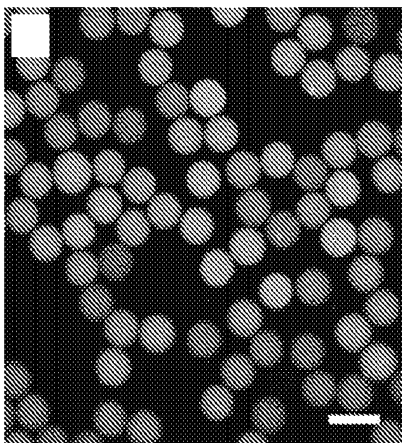
Fig. 21C

Fig. 21D

Fig. 21E

Top barcode fraction = 92±4.2% (n=11)
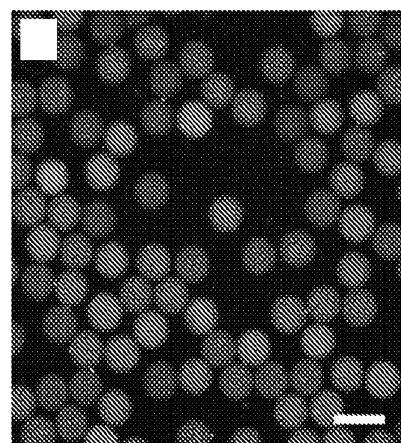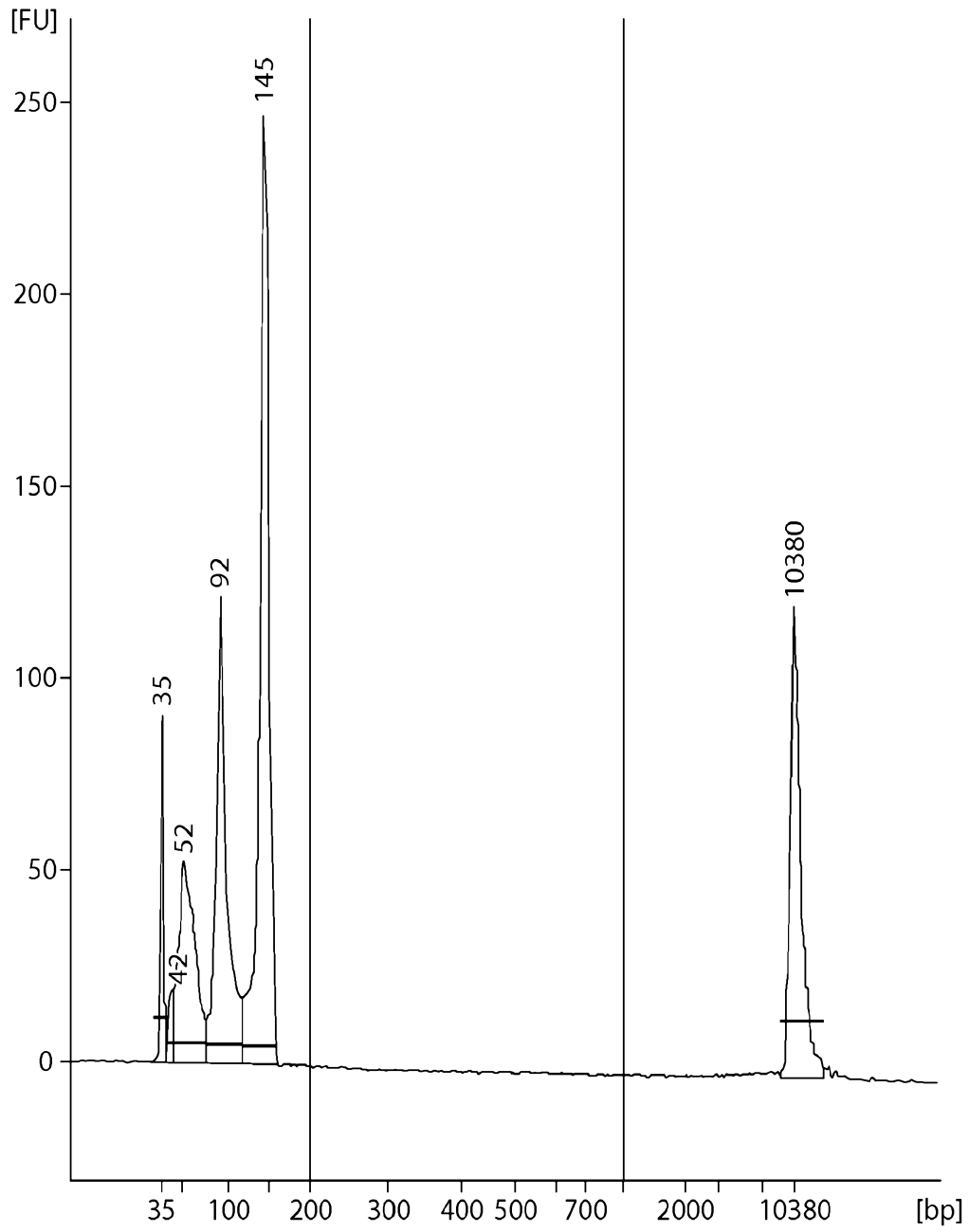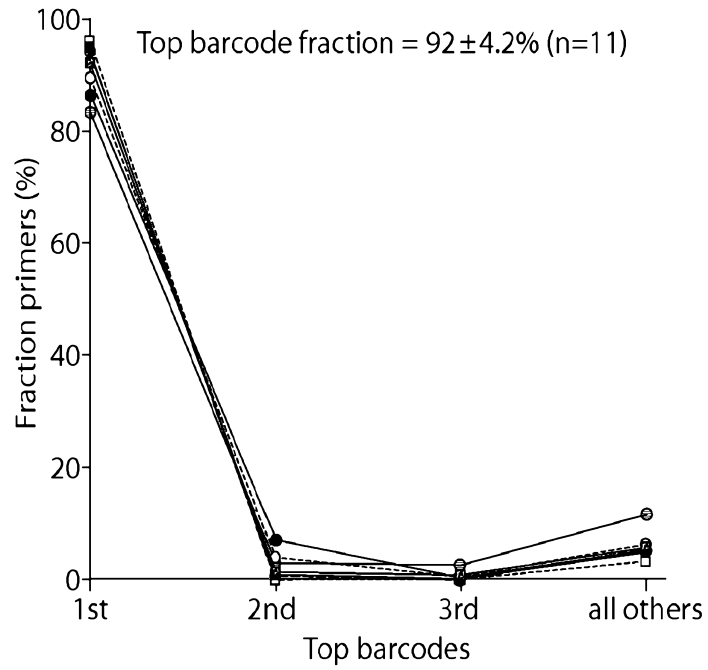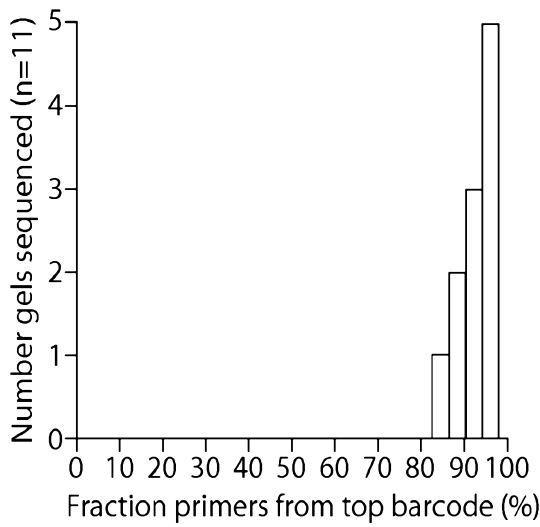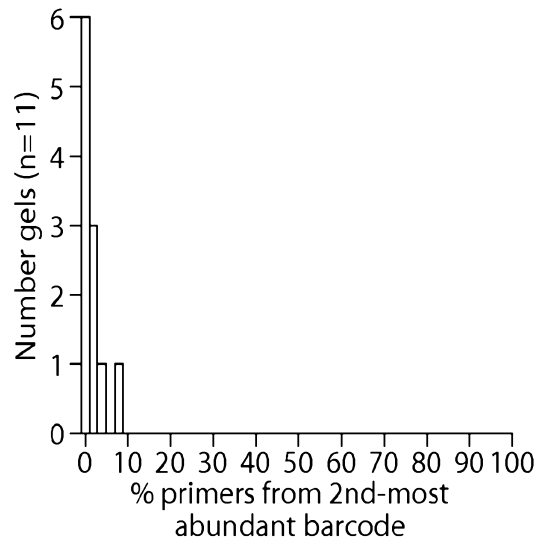
Fig. 21F

Fig. 21G

Fig. 21H

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Number
cells/controls   Number shared barcodes: observed/expected
barcoded

140	1/0.6	1/0.9	0/0.9	1/0.9	3/2.1	3/2.3	2/2.8
0.88	608	2/3.8	4/3.9	7/3.9	9/8.9	17/10	14/12.1
0.83	0.95	921	7/5.9	5/5.9	22/13.5	26/15.1	20/18.3
0.81	0.91	0.92	945	8/6.1	12/13.9	15/15.5	24/18.8
0.81	0.89	0.91	0.82	951	11/14	17/15.6	23/18.9
0.68	0.92	0.98	0.95	0.86	2,159	36/35.6	47/43.1
0.81	0.92	0.89	0.97	0.99	0.99	2,420	70/48.1
0.94	0.87	0.93	0.98	0.95	0.93	0.93	2,930

p-value random 384² barcoding
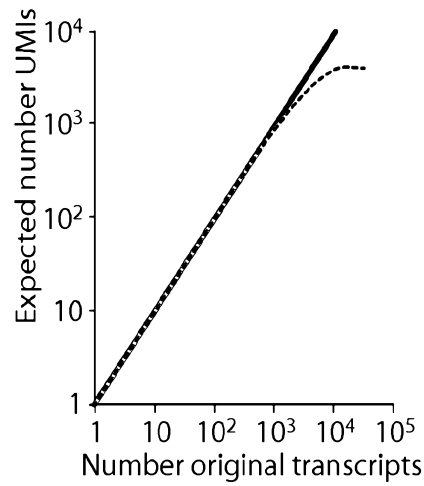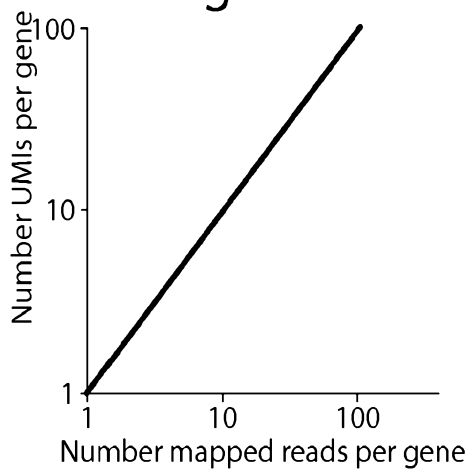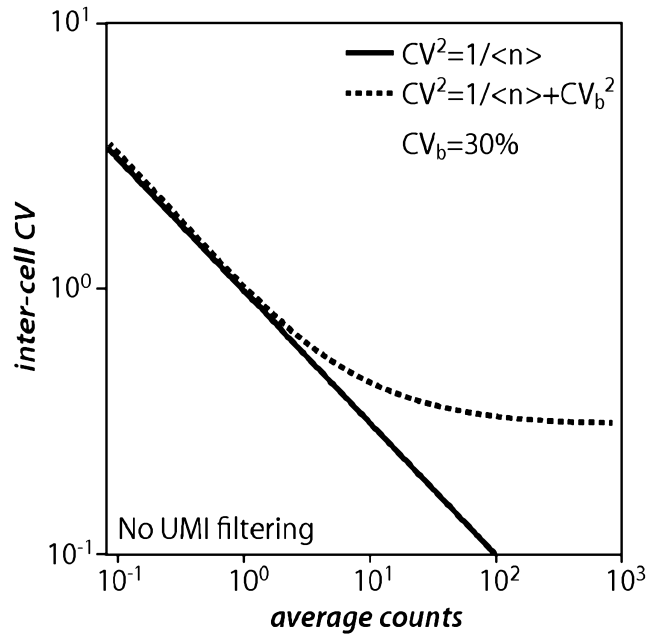
Fig.22A

