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(54) **Title**: GEL BEADS IN MICROFLUIDIC DROPLETS

(57) **Abstract**: This invention relates to the use of gel beads in microfluidic droplets to perform multi-step compartmentalised reactions in vitro. Methods may comprise emulsifying an aqueous reporter solution which comprises polynucleotides, a reporter substrate, and a gel-forming agent into microdroplets. Each polynucleotide encodes a product