Fig.22B

Fig.22C

Fig.22D
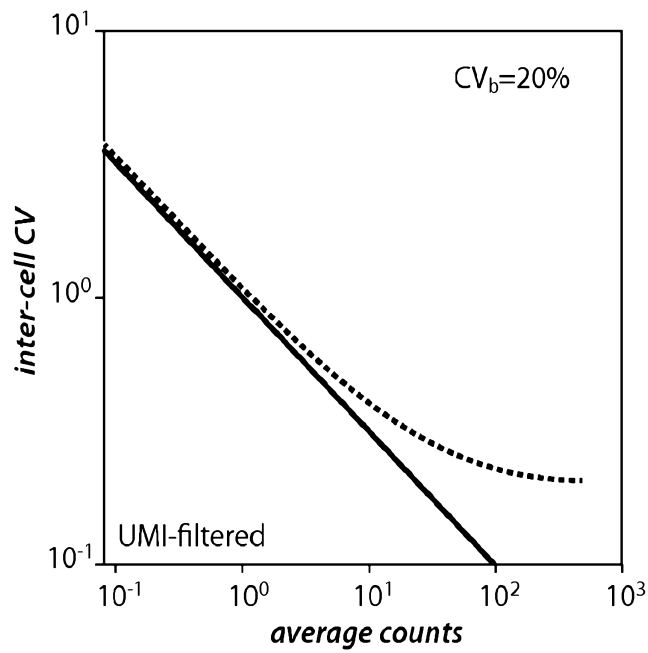
Fig.22E
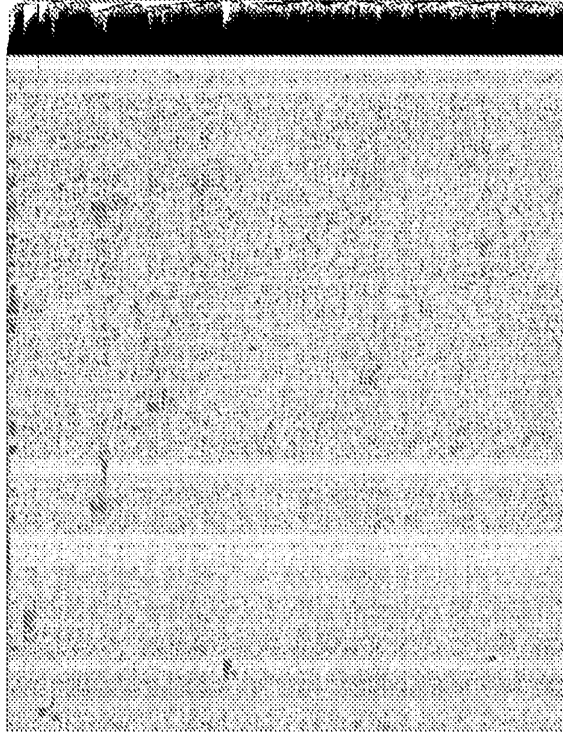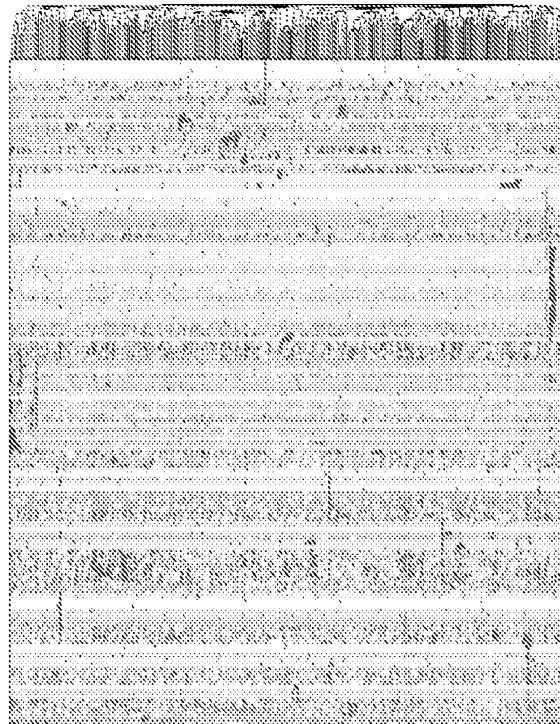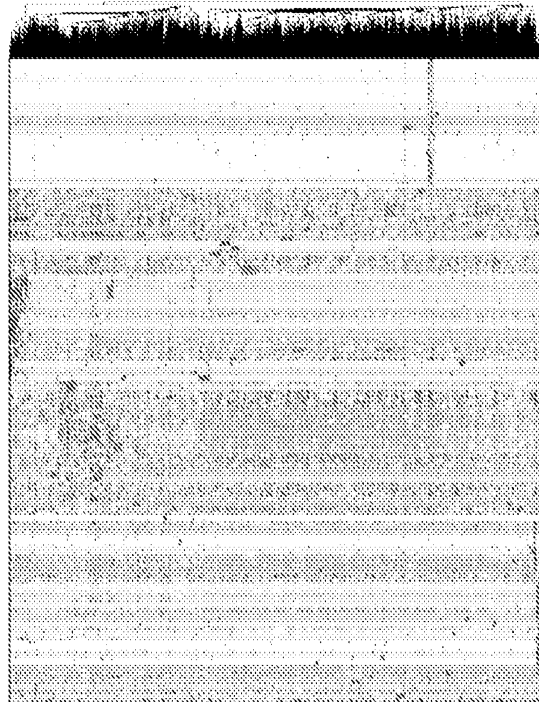
Fig. 23A
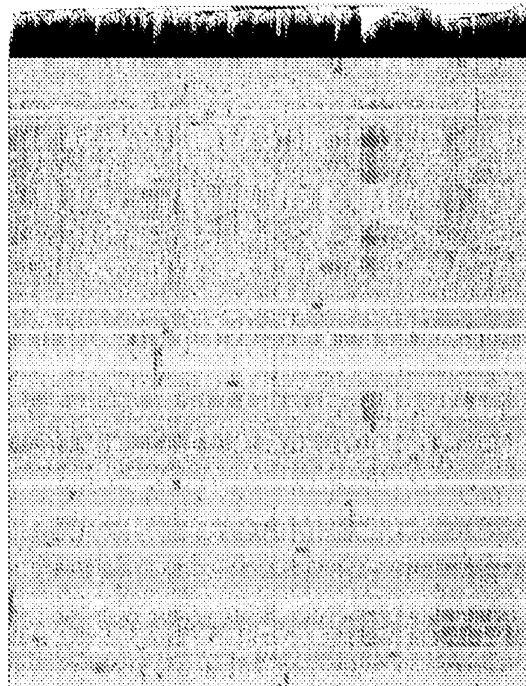
Fig. 23B

Fig. 23C

Fig. 23D
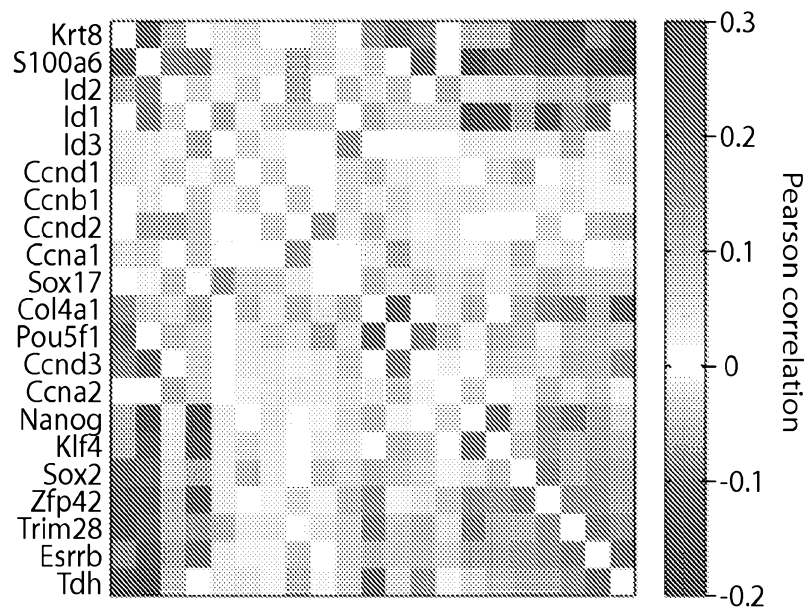
Fig. 24A

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Fig. 24B

Fig. 24C

Relative expression

Min   Max

Fig. 24D

Relative expression

Min   Max

Fig. 24E

Hsp90-correlated
HSP90b1/Hspa5/Manf/Pdia6/Calr/Hyou1/Creld2

Relative expression

Min   Max

Fig. 24F

Lin41/Trim7-correlated
Trim71/Cd44/Sbk1/Cep83/Med10

Relative expression

Min   Max

Fig. 24G

Ahnak   Cnn2
Tubb6   Tagln/2
Krt19   Fxyd6
Fn1   Tpm1
Flnb/c   Anxa3
Itga3   Igals1
S100a6   Tnfrsf12a
Tmsb4x   Krt8/18/19a
Wnk1 — Csrp1

Dusp14   Cnn1
Tgm2   Ngfrap1
Cald1   Bex1
Arl4c   Acsl6
Snx9   Lrrc58
Sfn   Acta1/b/c1/g1/g2/n1
Cgn   Dstn
Gsn   Wbp5
Anxa2

Kctd11
Nes
Ldn1

Hspb1
Dusp9
Krt/

H19/Igf2   Gata2
Rhox6/9   Zbtb10
Peg10   Phlda2
Cdkn1c — Ascl2

Sct   Arpc1b
Irx3   Tfap2c
Pfger3   Gjb3/5
Fxs1   Flnb1
Nrk   Sin3b
Hmgn5   Ldoc1
Psap   Plac1
  Basp1
  Hic2
  G630071F17Rik
  A530013C23Rik
  A3830417A13Rik

Hmga1•Sox2
Zfp42
Dppa5a
Esrrb
Serpina4-ps1
Prdm1
Heyl   Cox7a1
5730488B01Rik

Calcoco2
Klhl21
Klf2
Mylpf
Fgd1
Cpx4
Col18a1
Gm6644

Tmem92
Ccno
Zscan4a/b/c/d/f
Lgals4
Lgals6
Wdr92
Gm2016
Gm5039
Gm2022
Gm11544

Ddit4l
Tcstv1/3
Dcdc2c
Usp17la
Fbxo15
Gm/102
Gm8994
Gm20767
2310039L15Rik
B020031M1/Rik

Calcrl   Slc2a3
Car4   Igfbp2
Ctr9   Lrrc15
Pigp   Ifitm1
Apol11b   Gm6083

Crct1   Ddit
Trib3   Fem1b
Bbc3   Cldn4
Smgc   Cldn6
Mxd3   Hspb8
Klf4   Srrm4
Cmtm3   Sqstm1
Izumo1

9230116N13Rik
4930515L19Rik

Klhl7   Nanog   Ptma
Acsf2   Pou5f1   Tdgf1
Ppm1k   Trim28   Ranbp1
Glod4   Tpt1   Mgl2
Mtfmt   Mkrn1   Mi
Mfsd11   Mroh5   Dnmt3bf
Gm10941   Fabp5   Nlrp1a
Vmn2r   Ube2s   Gm5712
  Npm1   Gm5860
  4930567   K20Rik

Ark1b3

Eno1/1b

Snurf/Snrpn

Gata4   Fabp3
Col4a1/2   Cd63
Glul   Chn2
Glipr1   Serpinh1
Ctsl   Sparc
Foxq1   Lamc1
Ctsh   Lama1
Dab2   Lamb1
Lgmn   Foxa2

Lce1l   Acad9
Triml1   Fabp4
Gnmt   Neat1
Phkg1   Itga11
Gm7609   Cln3
Gm11569   Apoe
Vmn1r58   Nkain3
H2-Q4   Ppp1r12b
Krtap4-13
A130077B15Rik

Ahr
Acer2
Agbl2   9930111J21Rik1
Slfn9   5430427O19Rik1
Uhmk1   A630089N07Rik
Gm17821   D830005E20Rik
Kcnq1ot1   F830016B08Rik

Fig. 25

(Table 2)

Gene symbol	v-score	q-value (BH-FDR-corrected p-value)	Maximum abundance (normalized counts, rounded)
Sprr2b	21.646	0	94
Krt8	19.012	0	115
Sparc	14.051	0	259
Bc1	12.630	0	269
Clu	12.060	0	146
Krt18	9.637	0	82
H19	7.345	0	59
Ccdc36	7.124	0	248
Serpinh1	6.580	0	67
Hist1h2ao	6.577	0	104
Col4a1	6.194	0	53
Fam25c	5.457	0	92
Lamb1	4.808	0	55
Gm7102	4.557	0	59
S100a6	4.253	0	60
Sfn	4.209	0	44
Malat1	3.667	0	83
Lama1	3.666	0	44
Mt1	3.441	0	123
Hspa5	3.373	0	56
Fabp3	3.283	0	52
Tagln2	3.200	0	55
Hist1h2ah	3.005	0	38
Acaa1b	2.982	0	34
Ly6a	2.982	0	30
Sohlh2	2.966	0	47
Laptm5	1.967	0.024794	22
Dnmt3l	1.954	0.024794	35
Krt19	1.939	0.024794	11
Wtap	1.936	0.024794	65
Dusp9	1.924	0.024794	24
Gpx4	1.923	0.024794	53
Tdh	1.912	0.024794	41
P4hb	1.909	0.024794	32
Tspan17	1.898	0.024794	23
S100a10	1.897	0.024794	34
Slc2a3	1.895	0.032493	40
Ldha	1.891	0.032493	35
Peg10	1.871	0.038635	36
Rhox13	1.863	0.038635	15
Igfbp2	1.860	0.038635	24
Tdgf1	1.858	0.038635	31
Vim	1.858	0.038635	15
Tor4a	1.849	0.038635	53
Lamc1	1.840	0.053653	36
Ldhb	1.818	0.060827	14
Lgmn	1.809	0.067887	28
Klf5	1.806	0.071607	19
Upp1	1.805	0.071607	17
A630089N07Rik	1.805	0.071607	45
Ngfrap1	1.800	0.071607	37
Dusp1	1.791	0.071607	14
Slc2a1	1.790	0.071607	30
BC018473	1.786	0.071607	30
Pmvk	1.783	0.071607	18
Calr	1.780	0.071607	26
Eif4ebp1	1.577	0.075054	34
Ptcd2	1.574	0.075054	14
Marcksl1	1.573	0.075054	19
Actb	1.570	0.075054	94
Cep83	1.569	0.075054	16
Tceb2	1.569	0.075054	29
2410006H16Rik	1.567	0.075054	19
Pkp2	1.566	0.075054	17
Blvrb	1.562	0.075054	17
Ube2l6	1.561	0.075054	15
Pcolce	1.557	0.075054	14
Tcl1	1.553	0.075054	11
Klf2	1.553	0.075054	18
Hmces	1.550	0.075054	17
D11Wsu47e	1.548	0.075054	12
Mfge8	1.545	0.075054	16
Rpl39l	1.544	0.075054	12
Pdia6	1.544	0.075054	23
Creld2	1.543	0.075054	17
Utf1	1.541	0.075054	28
Surf4	1.539	0.075054	21
Gng5	1.539	0.075054	26
Stmn2	1.538	0.075054	15
Trib3	1.533	0.075054	16
Hist1h4i	1.532	0.075054	9
Hist1h3b	1.528	0.075054	6
Exoc3l	1.528	0.075054	12
Set	1.527	0.075054	67
Tpd52	1.527	0.075054	18
Gabarapl2	1.527	0.075054	14

Fig. 26-1

Gene	Value	Value	Count
Id1	2.965	0	24
Fgd1	2.927	0	73
Lrpap1	2.873	0	55
Gm6878	2.862	0	28
Cd63	2.840	0	51
Bscl2	2.821	0	44
Mt2	2.690	0	197
Gm20594	2.633	0	35
Papd5	2.629	0	51
Slc29a1	2.622	0.016109	36
Med10	2.605	0.016109	31
Actg1	2.560	0.016109	162
Dcdc2c	2.515	0.016109	38
Lgals1	2.451	0.01882	34
Anxa2	2.419	0.01882	26
Rhox5	2.381	0.01882	15
Mylpf	2.380	0.01882	27
Hsp90b1	2.365	0.01882	43
Meg3	2.362	0.01882	31
2700046A07Rik	2.317	0.01882	32
Slc25a4	2.315	0.01882	32
Ctgf	2.309	0.01882	34
Tmsb4x	2.279	0.01882	61
Gsn	2.264	0.01882	30
Bhmt	2.254	0.01882	20
Ccnd3	2.245	0.01882	39
Sept12	2.210	0.01882	17
Manf	2.206	0.01882	32
Trim71	2.184	0.01882	29
Tagln	2.138	0.01882	20
Col4a2	2.135	0.01882	35
Hsp90aa1	2.1215	0.01882	190

Gene	Value	Value	Count
Pbld1	1.777	0.071607	54
Fabp5	1.777	0.071607	16
Slc25a5	1.764	0.071607	21
Eif2s2	1.741	0.071607	52
Asb13	1.738	0.07173	28
Kif15	1.738	0.07173	22
Slc3a2	1.737	0.07173	39
Gm7325	1.726	0.07173	25
Atf5	1.724	0.07173	15
Id3	1.703	0.075054	15
Hspb1	1.684	0.075054	52
Ifitm1	1.681	0.075054	14
Erdr1	1.675	0.075054	40
Pgk1	1.669	0.075054	59
Htra1	1.660	0.075054	20
Kpna2	1.657	0.075054	24
Morf4l2	1.656	0.075054	26
Ctsd	1.652	0.075054	29
Cdc20	1.651	0.075054	20
Gpx1	1.649	0.075054	40
Id2	1.649	0.075054	14
Madd	1.648	0.075054	38
Rasgrp2	1.647	0.075054	12
Zfp286	1.644	0.075054	16
Pfdn2	1.637	0.075054	18
Aes	1.632	0.075054	25
Ccne1	1.631	0.075054	19
Sort1	1.629	0.075054	28
Kctd15	1.629	0.075054	24
Elp5	1.628	0.075054	23
Bcas2	1.617	0.075054	41
Plec	1.611	0.075054	21

Gene	Value	Value	Count
H2afx	1.526	0.075054	15
Nxn	1.526	0.075054	13
Cks2	1.522	0.075054	18
Nme6	1.521	0.075054	23
Cd9	1.519	0.075054	30
2810403A07Rik	1.519	0.075054	25
Arl6ip1	1.518	0.075054	17
Pim3	1.518	0.075054	23
Psap	1.518	0.075054	18
Sssca1	1.517	0.075054	21
Stmn1	1.516	0.075054	15
Zfp296	1.514	0.075054	11
Pdlim7	1.512	0.075054	17
Actn1	1.506	0.075054	24
Glrx5	1.506	0.075054	14
Epha2	1.505	0.075054	23
Exosc2	1.505	0.075054	29
Psmd7	1.504	0.075054	21
Gm6083	1.503	0.075054	30
Pkig	1.503	0.075054	10
Bloc1s1	1.502	0.075054	20
Supt4a	1.502	0.075054	19
Dnmt3b	1.501	0.075054	21
Apoe	1.500	0.075054	14
Clic4	1.496	0.075054	24
Bex1	1.496	0.075054	35
Cenpn	1.495	0.075054	12
Cox10	1.492	0.075054	15
Mdh2	1.492	0.075054	23
Nkain3	1.491	0.075054	66
Srm	1.490	0.075054	24
Tsc22d1	1.490	0.075054	27

Fig. 26-2

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Gene	Value	p-value	Count
Ahcyl2	2.105	0.01882	30
Best2	2.094	0.01882	19
Asns	2.091	0.01882	28
Calcoco2	2.064	0.01882	25
Ctsl	2.063	0.01882	28
Dppa5a	2.036	0.01882	133
Fam229b	2.024	0.01882	38
Chchd10	2.018	0.01882	29
Sbk1	1.987	0.024794	21
E130012A19Rik	1.975	0.024794	31
Parvb	1.481	0.075054	19
Imp3	1.479	0.075054	14
Thoc3	1.479	0.075054	21
Hn1	1.478	0.075054	16
1110008F13Rik	1.478	0.075054	18
2310036O22Rik	1.477	0.075054	20
Crip1	1.476	0.075054	16
Esrrb	1.475	0.075054	20
Gtf3a	1.475	0.075054	13
Aurka	1.474	0.075054	10
Ass1	1.473	0.075054	15
Rnaseh2c	1.473	0.075054	14
Tbc1d16	1.472	0.075054	23
Ube2s	1.471	0.075054	31
Cdk2ap1	1.470	0.075054	15
Tmem230	1.469	0.075054	21
Pdgfa	1.468	0.075054	10
Rrp7a	1.468	0.075054	13
Tyw1	1.468	0.075054	36
Erf	1.464	0.075054	22
Timm8b	1.464	0.075054	12

Value	p-value	Count	Gene	Value	p-value	Count
1.604	0.075054	35	Qsox1	1.490	0.075054	26
1.600	0.075054	28	Ulk1	1.489	0.075054	17
1.600	0.075054	65	Gpx3	1.488	0.075054	17
1.599	0.075054	48	Srgn	1.488	0.075054	12
1.598	0.075054	23	Xrcc1	1.488	0.075054	12
1.596	0.075054	19	Rpl10l	1.487	0.075054	13
1.596	0.075054	18	Ung	1.486	0.075054	17
1.595	0.075054	21	Itpk1	1.486	0.075054	14
1.594	0.075054	23	Mthfd2	1.485	0.075054	12
1.594	0.075054	20	Srrd	1.485	0.075054	16
1.589	0.075054	12	Hspa12b	1.483	0.075054	26
1.426	0.075054	18	Qdpr	1.396	0.075054	12
1.425	0.075054	14	Midn	1.396	0.075054	20
1.425	0.075054	15	Mars	1.395	0.075054	16
1.425	0.075054	20	Dap	1.395	0.075054	10
1.423	0.075054	19	2200002D01Rik	1.394	0.075054	11
1.423	0.075054	14	Zdhhc7	1.392	0.075054	10
1.422	0.075054	27	Hap1	1.392	0.075054	12
1.422	0.075054	18	Cdv3	1.392	0.075054	24
1.422	0.075054	16	Polr2e	1.392	0.075054	22
1.422	0.075054	17	Ctnnal1	1.390	0.075054	15
1.422	0.075054	15	Nlrp1a	1.390	0.075054	41
1.422	0.075054	24	Snx6	1.390	0.075054	10
1.421	0.075054	20	Xpnpep1	1.390	0.075054	18
1.421	0.075054	21	Gm10845	1.390	0.075054	19
1.421	0.075054	14	Trim25	1.390	0.075054	18
1.421	0.075054	15	Gpx2	1.389	0.075054	8
1.420	0.075054	14	Esd	1.389	0.075054	14
1.419	0.075054	12	Sgk1	1.389	0.075054	13
1.419	0.075054	17	Mrpl13	1.389	0.075054	12
1.419	0.075054	25	Stmn3	1.389	0.075054	12
1.417	0.075054	22	Mrpl53	1.389	0.075054	9

Fig. 26-3

Gene	Value	p	Count	Gene	Value	p	Count	Gene	Value	p	Count
D230025D16Rik	1.464	0.075054	14	Trmt1	1.417	0.075054	15	Rwdd1	1.388	0.075054	16
Scd2	1.463	0.075054	19	Fkbp1a	1.417	0.075054	32	Fkbp4	1.388	0.075054	19
Med13l	1.463	0.075054	19	Uchl1	1.417	0.075054	16	Txnl1	1.388	0.075054	20
Syce2	1.462	0.075054	13	Psmd11	1.416	0.075054	12	Asb6	1.387	0.075054	11
Arf1	1.462	0.075054	34	Mif4gd	1.416	0.075054	13	Chchd4	1.387	0.075054	13
Fcf1	1.461	0.075054	10	Pfdn6	1.416	0.075054	20	Sqstm1	1.387	0.075054	19
Etv4	1.459	0.075054	17	1700007K13Rik	1.416	0.075054	8	Guca1a	1.386	0.075054	7
Eid2	1.458	0.075054	8	Rabac1	1.416	0.075054	11	Glipr2	1.386	0.075054	13
Polr2h	1.456	0.075054	18	Snrpa1	1.415	0.075054	16	Prpsap1	1.386	0.075054	17
Rad51	1.456	0.075054	13	Dtd1	1.414	0.075054	11	Rbmxl1	1.386	0.075054	16
Dppa4	1.455	0.075054	17	Ddx56	1.413	0.075054	20	Eif3c	1.385	0.075054	18
Sertad1	1.454	0.075054	12	Srpk1	1.413	0.075054	15	Tspan4	1.385	0.075054	11
Lincenc1	1.453	0.075054	11	Krtcap2	1.413	0.075054	14	Dmkn	1.384	0.075054	10
Cox7a2l	1.451	0.075054	19	Gorasp2	1.412	0.075054	14	Rian	1.384	0.075054	11
Sf3b5	1.451	0.075054	13	Malsu1	1.412	0.075054	12	Copb2	1.384	0.075054	13
Tra2b	1.451	0.075054	19	Wbp5	1.411	0.075054	18	Ahsa1	1.384	0.075054	19
Tinagl1	1.450	0.075054	14	Mark3	1.409	0.075054	24	Ndufb6	1.384	0.075054	12
Tubb6	1.450	0.075054	14	Tsta3	1.408	0.075054	12	Atp6v0e	1.383	0.075054	13
Chchd3	1.450	0.075054	13	Tpx2	1.408	0.075054	16	Ccdc22	1.382	0.075054	11
Fam195a	1.449	0.075054	9	Sec11c	1.407	0.075054	15	Rbm42	1.381	0.075054	12
Abcd4	1.449	0.075054	27	H2afz	1.407	0.075054	34	Cyc1	1.380	0.075054	21
F830016B08Rik	1.448	0.075054	26	Nfyb	1.407	0.075054	13	Lmna	1.380	0.075054	15
U2af1	1.446	0.075054	21	Atg101	1.407	0.075054	14	Clic1	1.380	0.075054	10
Rbfa	1.444	0.075054	10	Wars	1.407	0.075054	17	Klf4	1.379	0.075054	14
Fn1	1.444	0.075054	23	Atp6v0d1	1.406	0.075054	12	Slc30a2	1.379	0.075054	11
Cope	1.444	0.075054	11	Coq7	1.406	0.075054	10	Psmd6	1.379	0.075054	16
Dym	1.443	0.075054	11	Ctsh	1.406	0.075054	16	Scamp3	1.378	0.075054	16
Calu	1.443	0.075054	18	2810428I15Rik	1.406	0.075054	15	Calm1	1.378	0.075054	24
Crlf2	1.442	0.075054	11	Fam96b	1.406	0.075054	14	Qars	1.376	0.075054	15
Top2a	1.441	0.075054	14	Hbegf	1.406	0.075054	15	Mrpl43	1.376	0.075054	38
Cstb	1.441	0.075054	10	Samm50	1.405	0.075054	15	Adamts13	1.376	0.075054	18
Ppa1	1.440	0.075054	17	Zc3hc1	1.404	0.075054	19	Ppie	1.376	0.075054	11

Fig. 26-4

Gene	Value	Score	N	Gene	Value	Score	N	Gene	Value	Score	N
Arl6ip4	1.440	0.075054	10	Rpap1	1.404	0.075054	17	Mea1	1.375	0.075054	11
Tuba4a	1.439	0.075054	24	Tpi1	1.403	0.075054	11	Prpf3	1.375	0.075054	16
Bzw2	1.438	0.075054	19	Elof1	1.403	0.075054	12	Atf4	1.375	0.075054	13
Churc1	1.438	0.075054	34	Tcea3	1.403	0.075054	13	Spata33	1.375	0.075054	11
Bud31	1.438	0.075054	20	1500012F01Rik	1.402	0.075054	14	Atp5j2	1.374	0.075054	19
Aifm1	1.437	0.075054	16	Cenpa	1.401	0.075054	14	Lrrc59	1.374	0.075054	23
Ndufa13	1.436	0.075054	12	Pecr	1.400	0.075054	13	Fam129b	1.374	0.075054	14
Dstn	1.435	0.075054	18	Myod1	1.400	0.075054	10	Nhp2	1.373	0.075054	22
Ocel1	1.435	0.075054	15	Degs1	1.400	0.075054	14	Aprt	1.372	0.075054	22
Galk1	1.435	0.075054	11	Ndrg4	1.399	0.075054	16	Cdc5l	1.371	0.075054	19
Hmox1	1.433	0.075054	12	Lonp1	1.399	0.075054	16	Gna11	1.371	0.075054	11
Ubald2	1.433	0.075054	15	Ppa2	1.399	0.075054	15	Spns1	1.370	0.075054	19
Romo1	1.432	0.075054	14	Apitd1	1.399	0.075054	10	Ntan1	1.370	0.075054	20
Phc1	1.432	0.075054	23	Dcun1d5	1.399	0.075054	11	Actl6a	1.369	0.075054	13
Emc10	1.432	0.075054	13	Ndufa4	1.398	0.075054	19	Ywhaq	1.369	0.075054	24
Ptov1	1.431	0.075054	17	Stx5a	1.398	0.075054	9	Chmp4b	1.369	0.075054	17
Snf8	1.430	0.075054	21	Rheb	1.398	0.075054	21	Fto	1.368	0.075054	24
Calm2	1.429	0.075054	21	Ddt	1.397	0.075054	14	Sdhc	1.368	0.075054	17
Mrpl14	1.429	0.075054	13	Trappc3	1.397	0.075054	12	Plin2	1.368	0.075054	11
Pih1d1	1.427	0.075054	14	Cbx3	1.397	0.075054	19	Eif3g	1.368	0.075054	19
Dhx16	1.427	0.075054	19	Slirp	1.396	0.075054	21	Mcm7	1.368	0.075054	16
Siva1	1.427	0.075054	18	Mrpl12	1.396	0.075054	14	Crip2	1.367	0.075054	11
Pop5	1.426	0.075054	11	Tcf15	1.396	0.075054	12	Slc6a8	1.334	0.075054	20
Saal1	1.367	0.075054	14	Gins4	1.349	0.075054	19	Rpl39	1.333	0.075054	53
Mrpl23	1.367	0.075054	17	Slc15a4	1.349	0.075054	14	Srp14	1.333	0.075054	15
Cdkn1a	1.367	0.075054	9	Agpat2	1.349	0.075054	9	Umps	1.333	0.075054	14
Emg1	1.366	0.075054	14	Cdca5	1.349	0.075054	11	Use1	1.332	0.075054	10
Ccdc124	1.366	0.075054	19	A430005L14Rik	1.348	0.075054	12	Nxt1	1.332	0.075054	11
Bri3	1.366	0.075054	14	Mrpl30	1.348	0.075054	15	Ehd1	1.331	0.075054	27
Csrp1	1.366	0.075054	17	Rcl1	1.348	0.075054	15	Timm10	1.331	0.075054	10
Glul	1.366	0.075054	20	Tmem216	1.347	0.075054	8	Srsf11	1.331	0.075054	15
Socs2	1.366	0.075054	10	Sall4	1.347	0.075054	20	Cyb5a	1.331	0.075054	13

Fig. 26-5

Gene	Value	p-value	Count
Ftl1	1.365	0.075054	185
Sox2	1.365	0.075054	21
Cdkn2aip	1.364	0.075054	15
Hs1bp3	1.364	0.075054	13
Fdft1	1.364	0.075054	13
Exosc8	1.364	0.075054	13
Guk1	1.364	0.075054	13
Deb1	1.363	0.075054	8
Ndufb10	1.362	0.075054	12
Rnf7	1.362	0.075054	19
Ndufb11	1.362	0.075054	13
Dynll1	1.362	0.075054	22
Gstm1	1.361	0.075054	9
Smn1	1.361	0.075054	14
Vdac3	1.361	0.075054	14
Cdkn2aipnl	1.360	0.075054	14
Safb2	1.360	0.075054	15
Dctn6	1.360	0.075054	9
Gnb2l1	1.360	0.075054	137
Psma5	1.360	0.075054	17
Dazap2	1.360	0.075054	9
Isyna1	1.360	0.075054	11
Odc1	1.360	0.075054	35
Psmb4	1.360	0.075054	25
Timm23	1.359	0.075054	23
1110038F14Rik	1.359	0.075054	9
Tc2n	1.359	0.075054	16
Srrt	1.359	0.075054	22
Defa6	1.359	0.075054	5
Hax1	1.359	0.075054	11
Epas1	1.358	0.075054	13
Socs3	1.358	0.075054	12

Count	p-value	Value	Gene
14	0.075054	1.347	Bex4
14	0.075054	1.347	Pttg1ip
21	0.075054	1.347	Immt
15	0.075054	1.347	Wdr74
10	0.075054	1.347	Pttg1
10	0.075054	1.346	Bub3
9	0.075054	1.346	Nodal
10	0.075054	1.346	Ppdpf
24	0.075054	1.346	Atp5g1
16	0.075054	1.345	Eftud2
15	0.075054	1.345	Nop10
13	0.075054	1.344	Rbpms2
19	0.075054	1.344	Impact
14	0.075054	1.344	Vbp1
13	0.075054	1.344	Plk1
26	0.075054	1.344	Pebp1
11	0.075054	1.343	Skap2
16	0.075054	1.343	Atp6v0b
12	0.075054	1.343	Maged1
14	0.075054	1.343	Trmt112
11	0.075054	1.343	Tfg
15	0.075054	1.342	Tyw3
17	0.075054	1.342	Ubqln1
28	0.075054	1.341	Ddost
13	0.075054	1.341	Ccnb1
10	0.075054	1.341	Slc25a20
9	0.075054	1.340	Apoc1
17	0.075054	1.340	Dscr3
14	0.075054	1.340	Ssr2
12	0.075054	1.340	Fgf4
26	0.075054	1.340	Myl12a
22	0.075054	1.340	Mcm6

Count	p-value	Value	Gene
7	0.075054	1.331	Cox7a1
8	0.075054	1.331	Taf11
16	0.075054	1.331	Pdhb
11	0.075054	1.331	Eif2b2
14	0.075054	1.330	Lcmt1
9	0.075054	1.330	Fzr1
22	0.075054	1.330	Cggbp1
18	0.075054	1.330	Prdx6
8	0.075054	1.330	Fbxo6
22	0.075054	1.330	Tomm70a
12	0.075054	1.330	Serpinb6a
5	0.075054	1.330	Rmrp
18	0.075054	1.330	Psma1
11	0.075054	1.329	Ppm1a
13	0.075054	1.329	Chtf18
11	0.075054	1.329	Sec13
18	0.075054	1.329	Ndufa10
15	0.075054	1.328	Slc7a3
10	0.075054	1.328	Dapk3
17	0.075054	1.328	Uqcc2
12	0.075054	1.327	Rarg
13	0.075054	1.327	Polr2k
12	0.075054	1.327	Mfsd3
12	0.075054	1.326	Gm9855
7	0.075054	1.326	Scand1
14	0.075054	1.326	Polr2i
18	0.075054	1.326	Atp5e
15	0.075054	1.326	Ppp1r8
8	0.075054	1.326	Rilpl1
14	0.075054	1.326	Podxl
4	0.075054	1.325	Rpph1
10	0.075054	1.325	Lsr

Fig. 26-6

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Gene	Ratio	p-value	Count
Rtcb	1.358	0.075054	21
Psmd2	1.358	0.075054	26
Llgl2	1.358	0.075054	12
Mcl1	1.358	0.075054	16
Cct4	1.357	0.075054	21
Suclg1	1.357	0.075054	13
1110058L19Rik	1.357	0.075054	9
Ybx3	1.356	0.075054	24
P4ha2	1.356	0.075054	11
Fkbp2	1.356	0.075054	12
Fblim1	1.355	0.075054	15
Sf3b1	1.355	0.075054	31
Psmb3	1.355	0.075054	34
Acer2	1.355	0.075054	26
Cacng7	1.354	0.075054	14
Mrps36	1.354	0.075054	10
Phf23	1.354	0.075054	12
Eif2b5	1.353	0.075054	14
Ndufs7	1.352	0.075054	13
Tubg1	1.352	0.075054	9
Dnajc2	1.352	0.075054	17
Capns1	1.351	0.075054	22
Utp3	1.351	0.075054	11
Ctnna1	1.351	0.075054	17
Prr13	1.351	0.075054	11
Psph	1.351	0.075054	10
Prkcsh	1.351	0.075054	21
Mrpl54	1.351	0.075054	13
Slmo2	1.351	0.075054	9
Mrpl46	1.350	0.075054	10
Tprgl	1.350	0.075054	13
Trim28	1.350	0.075054	50
Ctc1	1.350	0.075054	11
Ube2c	1.349	0.075054	26
Txn2	1.349	0.075054	15

Gene	Ratio	p-value	Count
Mrps12	1.339	0.075054	14
Ubl5	1.339	0.075054	14
Trap1	1.339	0.075054	11
Atp5o	1.339	0.075054	21
Gtf2f1	1.339	0.075054	17
Pacsin2	1.339	0.075054	26
Dcaf7	1.339	0.075054	15
2410004N09Rik	1.338	0.075054	11
Cisd3	1.338	0.075054	10
Asnsd1	1.338	0.075054	13
Pomgnt1	1.337	0.075054	9
Rpa1	1.337	0.075054	17
Mrpl4	1.337	0.075054	12
Adprh	1.337	0.075054	10
Tomm40l	1.336	0.075054	13
Ndufa6	1.336	0.075054	21
B4galt3	1.336	0.075054	12
Mvd	1.336	0.075054	12
Rbmxl2	1.336	0.075054	13
Hsph1	1.336	0.075054	22
Lin28a	1.336	0.075054	12
Got1	1.336	0.075054	15
Utp14a	1.336	0.075054	9
Efcc1	1.336	0.075054	21
Ndufs3	1.336	0.075054	15
Fam64a	1.335	0.075054	9
Txnip	1.335	0.075054	13
AU022252	1.335	0.075054	12
Tbrg1	1.335	0.075054	15
Blmh	1.335	0.075054	21
Uqcr10	1.335	0.075054	25
Hexa	1.334	0.075054	9
Mrpl44	1.334	0.075054	10
Add1	1.334	0.075054	11
Mrps24	1.334	0.075054	16

Gene	Ratio	p-value	Count
Psmc1	1.325	0.075054	18
Lin7b	1.325	0.075054	6
Gabarapl1	1.325	0.075054	13
Zfp42	1.325	0.075054	17
Snrpf	1.325	0.075054	24
Bcl3	1.324	0.075054	10
Atic	1.324	0.075054	17
Ddx39b	1.324	0.075054	17
Ppp2r5a	1.324	0.075054	15
Rangap1	1.323	0.075054	15
Abhd17a	1.323	0.075054	10
Smyd5	1.323	0.075054	11
Dpp7	1.323	0.075054	8
Dnpep	1.323	0.075054	11
Bms1	1.323	0.075054	16
Ptp4a2	1.323	0.075054	15
Lage3	1.323	0.075054	14
Cdca8	1.323	0.075054	16
Ankrd46	1.323	0.075054	8
Ddx23	1.323	0.075054	13
Rfc2	1.323	0.075054	14
Uqcrb	1.323	0.075054	13
Txn1	1.322	0.075054	27
Snhg5	1.322	0.075054	11
Bcat1	1.322	0.075054	31
Yrdc	1.322	0.075054	16
Tuba1b	1.322	0.075054	43
Vipas39	1.321	0.075054	8
Cops8	1.321	0.075054	15
Pcna	1.321	0.075054	17
Ydjc	1.320	0.075054	12
Flna	1.320	0.075054	19
Ampd2	1.320	0.075054	12
Gpn1	1.320	0.075054	11
Sars	1.320	0.075054	11

Fig. 26-7

Gene	Val1	Val2	Count	Gene	Val1	Val2	Count	Gene	Val1	Val2	Count
Pfdn4	1.319	0.075054	13	Tnk2	1.308	0.075054	12	Coro1a	1.297	0.075054	10
Mfsd5	1.319	0.075054	11	Nutf2	1.308	0.075054	12	H1fx	1.297	0.075054	8
Igf2bp1	1.319	0.075054	45	Ddx47	1.307	0.075054	10	Psma6	1.297	0.075054	21
Pgp	1.318	0.075054	13	Gpatch4	1.307	0.075054	16	Stra13	1.297	0.075054	13
Ear10	1.318	0.075054	6	Rrp12	1.307	0.075054	11	N6amt2	1.297	0.075054	10
Cldn7	1.318	0.075054	14	Tma7	1.307	0.075054	23	Dus1l	1.297	0.075054	11
Cpsf4l	1.318	0.075054	10	Cuta	1.307	0.075054	11	Smarcb1	1.296	0.075054	13
Mif	1.317	0.075054	29	Cox14	1.307	0.075054	10	Tax1bp1	1.296	0.075054	12
Nsmce4a	1.317	0.075054	14	Mdh1	1.307	0.075054	16	Mtdh	1.296	0.075054	21
Pold1	1.317	0.075054	16	Tufm	1.307	0.075054	16	Rlim	1.296	0.075054	23
Pnp	1.316	0.075054	15	Tomm5	1.306	0.075054	11	S100a11	1.296	0.075054	8
Cib1	1.316	0.075054	9	Cog8	1.306	0.075054	13	Gde1	1.295	0.075054	8
Tipin	1.316	0.075054	12	Mbd3	1.306	0.075054	24	Plrg1	1.295	0.075054	10
Vdac1	1.316	0.075054	26	Uqcr11	1.306	0.075054	21	Gabarap	1.295	0.075054	12
4833439L19Rik	1.316	0.075054	12	Ap4m1	1.306	0.075054	11	Ilk	1.295	0.075054	8
L1td1	1.315	0.075054	24	Rbpj	1.306	0.075054	18	Fads1	1.294	0.075054	12
Slc25a1	1.315	0.075054	9	Nedd8	1.306	0.075054	18	Dnajc21	1.294	0.075054	10
Ints3	1.315	0.075054	13	Gtsf1l	1.305	0.075054	11	Tpst2	1.294	0.075054	10
Cdc16	1.315	0.075054	11	2500004C02Rik	1.305	0.075054	21	Lamtor4	1.294	0.075054	13
Sdhb	1.315	0.075054	10	Uqcrh	1.305	0.075054	20	Skp1a	1.294	0.075054	19
Sfr1	1.315	0.075054	13	Myl9	1.305	0.075054	14	Msn	1.294	0.075054	17
Phlda3	1.315	0.075054	12	Zfp771	1.305	0.075054	8	Grhpr	1.294	0.075054	9
Ndufs2	1.314	0.075054	12	Ifi30	1.305	0.075054	10	Rsl1d1	1.293	0.075054	17
Psrc1	1.314	0.075054	10	Lsm4	1.304	0.075054	19	Nrp	1.293	0.075054	8
Prdx2	1.314	0.075054	18	Ywhab	1.304	0.075054	20	Psmc2	1.293	0.075054	15
Fam168b	1.314	0.075054	13	Dnajb1	1.304	0.075054	10	Mel	1.293	0.075054	12
Ebp	1.314	0.075054	10	Polr2g	1.304	0.075054	9	Mrps35	1.293	0.075054	7
Ss18	1.314	0.075054	10	Oaz2	1.304	0.075054	14	Gsta4	1.292	0.075054	18
Sumo2	1.314	0.075054	22	Pycr1	1.304	0.075054	8	Ap1s1	1.292	0.075054	13
Hdgf	1.314	0.075054	41	Dpy30	1.304	0.075054	19	Zdhhc4	1.292	0.075054	8
Ik	1.314	0.075054	16	Parp1	1.303	0.075054	16	Phf10	1.292	0.075054	9
Farsa	1.314	0.075054	10	Ugp2	1.303	0.075054	12	Tfpi	1.292	0.075054	21

Fig. 26-8

Gene	Value		Count
Aimp2	1.314	0.075054	12
Pgls	1.314	0.075054	10
B9d1	1.314	0.075054	9
Chmp6	1.314	0.075054	8
Aup1	1.314	0.075054	11
Aldh2	1.314	0.075054	14
Dnajc9	1.314	0.075054	15
Tmed10	1.314	0.075054	13
Sap30	1.313	0.075054	10
Maf1	1.313	0.075054	11
Drg1	1.313	0.075054	17
Snd1	1.313	0.075054	18
Acaa1a	1.312	0.075054	12
Polr2j	1.312	0.075054	16
Pes1	1.312	0.075054	17
N4bp3	1.312	0.075054	9
Gars	1.312	0.075054	17
Ccz1	1.312	0.075054	9
Tuba1c	1.312	0.075054	37
Nfkbia	1.312	0.075054	9
Nono	1.312	0.075054	29
Mtch1	1.312	0.075054	21
Ndufb8	1.312	0.075054	16
Sec61b	1.311	0.075054	17
Ppm1g	1.311	0.075054	18
Mrps2	1.311	0.075054	11
Gnai2	1.311	0.075054	14
Zw10	1.311	0.075054	9
Sfxn1	1.311	0.075054	12
Atp9a	1.310	0.075054	15
Ccdc93	1.309	0.075054	22
2010107E04Rik	1.309	0.075054	18

Gene	Value		Count
Wdr5	1.303	0.075054	17
Pold2	1.303	0.075054	11
Ergic3	1.303	0.075054	10
Rhoc	1.303	0.075054	12
Bub1b	1.303	0.075054	14
Pbk	1.303	0.075054	20
Phgdh	1.302	0.075054	11
Cnn2	1.302	0.075054	12
Slc50a1	1.302	0.075054	13
Ak4	1.302	0.075054	15
Ndufb4	1.302	0.075054	10
Ptrf	1.302	0.075054	10
Scyl1	1.302	0.075054	12
Cyth2	1.302	0.075054	10
Xrcc5	1.301	0.075054	14
Cox6a1	1.301	0.075054	26
Zdhhc18	1.301	0.075054	14
Bloc1s2	1.301	0.075054	8
Mast2	1.301	0.075054	10
1110038B12Rik	1.301	0.075054	18
Aspscr1	1.300	0.075054	15
Cops3	1.300	0.075054	13
Nenf	1.300	0.075054	11
Ndufs6	1.299	0.075054	18
1190005I06Rik	1.299	0.075054	6
Hsbp1	1.299	0.075054	12
Tmed9	1.299	0.075054	10
Gm3258	1.299	0.075054	10
Rpl36al	1.299	0.075054	43
Jam2	1.299	0.075054	21
Eci2	1.299	0.075054	10
Gm16062	1.299	0.075054	14

Gene	Value		Count
Fdx1	1.292	0.075054	10
Ccng1	1.292	0.075054	12
Rab2a	1.291	0.075054	11
Bax	1.291	0.075054	27
Ecsit	1.291	0.075054	10
Mrps30	1.290	0.075054	10
Etv5	1.290	0.075054	15
Ino80e	1.290	0.075054	12
2810008D09Rik	1.290	0.075054	5
Ubxn1	1.290	0.075054	21
Hspa9	1.290	0.075054	24
Nudt1	1.289	0.075054	12
2700094K13Rik	1.289	0.075054	18
Clstn1	1.289	0.075054	10
Psmd12	1.289	0.075054	14
Cmtm7	1.289	0.075054	10
Kat2a	1.289	0.075054	15
Phkg1	1.289	0.075054	20
Arhgap8	1.288	0.075054	12
Atp5k	1.288	0.075054	31
Tdrp	1.288	0.075054	12
Nutf2-ps1	1.288	0.075054	16
Mrpl47	1.288	0.075054	10
Pdia3	1.288	0.075054	13
Iscu	1.288	0.075054	12
Jmjd6	1.288	0.075054	10
Ndufs1	1.288	0.075054	17
Kif22	1.287	0.075054	12
Scamp4	1.287	0.075054	11
Drosha	1.287	0.075054	12
Snrnp25	1.287	0.075054	9
Rrp15	1.287	0.075054	12

Fig. 26-9

Gene				Gene				Gene			
Rpp25	1.309	0.075054	11	Bola1	1.299	0.075054	8	Ruvbl1	1.287	0.075054	16
Cd99	1.309	0.075054	10	Mrpl20	1.298	0.075054	15	Clta	1.287	0.075054	19
Pfn1	1.309	0.075054	10	Ube2q1	1.298	0.075054	9	Pdlim1	1.287	0.075054	11
Dcaf15	1.309	0.075054	11	Csrp2	1.298	0.075054	15	Eras	1.287	0.075054	9
Dus2	1.309	0.075054	18	Map1lc3b	1.298	0.075054	16	Prdx4	1.287	0.075054	11
Ezh2	1.308	0.075054	13	Hnrnph1	1.298	0.075054	17	Rab1b	1.287	0.075054	13
Cnn3	1.308	0.075054	22	Ccdc12	1.298	0.075054	8	Idh3g	1.287	0.075054	10
Sumo1	1.308	0.075054	15	Ddx1	1.298	0.075054	13	Rcor2	1.287	0.075054	14
Lsm10	1.308	0.075054	9	Sar1b	1.298	0.075054	8	1110008P14Rik	1.287	0.075054	10
Pa2g4	1.308	0.075054	30	Elp3	1.298	0.075054	17	Csnk1e	1.287	0.075054	24
Tomm20	1.308	0.075054	41	Mafg	1.297	0.075054	12	Grcc10	1.287	0.075054	10
Tmem147	1.308	0.075054	9	Irf3	1.297	0.075054	22	Hras	1.287	0.075054	15
Nudt21	1.287	0.075054	11	Ier3ip1	1.278	0.075054	17	Cap1	1.268	0.075698	14
Gjb3	1.286	0.075054	8	Stau1	1.278	0.075054	21	Dcakd	1.268	0.075698	10
Acaa2	1.286	0.075054	11	Tmem208	1.278	0.075054	9	Pabpc4	1.268	0.075698	16
Ppp2r2d	1.286	0.075054	13	Arrb2	1.278	0.075054	16	Gak	1.268	0.075698	13
Nthl1	1.286	0.075054	9	Aldh7a1	1.277	0.07553	10	Mpdu1	1.267	0.075698	15
Chmp2a	1.286	0.075054	11	Eif6	1.277	0.07553	15	Pomp	1.267	0.075698	20
Gatsl3	1.286	0.075054	8	Vmp1	1.277	0.07553	10	Idh1	1.267	0.075698	13
Erp29	1.285	0.075054	14	Trpt1	1.277	0.07553	11	Poldip2	1.267	0.075698	12
Hsd17b10	1.285	0.075054	10	Ddx21	1.277	0.075698	17	Rpusd1	1.267	0.075698	10
Plaur	1.285	0.075054	10	Rps19bp1	1.277	0.075698	12	Agl	1.267	0.075698	43
Capza1	1.285	0.075054	15	Gdi2	1.277	0.075698	18	Sept1	1.267	0.075698	8
Mtch2	1.285	0.075054	10	Sephs2	1.276	0.075698	15	Lsm7	1.267	0.075698	13
Bcap31	1.284	0.075054	12	Gbx2	1.276	0.075698	7	Nubp2	1.267	0.075698	8
Eed	1.284	0.075054	15	Sdha	1.276	0.075698	15	Atp5l	1.267	0.075698	20
Ccnb2	1.284	0.075054	11	Nop2	1.276	0.075698	12	Fxr1	1.267	0.075698	11
Ddit4	1.284	0.075054	10	H2afy	1.275	0.075698	15	Pam16	1.267	0.075698	15
Vps4b	1.284	0.075054	16	Pld3	1.275	0.075698	13	Epcam	1.267	0.075698	13
Apex1	1.284	0.075054	11	Gss	1.275	0.075698	11	Edf1	1.267	0.075698	19
Thap4	1.284	0.075054	11	Map7d2	1.275	0.075698	16	Parl	1.267	0.075698	10
Imp4	1.284	0.075054	12	Cox7a2	1.275	0.075698	21	Rpn1	1.266	0.075698	14

Fig. 26-10

Gene	Value	Score	Count	Gene	Value	Score	Count	Gene	Value	Score	Count
Atp5g3	1.284	0.075054	29	Med24	1.275	0.075698	11	Gcat	1.266	0.075698	13
3110001I22Rik	1.284	0.075054	17	Ccrn4l	1.275	0.075698	10	Slc9a3r1	1.266	0.075698	11
Herpud1	1.283	0.075054	8	Stmn1-rs1	1.275	0.075698	12	Sf3b3	1.266	0.075698	18
Cirbp	1.283	0.075054	9	Mgea5	1.274	0.075698	15	Gon4l	1.266	0.075698	14
Rrm2	1.283	0.075054	14	Bnip3l	1.274	0.075698	16	Uba2	1.266	0.075698	14
Pef1	1.283	0.075054	11	Hyou1	1.274	0.075698	13	Stoml1	1.266	0.075698	12
Ctps	1.283	0.075054	13	Chrac1	1.274	0.075698	8	Sod2	1.266	0.075698	11
Ly6e	1.283	0.075054	13	Apol7a	1.274	0.075698	13	Rangrf	1.265	0.075698	13
Gldc	1.283	0.075054	11	Trp53	1.274	0.075698	18	Slc20a2	1.265	0.075698	13
Gsr	1.283	0.075054	12	Adck5	1.274	0.075698	8	Usmg5	1.265	0.075698	20
Spcs2	1.283	0.075054	15	Nid2	1.274	0.075698	14	Gm561	1.265	0.075698	9
Eif3b	1.283	0.075054	34	Zfp451	1.273	0.075698	14	Polr1d	1.265	0.075698	19
Usp10	1.283	0.075054	13	Tnip1	1.273	0.075698	10	Uhrf1	1.265	0.075698	15
Rpa2	1.283	0.075054	19	Mgat4b	1.273	0.075698	10	Atp6v1g1	1.265	0.075698	13
Mrpl55	1.283	0.075054	13	Snrpd2	1.273	0.075698	30	Rps27l	1.265	0.075698	29
Mrps15	1.282	0.075054	13	Timm13	1.273	0.075698	18	Igf2	1.265	0.075698	12
Rab35	1.282	0.075054	9	Fbxo15	1.273	0.075698	12	Phb2	1.265	0.075698	19
Ubc	1.282	0.075054	16	Syngr2	1.273	0.075698	10	Arpc2	1.265	0.075698	14
0610007P14Rik	1.282	0.075054	8	Cox7b	1.273	0.075698	24	Plvap	1.265	0.075698	11
Cpsf3	1.282	0.075054	11	Atp6v1e1	1.273	0.075698	10	Cd81	1.265	0.075698	15
Tceb1	1.282	0.075054	13	Card10	1.273	0.075698	8	Mapkapk2	1.264	0.075698	11
Wnt7b	1.282	0.075054	10	Mpzl1	1.273	0.075698	11	Nme4	1.264	0.075698	8
G3bp2	1.282	0.075054	24	Slc29a4	1.272	0.075698	7	Lgals3	1.264	0.075698	11
Ubqln4	1.282	0.075054	13	Mfsd11	1.272	0.075698	22	Lrrc14	1.264	0.075698	11
Unc45a	1.281	0.075054	9	Mrps9	1.272	0.075698	12	Ccdc3	1.264	0.075698	9
Wdr61	1.281	0.075054	9	Ipo4	1.272	0.075698	11	Bet1l	1.264	0.075698	9
Zfpl1	1.281	0.075054	10	6430706D22Rik	1.272	0.075698	11	Hist1h1b	1.264	0.075698	8
Nup62	1.281	0.075054	17	Ppp1r14b	1.271	0.075698	14	Hnrnph3	1.264	0.075698	11
Cox6c	1.281	0.075054	16	Msh2	1.271	0.075698	20	Tbc1d13	1.264	0.075698	15
Nars	1.281	0.075054	12	Acta1	1.271	0.075698	14	Lamtor2	1.263	0.075698	15
Cipc	1.281	0.075054	13	Sec23b	1.271	0.075698	10	Aph1a	1.263	0.075698	14
Psmd13	1.281	0.075054	22	Ninj1	1.271	0.075698	8	Nme1	1.263	0.075698	16

Fig. 26-11

Gene	Ratio	p-value	Count
Slc25a3	1.281	0.075054	28
Sin3b	1.281	0.075054	12
Pde6d	1.281	0.075054	12
Ndufaf2	1.281	0.075054	11
Klf9	1.280	0.075054	17
Znrd1	1.280	0.075054	12
Cox6b1	1.280	0.075054	13
Ece2	1.280	0.075054	11
Sh3bgrl3	1.280	0.075054	12
Icam1	1.280	0.075054	9
Mageb16	1.280	0.075054	11
Myo9b	1.279	0.075054	12
Sra1	1.279	0.075054	10
Ddx49	1.279	0.075054	12
Ptms	1.279	0.075054	15
Psmc4	1.279	0.075054	18
Bsg	1.279	0.075054	20
Ak1	1.279	0.075054	16
Dohh	1.279	0.075054	12
Wdr75	1.279	0.075054	11
Snai3	1.279	0.075054	8
Aen	1.279	0.075054	13
Tbc1d10b	1.279	0.075054	12
Riok2	1.278	0.075054	12
Tfdp1	1.259	0.077442	10
Rhebl1	1.259	0.077442	13
Ndufb7	1.259	0.077442	14
Ltv1	1.259	0.077442	13
Mtfr1l	1.259	0.077442	10
Tram1	1.258	0.077442	9
Ssr4	1.258	0.077442	9
Arl3	1.258	0.077442	8

Gene	Ratio	p-value	Count
Gps1	1.270	0.075698	13
Cryz	1.270	0.075698	10
Eif3m	1.270	0.075698	14
F3	1.270	0.075698	16
Mrpl2	1.270	0.075698	9
Lta4h	1.270	0.075698	16
Cnpy2	1.270	0.075698	12
Nsmce1	1.270	0.075698	11
Rfc5	1.270	0.075698	14
Tomm22	1.270	0.075698	22
Ssrp1	1.269	0.075698	20
Psmd4	1.269	0.075698	21
Timm50	1.269	0.075698	7
Elovl6	1.269	0.075698	15
Snhg1	1.269	0.075698	13
Polr1c	1.269	0.075698	12
Zmynd19	1.269	0.075698	11
Lphn1	1.269	0.075698	21
Atp1b1	1.269	0.075698	12
Ssbp1	1.268	0.075698	18
Dusp12	1.268	0.075698	11
Spr	1.268	0.075698	13
Cir1	1.268	0.075698	15
Ywhah	1.268	0.075698	13
Noc4l	1.252	0.077442	13
Zswim7	1.252	0.077442	8
Coro1b	1.252	0.077442	11
Ier2	1.252	0.077442	9
Timm10b	1.251	0.077442	11
Arhgap21	1.251	0.077442	12
Pxn	1.251	0.077442	10
Mrpl52	1.251	0.077442	9

Gene	Ratio	p-value	Count
Hnrnpdl	1.263	0.075698	16
Dynlrb1	1.263	0.075698	10
Prmt7	1.263	0.075698	13
Pwp2	1.263	0.075698	12
Lamp1	1.262	0.075698	24
Ndufa9	1.262	0.075698	10
Zfp511	1.262	0.075698	10
Rexo2	1.262	0.075698	16
Nol7	1.262	0.075698	13
Dlst	1.262	0.075698	18
Atox1	1.261	0.076367	20
Ccm2	1.261	0.076367	11
Poli	1.261	0.076367	13
Gtf2h4	1.261	0.076367	12
Copz1	1.260	0.076367	16
Ift43	1.260	0.076367	10
Fsd1	1.260	0.076367	8
Hdac1	1.260	0.076367	14
Prss50	1.260	0.076367	14
Arl6ip5	1.260	0.076367	10
Cyb5r3	1.260	0.076367	14
Myl6	1.260	0.076367	32
Hs2st1	1.259	0.077442	19
Nup85	1.259	0.077442	15
Rapgef1	1.245	0.079253	20
3110040N11Rik	1.245	0.079253	8
Akt1	1.245	0.079253	11
Hlrip3	1.245	0.079508	11
Samd1	1.245	0.079508	11
AK010878	1.245	0.079508	21
Mrpl16	1.245	0.079508	9
Rgl2	1.245	0.079508	8

Fig. 26-12

Gene			Count
Snrpc	1.258	0.077442	16
Park7	1.258	0.077442	27
Eif4ebp3	1.258	0.077442	8
Drap1	1.258	0.077442	19
Rab33b	1.258	0.077442	24
Flot1	1.258	0.077442	9
Fntb	1.258	0.077442	11
Wdr45b	1.257	0.077442	11
Odf2	1.257	0.077442	11
Slc10a1	1.257	0.077442	17
Cenpt	1.257	0.077442	11
Nrde2	1.257	0.077442	14
Ndufab1	1.257	0.077442	12
Gcsh	1.257	0.077442	10
Msantd2	1.257	0.077442	7
Haus4	1.257	0.077442	9
Itm2b	1.257	0.077442	14
Ict1	1.256	0.077442	9
Ndufa3	1.256	0.077442	11
Dnajc8	1.256	0.077442	13
Akap8l	1.256	0.077442	12
Dnajc19	1.256	0.077442	18
1810022K09Rik	1.256	0.077442	10
Smim11	1.256	0.077442	8
Dhx30	1.256	0.077442	10
Ccnd1	1.256	0.077442	9
Mgl2	1.256	0.077442	23
Atp6v1f	1.256	0.077442	12
Rpl30	1.255	0.077442	49
Ubap2	1.255	0.077442	12
Rhoa	1.255	0.077442	26
Map2k2	1.255	0.077442	22

Count			Gene
14	1.251	0.077442	Dynll2
11	1.251	0.077442	Fmr1nb
13	1.251	0.077442	Bag1
9	1.251	0.077442	Tmem11
15	1.251	0.077442	Cdt1
11	1.251	0.077442	Me2
8	1.251	0.077442	Coa3
9	1.251	0.077442	Nipsnap3b
12	1.250	0.077442	Bag6
16	1.250	0.077442	Mcm5
15	1.250	0.077442	Zfp13
15	1.250	0.077442	Rad21
14	1.250	0.077442	Laptm4a
24	1.250	0.077442	Hat1
21	1.250	0.077442	Fbl
12	1.250	0.077442	Ppil3
13	1.250	0.077442	Mest
16	1.250	0.077442	Dtymk
11	1.249	0.07782	Slbp
20	1.249	0.07782	Llph
9	1.249	0.07782	Perp
11	1.249	0.07782	Pagr1a
12	1.249	0.078195	Ercc3
15	1.249	0.078195	1110004F10Rik
10	1.249	0.078195	Rp9
9	1.249	0.078195	Pop4
11	1.249	0.078195	Arg2
24	1.249	0.078255	Eif4b
13	1.249	0.078255	Pltrm1
9	1.249	0.078255	Jagn1
8	1.249	0.078255	Utp11l
10	1.249	0.078255	Snupn

Count			Gene
26	0.079508	1.245	Psma4
9	0.079508	1.244	Ogfod3
12	0.080016	1.244	Mtx1
17	0.080453	1.244	Ahcy
11	0.080453	1.244	Nelfe
10	0.080453	1.244	Tmem60
15	0.080453	1.244	Phax
12	0.080453	1.244	Rab14
9	0.080453	1.244	Fam89b
13	0.080453	1.244	2410015M20Rik
10	0.080453	1.244	Acadvl
10	0.080453	1.244	Pmf1
15	0.080453	1.243	Psmb2
16	0.080453	1.243	Farsb
11	0.080453	1.243	Ube2a
12	0.080453	1.243	Pced1b
7	0.080453	1.243	Hspb8
26	0.080453	1.243	Ywhae
10	0.080453	1.243	BC031181
8	0.080453	1.243	Zcchc17
9	0.080453	1.242	Fam162a
14	0.080453	1.242	Otud6b
9	0.080453	1.242	Sap30l
29	0.080515	1.242	Nop56
18	0.080515	1.242	Anxa7
9	0.080515	1.242	Rbm12b2
9	0.080515	1.242	Hiatl1
12	0.080515	1.241	Dclk2
10	0.080515	1.241	Akr1e1
6	0.080515	1.241	Blvra
25	0.080515	1.241	Pgam1
16	0.080515	1.241	Irf2bpl

Fig. 26-13

Gene	Value 1	Value 2	Count
Mrps34	1.255	0.077442	6
Zdhhc12	1.255	0.077442	10
Wbp1l	1.255	0.077442	16
Birc5	1.255	0.077442	18
Shmt1	1.255	0.077442	15
Cst3	1.255	0.077442	14
Maea	1.254	0.077442	15
Gadd45gip1	1.254	0.077442	13
Ppib	1.254	0.077442	16
Gpr108	1.254	0.077442	8
Chchd6	1.254	0.077442	7
Eif2d	1.254	0.077442	13
Zmiz2	1.254	0.077442	16
Clpp	1.254	0.077442	22
Skiv2l2	1.254	0.077442	10
Anp32e	1.253	0.077442	15
Wdr46	1.253	0.077442	17
Rpn2	1.253	0.077442	12
Bcam	1.253	0.077442	8
Abce1	1.253	0.077442	13
Slc25a12	1.253	0.077442	14
Anxa5	1.253	0.077442	14
Zc3h10	1.253	0.077442	11
Dnttip2	1.253	0.077442	16
Plscr1	1.252	0.077442	10
Cdk2ap2	1.252	0.077442	9
Gm12669	1.252	0.077442	12
Smpd1	1.252	0.077442	8
Mff	1.252	0.077442	11
Mogs	1.252	0.077442	7
Atp1a1	1.252	0.077442	15
Rsrp1	1.252	0.077442	9
Atpif1	1.252	0.077442	13
Srp9	1.252	0.077442	14
Wbp11	1.252	0.077442	16
Rnf220	1.252	0.077442	11

Gene	Value 1	Value 2	Count
Drg2	1.249	0.078255	10
Pecam1	1.248	0.078255	8
Psat1	1.248	0.078255	16
Nucks1	1.248	0.078255	27
Evl	1.248	0.078255	8
Uhrf2	1.248	0.078255	9
Ap2m1	1.248	0.078686	17
Tra2a	1.248	0.078686	11
Tfcp2l1	1.248	0.078686	22
Tex264	1.248	0.078686	9
Csnk1g2	1.248	0.078686	10
Myeov2	1.247	0.078686	16
Ints9	1.247	0.078686	14
Myadm	1.247	0.078686	16
Tns3	1.247	0.078686	12
Tecr	1.247	0.078686	11
Pycr2	1.247	0.078686	9
Arpc5l	1.247	0.078686	10
Cox5b	1.247	0.078686	26
Adap1	1.247	0.078686	11
Lypla2	1.247	0.078686	10
Hk2	1.247	0.078686	17
Mrpl40	1.246	0.078686	11
Msmo1	1.246	0.078686	6
Ndufa8	1.246	0.078686	10
Nifk	1.246	0.078686	15
St6galnac2	1.246	0.078686	11
Nradd	1.246	0.078686	13
Mcts2	1.246	0.078792	8
Cramp1l	1.246	0.078792	19
Nacc1	1.246	0.078792	20
Magoh	1.246	0.078792	13
Tspo	1.246	0.078792	11
Prss43	1.246	0.078792	26
Ing1	1.245	0.078792	11
Sept11	1.245	0.078792	13

Gene	Value 1	Value 2	Count
Wbp1	1.241	0.080515	10
Serf2	1.241	0.080515	24
2810004N23Rik	1.241	0.080515	10
Msh6	1.241	0.080515	13
Bap1	1.241	0.080515	11
Morc1	1.240	0.080515	12
Ccdc163	1.240	0.080515	9
Tmem97	1.240	0.080515	9
Trappc1	1.240	0.080515	9
Bcat2	1.240	0.080515	8
Cenpm	1.240	0.080515	9
Cotl1	1.240	0.080515	9
Mpv17l2	1.240	0.080515	7
Nob1	1.240	0.080515	13
1810037I17Rik	1.240	0.080515	9
Pno1	1.240	0.080515	9
Kif4	1.240	0.080515	15
Rrp9	1.240	0.080515	14
Ostc	1.240	0.080515	10
Nit2	1.239	0.080515	12
Ftsj2	1.239	0.080515	12
Uchl3	1.239	0.080515	9
Yeats4	1.239	0.080515	12
Dppa3	1.239	0.080515	15
1110001J03Rik	1.239	0.080515	7
2310009B15Rik	1.239	0.080515	7
Atp5c1	1.239	0.080515	22
Ndufb9	1.239	0.080515	16
Pet112	1.239	0.080515	11
Egfl7	1.238	0.080515	14
Taf9	1.238	0.080515	8
2610507B11Rik	1.238	0.080515	16
Arl2	1.238	0.080515	11
Rabggtb	1.238	0.080515	14
Mat2a	1.238	0.080515	19
Avpi1	1.238	0.080515	10

Fig. 26-14

Gene				Gene				Gene			
Dctpp1	1.238	0.080515	20	Cdk4	1.231	0.08545	13	Mt3	1.224	0.089235	10
Dctn3	1.238	0.080999	12	Mtg2	1.231	0.08545	7	Chmp1a	1.224	0.089235	11
Mrps18a	1.238	0.081183	9	Inpp5d	1.231	0.08545	11	Msc	1.224	0.089235	10
Slc35a4	1.238	0.081183	11	Cct2	1.231	0.08545	27	Mcm4	1.224	0.089235	17
Metap1	1.238	0.081183	11	Actr3	1.231	0.08545	17	Ube2d3	1.224	0.089235	17
Mrps10	1.237	0.081183	11	Top3b	1.230	0.08545	10	Nhp2l1	1.224	0.089235	19
Lmnb1	1.237	0.081183	20	Ube2g2	1.230	0.08545	9	Ranbp1	1.224	0.089235	24
Rnf167	1.237	0.081183	10	Ergic1	1.230	0.08545	12	Ifrd1	1.224	0.089235	7
Ywhag	1.237	0.081515	23	Ndufa11	1.230	0.08545	18	Ndufa2	1.224	0.089235	21
Zfp706	1.237	0.081515	24	Rer1	1.230	0.08545	10	Pou5f1	1.224	0.089235	55
Plod1	1.237	0.081515	7	Aco2	1.230	0.085902	14	Timm17a	1.224	0.089235	10
Gtf2f2	1.237	0.081515	12	Pspc1	1.230	0.087351	9	Cks1b	1.224	0.089235	16
Farp1	1.237	0.081646	10	Gltscr2	1.230	0.087351	15	Gmppa	1.224	0.089235	9
D8Ertd738e	1.237	0.081646	11	1700037H04Rik	1.230	0.087351	14	Fbxo45	1.224	0.089235	9
Ndufa12	1.237	0.081646	13	Mphosph8	1.229	0.087439	11	Dppa2	1.224	0.089235	12
Jarid2	1.237	0.081646	20	Urah	1.229	0.087439	10	Orai1	1.224	0.089235	7
Ntmt1	1.237	0.081646	9	Stk11	1.229	0.087439	10	Prr5	1.224	0.089235	7
Naca	1.237	0.081646	38	Rpl7l1	1.229	0.087439	48	Nrbp1	1.224	0.089235	10
Aldoa	1.237	0.081646	19	Aqp3	1.229	0.087439	6	Uqcrfs1	1.223	0.089235	10
Zswim1	1.236	0.081646	9	Ascc1	1.229	0.087439	10	Gm10094	1.223	0.089235	10
Pgs1	1.236	0.081646	10	Polr2f	1.229	0.087439	16	Josd2	1.223	0.089235	8
Phldb1	1.236	0.081973	11	Kif11	1.229	0.087439	16	Ndufb3	1.223	0.089235	8
Cers5	1.236	0.081973	8	Mapre1	1.229	0.087439	24	Coq2	1.223	0.089235	9
Cul7	1.236	0.081973	11	Nr4a1	1.229	0.087439	9	Upf3a	1.223	0.089235	8
Tspan3	1.236	0.081973	9	Ints5	1.228	0.087439	11	Pxk	1.223	0.089235	13
F2rl1	1.236	0.081973	12	Uspl1	1.228	0.087439	11	Pgd	1.222	0.089235	17
Lsm3	1.236	0.082051	15	Usp22	1.228	0.087439	10	Yars	1.222	0.089235	12
Elp2	1.236	0.082051	10	Rad23b	1.228	0.087439	17	Cdc45	1.222	0.089235	9
Ift20	1.236	0.082051	12	Eif3l	1.228	0.087439	10	Dnajc11	1.222	0.089235	11
Rev1	1.236	0.082051	13	Dut	1.228	0.087439	15	Gtf2e2	1.222	0.089235	11
Tbcb	1.236	0.082051	10	Cep89	1.228	0.087439	8	Tax1bp3	1.222	0.089235	8
Ankrd54	1.236	0.0820515	11	Tmbim6	1.228	0.087439	17	Tarbp2	1.222	0.089235	14

Fig. 26-15

Gene	Val1	Val2	N
Nckap1l	1.236	0.082051	11
Kcmf1	1.236	0.082051	17
Mkrn1	1.236	0.082374	25
Arhgdia	1.236	0.082374	19
Gldnos	1.236	0.082374	19
Tbrg4	1.235	0.082374	18
Tjap1	1.235	0.082374	7
Chmp3	1.235	0.082426	10
Dtnbp1	1.235	0.082426	5
Nsf	1.235	0.082426	15
Grtp1	1.235	0.082426	8
Supt5	1.235	0.082426	17
Morf4l1	1.235	0.082426	27
Tcf3	1.235	0.082426	11
Enoph1	1.235	0.082426	9
Pole4	1.235	0.082426	14
Prelid1	1.234	0.082426	23
Atxn10	1.234	0.082426	14
Ppp2r2a	1.234	0.082426	18
Slc39a7	1.234	0.082426	11
U2af2	1.234	0.082426	15
BC030867	1.234	0.082426	10
Ccndbp1	1.234	0.082426	7
Bod1	1.234	0.082426	9
Dctd	1.233	0.082426	9
Tpm1	1.233	0.082426	15
Chchd1	1.233	0.082426	10
Preb	1.233	0.082938	10
Taldo1	1.233	0.083351	14
Cox5a	1.233	0.083351	27
Myo1c	1.233	0.083351	17
Hs6st1	1.233	0.084271	14
Sugt1	1.228	0.088122	11
Fxr2	1.228	0.088122	15
Zglp1	1.228	0.088122	8
Slc35b1	1.228	0.088122	10
H2afv	1.228	0.088122	17
Rhot2	1.228	0.088122	8
Eif1	1.228	0.088122	35
Kdelr1	1.227	0.088486	12
Unc119	1.227	0.088486	8
Lmnb2	1.227	0.088486	10
Mphosph6	1.227	0.088486	11
Hmgn2	1.227	0.088486	15
Hsdl1	1.227	0.088486	12
Dnmt1	1.227	0.088486	14
Rdh14	1.227	0.088486	13
Isca2	1.226	0.088486	7
Tmem126a	1.226	0.088486	6
Dhrs4	1.226	0.088486	11
Mak16	1.226	0.088486	10
Ddx24	1.226	0.088486	11
Pvrl2	1.226	0.088486	11
Man2c1	1.226	0.088486	8
Creld1	1.226	0.088486	9
Lgsn	1.226	0.088486	15
Uck2	1.226	0.088486	8
Ldlrap1	1.226	0.088486	7
Ndufv3	1.226	0.088486	10
Banf1	1.226	0.088486	18
Phb	1.225	0.088486	18
Prkar1a	1.225	0.088919	14
Timm8a1	1.225	0.088919	10
Rps6ka1	1.225	0.089235	14
Cops5	1.222	0.089235	16
Lsm6	1.222	0.089235	12
Rce1	1.222	0.089235	8
Gadd45b	1.222	0.089235	8
Unc50	1.222	0.089235	9
Akap8	1.222	0.089235	10
Bckdha	1.221	0.089235	8
Isca1	1.221	0.089235	9
Derl1	1.221	0.089235	10
Nusap1	1.221	0.089235	10
Abtb1	1.221	0.089235	8
Evi5	1.221	0.089235	24
Lpar6	1.221	0.089235	9
Rgs19	1.221	0.089235	12
Eprs	1.221	0.089235	16
Oser1	1.221	0.089235	8
3110062M04Rik	1.221	0.089235	8
2700060E02Rik	1.221	0.089235	20
Tagap1	1.221	0.089235	7
Tmem192	1.221	0.089235	7
Trappc2l	1.220	0.089235	8
Tbx3	1.220	0.089235	18
Adipor2	1.220	0.089235	16
Dpm3	1.220	0.089235	10
Ddx41	1.220	0.089235	8
Tmem69	1.220	0.089235	12
Hadh	1.220	0.089235	9
Cstf2t	1.220	0.089235	21
Vps36	1.220	0.089235	7
Acads	1.220	0.089235	10
Cacybp	1.220	0.089235	17
Tjp2	1.220	0.089235	13

Fig. 26-16

Gene	Ratio	p-value	Count
Polr2d	1.233	0.084271	9
Mrpl33	1.232	0.084271	10
Gadd45a	1.232	0.084271	9
Fdps	1.232	0.084581	12
Orc2	1.232	0.084581	17
Fam207a	1.232	0.084581	14
Smg7	1.232	0.084581	10
Haus1	1.231	0.08545	9
Snrpg	1.231	0.08545	38
Ddx39	1.231	0.08545	15
Glrx3	1.231	0.08545	15
Tsen15	1.231	0.08545	8
Stt3a	1.219	0.089235	12
Rab3gap1	1.219	0.089235	20
Dync1i2	1.219	0.089235	11
Champ1	1.219	0.089235	10
Exosc5	1.219	0.089235	11
Ascc2	1.219	0.089235	8
Cnot10	1.219	0.089235	17
Mrps17	1.219	0.089235	8
Tma16	1.218	0.089235	10
Gprc5a	1.218	0.089235	11
Dok2	1.218	0.089235	9
Snrpa	1.218	0.089235	18
Rnf115	1.218	0.089235	10
Eml2	1.218	0.089235	9
Cox6b2	1.218	0.089235	8
Prdm15	1.218	0.089235	14
Itga3	1.217	0.089235	11
Rnf126	1.217	0.089235	13
Idh3b	1.217	0.089235	10
Cisd1	1.217	0.089235	10

Gene	Ratio	p-value	Count
Trmt6	1.225	0.089235	12
Dmwd	1.225	0.089235	9
Ndufaf3	1.225	0.089235	7
Rassf7	1.225	0.089235	9
Gtf2i	1.225	0.089235	11
Baz2b	1.225	0.089235	17
Scgb3a1	1.225	0.089235	8
Egln2	1.225	0.089235	10
Naa38	1.225	0.089235	17
Grb7	1.224	0.089235	10
Ndufs8	1.224	0.089235	9
Wdr83	1.224	0.089235	9
Etf1	1.213	0.089235	16
Dctn1	1.213	0.089235	12
Arih2	1.213	0.089235	14
Nck1	1.213	0.089235	7
Bst2	1.213	0.089235	8
Ppp1r10	1.213	0.089235	9
Smyd2	1.213	0.089235	10
Cpsf3l	1.212	0.089235	13
Smad4	1.212	0.089235	11
Klc3	1.212	0.089235	9
4930444M15Rik	1.212	0.089235	9
Ccnt1	1.212	0.089235	7
Atrx	1.212	0.089235	17
Rbbp7	1.212	0.089235	12
Mbnl2	1.212	0.089235	12
Phlda1	1.212	0.089235	11
Rbm17	1.212	0.089235	12
Tox4	1.212	0.089235	16
Srp72	1.212	0.089235	11
Uqcrq	1.212	0.089235	10

Gene	Ratio	p-value	Count
Mad2l2	1.220	0.089235	10
Pdk1	1.220	0.089235	16
Nop14	1.220	0.089235	13
Asna1	1.220	0.089235	13
Taf13	1.220	0.089235	8
Ube2e3	1.219	0.089235	11
Rnf25	1.219	0.089235	9
Angptl4	1.219	0.089235	7
Csnk1a1	1.219	0.089235	10
Gns	1.219	0.089235	8
Snord64	1.219	0.089235	4
Pdcd5	1.219	0.089235	14
Sqle	1.208	0.092108	8
Rab3ip	1.208	0.092108	10
Slc25a28	1.207	0.092108	9
Srf	1.207	0.092108	9
Pisd-ps3	1.207	0.092108	6
E430025E21Rik	1.207	0.092108	12
Mrpl15	1.207	0.092108	21
Echs1	1.207	0.092108	8
Fmnl3	1.207	0.092108	8
Mbtd1	1.207	0.092108	11
Atp5d	1.207	0.092108	21
Crls1	1.207	0.092108	11
Exosc6	1.207	0.092108	10
Pcbp4	1.206	0.092108	8
Psme4	1.206	0.092108	20
Syf2	1.206	0.092108	11
Nkain1	1.206	0.092108	11
Psmd3	1.206	0.092108	19
Srp68	1.206	0.092108	12
Mrps21	1.206	0.092108	10

Fig. 26-17

Gene				Gene				Gene			
Fam107b	1.217	0.089235	10	Mreg	1.212	0.089235	11	Lasp1	1.206	0.092108	15
Eif4e	1.217	0.089235	12	Epm2aip1	1.211	0.089235	22	Otx2	1.206	0.092108	8
Aars	1.217	0.089235	12	Zscan10	1.211	0.089235	10	Amn	1.206	0.092108	9
Nsfl1c	1.217	0.089235	11	Lmo4	1.211	0.089235	15	2510039O18Rik	1.206	0.092108	9
Nup43	1.217	0.089235	10	Cenph	1.211	0.089235	9	Dbf4	1.206	0.092108	13
Mrps16	1.217	0.089235	15	Fgfr1	1.211	0.089235	11	Triml2	1.206	0.092108	8
Psmg4	1.217	0.089235	8	Apmap	1.211	0.089235	10	Hagh	1.206	0.092108	10
Zmat2	1.216	0.089235	13	E2f6	1.211	0.089235	10	Pigp	1.206	0.092108	27
Fxyd6	1.216	0.089235	11	Thap7	1.211	0.089235	10	LOC101056043	1.205	0.092108	10
Mcm3	1.216	0.089235	21	Itga6	1.211	0.089235	13	Glo1	1.205	0.092108	16
Rtn4	1.216	0.089235	14	Tppp3	1.211	0.089235	8	Tnfrsf12a	1.205	0.092384	8
Frat2	1.216	0.089235	10	Ube2m	1.211	0.089235	18	Taf12	1.205	0.092384	8
Adss	1.216	0.089235	14	Gm13051	1.211	0.089352	13	Tssc4	1.205	0.092384	10
Gemin2	1.216	0.089235	9	Nsun2	1.211	0.089352	15	Ptdss2	1.205	0.092384	9
Cct3	1.216	0.089235	29	Snora73b	1.211	0.089352	3	Ciapin1	1.205	0.092583	21
Copa	1.216	0.089235	15	Gm5801	1.211	0.089352	13	Hint2	1.204	0.092583	8
Shfm1	1.216	0.089235	20	Tubb4b	1.210	0.089352	42	Znhit3	1.204	0.092583	11
Lamtor5	1.215	0.089235	10	Slc25a33	1.210	0.089352	9	Ola1	1.204	0.092583	14
Rcn1	1.215	0.089235	12	Strip1	1.210	0.089352	10	Lamtor3	1.204	0.092583	8
Rras2	1.215	0.089235	9	Chd4	1.210	0.090159	26	Cct7	1.204	0.092583	31
Khdrbs3	1.215	0.089235	9	Tuba1a	1.210	0.090584	43	Faf1	1.204	0.092583	11
Rab1	1.215	0.089235	12	Taf1c	1.210	0.09065	8	Emc4	1.204	0.092583	8
Psme1	1.215	0.089235	7	Ncapg	1.210	0.09065	11	Tyro3	1.204	0.092583	16
Ap1m1	1.215	0.089235	9	Dpp3	1.210	0.09065	9	Dpp9	1.204	0.092583	9
Vps28	1.215	0.089235	7	Myd88	1.210	0.09065	9	Phlda2	1.204	0.092583	6
Rtca	1.215	0.089235	8	Snrnp200	1.210	0.09065	17	Ap4s1	1.204	0.092583	7
Fars2	1.215	0.089235	10	Folr1	1.210	0.09065	11	Ttc1	1.204	0.092583	8
Phtf2	1.215	0.089235	12	Mrps28	1.209	0.09065	9	Ccnf	1.204	0.092583	10
Cd2bp2	1.215	0.089235	12	Lrch4	1.209	0.09065	11	Noc2l	1.203	0.092811	12
Nudt4	1.215	0.089235	12	Cd151	1.209	0.090849	8	Gadd45g	1.203	0.092811	9
Fam13b	1.215	0.089235	8	Mcts1	1.209	0.090849	8	Numa1	1.203	0.092811	15
Ap2b1	1.215	0.089235	13	Nudc	1.209	0.090849	22	Ssbp3	1.203	0.092811	13

Fig. 26-18

Gene				Gene				Gene			
Rraga	1.215	0.089235	9	Phyhd1	1.209	0.090849	12	Mrpl37	1.203	0.093266	10
Tmem41a	1.215	0.089235	8	Mtfmt	1.209	0.091736	20	Xrcc6	1.203	0.093266	10
Msantd3	1.215	0.089235	8	Hdgfrp2	1.209	0.091842	12	Ap2a2	1.203	0.093266	12
Zdhhc9	1.215	0.089235	8	Mapk13	1.209	0.091842	13	Mbtps1	1.203	0.093266	12
Aven	1.214	0.089235	9	Lman2	1.209	0.091842	13	Atp5j	1.203	0.093266	17
Ralbp1	1.214	0.089235	14	1500011K16Rik	1.209	0.091842	10	Akirin1	1.203	0.093266	11
Pdcd6	1.214	0.089235	8	Tmem134	1.209	0.091842	10	Rnps1	1.203	0.093266	15
Sptan1	1.214	0.089235	19	Wdr3	1.209	0.091842	11	Fam192a	1.203	0.093266	8
Gnb2	1.214	0.089235	18	Bloc1s4	1.209	0.091842	7	Jak3	1.203	0.093266	9
Ptpn6	1.214	0.089235	8	Ppp5c	1.209	0.091842	12	Tars	1.203	0.093623	15
Plekhj1	1.214	0.089235	9	Mdk	1.208	0.092006	12	Sept2	1.203	0.093623	18
Vmn2r15	1.214	0.089235	7	Cog4	1.208	0.092006	9	Cnih1	1.202	0.09376	10
Jam3	1.214	0.089235	9	2410003L11Rik	1.208	0.092006	6	Zic3	1.202	0.09376	17
Rbm19	1.214	0.089235	12	Ddx50	1.208	0.092006	13	Zfp428	1.202	0.09376	10
H1f0	1.214	0.089235	8	Selk	1.208	0.092006	10	Foxk2	1.202	0.09376	11
Ddx54	1.214	0.089235	11	Ddah1	1.208	0.092006	13	Txndc15	1.202	0.09376	7
Tmem115	1.214	0.089235	8	Fem1b	1.208	0.092006	15	Mcm10	1.202	0.094119	11
Klk8	1.214	0.089235	9	Ilf2	1.208	0.092006	10	Rmnd5a	1.202	0.094119	16
Ankfy1	1.214	0.089235	30	St14	1.208	0.092006	13	Arfgap1	1.202	0.094119	9
Rnh1	1.214	0.089235	8	Klhl13	1.208	0.092006	9	D2Wsu81e	1.202	0.094119	7
Sfswap	1.214	0.089235	9	Eif3f	1.208	0.092006	15	Rae1	1.202	0.094119	14
Scrib	1.213	0.089235	15	Sucla2	1.208	0.092006	9	Mrpl11	1.202	0.094119	13
Asrgl1	1.213	0.089235	7	Smdt1	1.208	0.092006	14	Timp1	1.202	0.094119	7
Ctbp2	1.213	0.089235	19	Nfatc2ip	1.208	0.092006	15	Ckap2l	1.202	0.094119	8
Ttc13	1.202	0.094119	8	Mrps33	1.197	0.095294	12	Nicn1	1.192	0.099753	15
Ccna2	1.202	0.094119	12	Timm22	1.197	0.095294	10	Nfu1	1.192	0.099753	7
Fam63a	1.202	0.094119	10	Lrwd1	1.197	0.095294	8	Rhox6	1.192	0.099753	6
Pafah1b3	1.202	0.094119	7	Mien1	1.197	0.095294	10	Vps4a	1.192	0.099753	9
Dpm2	1.201	0.094253	10	Golph3	1.197	0.095294	8	Zdhhc16	1.192	0.099753	9
Dhcr24	1.201	0.094253	13	Srsf7	1.197	0.095294	17	Plp2	1.191	0.099753	9
Uqcrc2	1.201	0.094253	15	Dqx1	1.197	0.095294	12	Crl	1.191	0.099753	12
Myo10	1.201	0.094253	11	Ncbp1	1.197	0.095294	9	Zfp330	1.191	0.099753	9

Fig. 26-19

Gene				Gene				Gene			
Clasrp	1.201	0.094253	10	Grpel1	1.196	0.095294	12	Elf3	1.191	0.099753	10
Pstk	1.201	0.094253	9	Mpc2	1.196	0.095294	9	Tomm34	1.191	0.099753	9
Rbm14	1.201	0.094399	8	Smc4	1.196	0.095294	11	Fam110a	1.191	0.099753	11
Brd2	1.201	0.094399	20	Rcc2	1.196	0.095294	29	Dguok	1.191	0.099753	8
Fastk	1.201	0.094399	9	BC005624	1.196	0.095294	11	Cdkal1	1.191	0.099753	9
Sec63	1.201	0.094399	13	Mrpl22	1.196	0.095294	7	1110007C09Rik	1.191	0.099753	7
Flot2	1.201	0.094399	9	Naa10	1.196	0.095294	11	Ncoa5	1.191	0.099753	10
Tmem259	1.201	0.094399	14	Mrps18b	1.196	0.095294	12	Anxa11	1.191	0.099753	7
Mob4	1.201	0.094399	12	Enah	1.196	0.095294	20	Sirt4	1.191	0.099753	8
Dnajc15	1.201	0.094399	7	Rbbp5	1.196	0.095459	9	Hmbs	1.191	0.099753	9
Trim35	1.201	0.094399	10	Fam96a	1.196	0.095459	8	Anxa3	1.191	0.099753	10
Pla2g1b	1.201	0.094399	7	Gm5860	1.196	0.095459	21	Ppp1cc	1.191	0.099753	18
Asun	1.200	0.094399	17	Arglu1	1.196	0.095459	11	Fbxo42	1.191	0.099753	13
Mtap	1.200	0.094399	10	Rpusd4	1.196	0.095459	7	Ufc1	1.190	0.099753	11
Znhit1	1.200	0.094399	9	Anp32b	1.196	0.095459	26	Nt5dc2	1.190	0.099753	8
Pepd	1.200	0.094399	10	Tmx2	1.196	0.095459	12	Fh1	1.190	0.099753	8
Ngdn	1.200	0.094399	8	Mettl9	1.196	0.095459	8	Polr2c	1.190	0.099753	12
Coq6	1.200	0.094399	7	Med28	1.196	0.095459	15	Vdac2	1.190	0.099753	23
Grwd1	1.200	0.094399	10	Mcm2	1.196	0.095459	20	Atxn7l3b	1.190	0.099753	15
Fig4	1.200	0.094399	11	Get4	1.196	0.095459	15	Afg3l2	1.190	0.099753	9
Cox20	1.200	0.094399	9	**Tcfl5**	1.195	0.095459	9	Tex13	1.190	0.099753	12
Abcg2	1.200	0.094399	9	Ubr7	1.195	0.095459	11	Mrto4	1.190	0.099753	10
Ntpcr	1.200	0.094399	7	Srsf3	1.195	0.095459	21	Tspyl2	1.190	0.099753	8
Canx	1.200	0.094399	20	Xbp1	1.195	0.096124	11	Otulin	1.190	0.099753	8
Ptbp1	1.200	0.094399	29	Phyh	1.195	0.096124	8	Mrps11	1.190	0.099753	8
Etaa1	1.200	0.094399	12	Ddah2	1.195	0.096372	8	Vkorc1	1.190	0.099753	9
Atad3a	1.200	0.094399	13	Rbm39	1.195	0.096372	16	Ppp1r15b	1.190	0.099753	13
Taf10	1.199	0.094399	9	Rap1b	1.195	0.096372	10	Ngrn	1.190	0.099753	9
Klhl21	1.199	0.094399	14	Basp1	1.195	0.096372	12	H3f3a	1.190	0.099753	25
Zcrb1	1.199	0.094399	11	Alg3	1.195	0.09666	9	Cwc15	1.189	0.099753	10
Mrpl18	1.199	0.094399	13	NrOb1	1.195	0.09666	7	Grb2	1.189	0.099753	16
Sdf2l1	1.199	0.094399	12	Polg	1.194	0.096948	9	Loxl2	1.189	0.099753	28

Fig. 26-20

Athl1	1.199	0.094399	12	Rpp25l	1.194	0.096948	9	Snx32	1.189	0.099753	8
Cmtm6	1.199	0.094399	12	Lin37	1.194	0.096948	5	Npdc1	1.189	0.099753	8
Emc3	1.199	0.094399	9	R3hdm4	1.194	0.097607	8	2310011J03Rik	1.189	0.099753	9
Metrn	1.199	0.094399	7	Gemin4	1.194	0.097607	10	Zfp936	1.189	0.099753	8
Thoc7	1.199	0.094399	13	BC052040	1.194	0.097607	9	Pdap1	1.189	0.099753	26
Prc1	1.199	0.094399	9	Zfp598	1.194	0.097935	8	Rgs2	1.189	0.099753	13
Srrm1	1.199	0.094399	17	Prmt1	1.194	0.097935	20	Cdc34	1.189	0.099753	22
Glt25d1	1.199	0.094399	11	Rfc3	1.194	0.09805	11	Dgkd	1.189	0.099753	9
Hars	1.199	0.094399	13	Cops4	1.194	0.09805	12	Snord47	1.189	0.099753	5
Rexo4	1.199	0.094399	10	Aplp2	1.194	0.09805	12	Tagln3	1.189	0.099753	6
Lpar5	1.199	0.094399	9	Mrps18c	1.194	0.09805	8	Gm12504	1.189	0.099753	41
Rab40c	1.199	0.094616	12	Pnpo	1.194	0.09805	11	Lamtor1	1.189	0.099753	13
Cnot2	1.198	0.094616	15	Vat1	1.193	0.09805	9	Suds3	1.189	0.099753	11
Thop1	1.198	0.094616	9	Nkiras2	1.193	0.09805	9	Prdx5	1.189	0.099753	7
Rad17	1.198	0.094616	9	Etfb	1.193	0.09805	11	Gar1	1.189	0.099753	15
Rela	1.198	0.094616	9	Esyt1	1.193	0.098419	9	1110065P20Rik	1.188	0.099753	7
Syce1	1.198	0.094616	8	Tatdn2	1.193	0.098985	9	Adck4	1.188	0.099753	8
Jkamp	1.198	0.094616	9	Zfp787	1.193	0.098985	10	Mrpl10	1.188	0.099753	10
Rbm38	1.198	0.094616	9	Sdhd	1.193	0.098985	11	Cpsf4	1.188	0.099753	9
Bckdhb	1.198	0.094616	7	Vars	1.193	0.098985	12	Pbdc1	1.188	0.099921	22
Mfsd10	1.198	0.094616	8	Abcf1	1.193	0.098985	14	Gclm	1.188	0.099921	12
Mthfd1	1.198	0.094616	13	Prkab1	1.193	0.099182	9	Fam136a	1.188	0.099921	10
Exosc4	1.198	0.094616	12	Khsrp	1.193	0.099182	17	Mlh1	1.188	0.099921	7
Uqcrc1	1.198	0.094618	15	Acvr2b	1.192	0.099182	7	Usp48	1.188	0.099921	11
Zc3h15	1.198	0.094618	13	Nr1h4	1.192	0.099182	9	Dctn2	1.188	0.099921	14
Ccdc181	1.198	0.094618	8	Ccdc166	1.192	0.09942	7	Timeless	1.188	0.099921	14
Fbxw11	1.198	0.094618	14	Ehd4	1.192	0.09942	10	U2af1l4	1.188	0.099921	8
Rrbp1	1.198	0.094618	16	Sec11a	1.192	0.09942	14	Snhg8	1.187	0.099921	12
Mzt2	1.198	0.094618	9	Cpsf1	1.192	0.09942	10	Cdc42	1.187	0.099921	22
Surf2	1.198	0.094618	7	Ecd	1.192	0.099753	13	Hint1	1.187	0.099921	26
Las1l	1.198	0.094618	15	Mrps6	1.192	0.099753	7	Arpc3	1.187	0.099921	10
Pla2g6	1.198	0.094618	12	Hsf1	1.192	0.099753	9	Mfap3	1.187	0.099921	18

Fig. 26-21

Mknk2	1.198	0.094618	8
Ttc27	1.198	0.094958	11
Ppp2r5d	1.197	0.094958	13
Prpf38a	1.197	0.095294	8
Abcf2	1.187	0.099921	15
Manbal	1.187	0.099921	10
Fis1	1.186	0.099921	10
Pgrmc1	1.186	0.099921	8

Wdr45	1.192	0.099753	11
Smarca4	1.192	0.099753	14
Sar1a	1.192	0.099753	16
Csnk2a1	1.192	0.099753	24
Mbip	1.186	0.099921	7
Trmt61a	1.186	0.099921	7
Ppp1r18	1.186	0.099921	8
Gm6792	1.186	0.099921	6

Nif3l1	1.187	0.099921	12
Pdia4	1.187	0.099921	9
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Nt5c	1.186	0.099921	7

Fig. 26-22
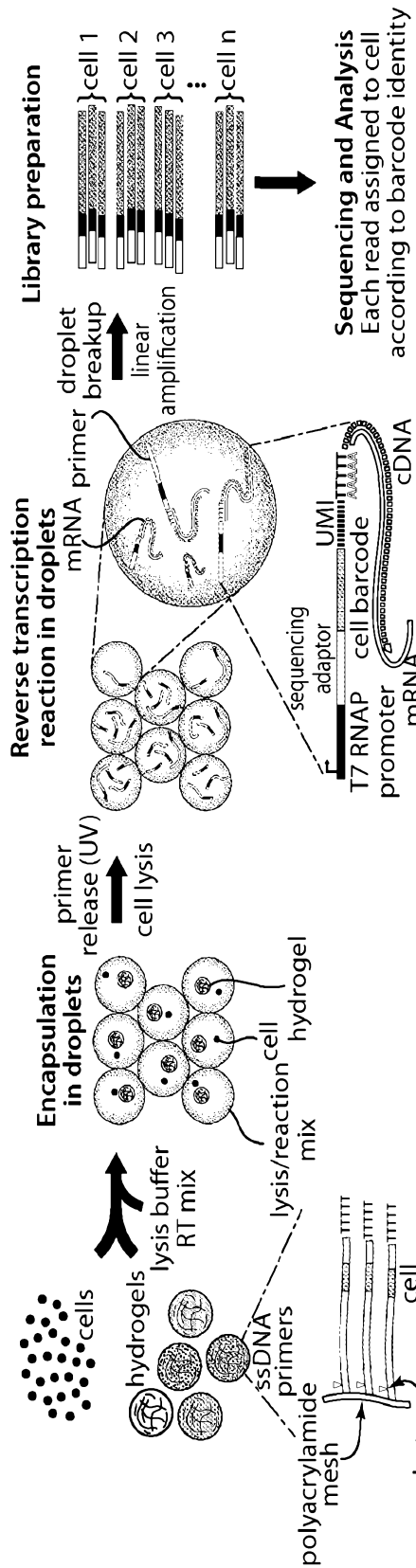
Fig.27

<110>   PRESIDENT AND FELLOWS OF HARVARD COLLEGE
         VILNIUS UNIVERSITY

<120>   SYSTEMS AND METHODS FOR BARCODING NUCLEIC ACIDS

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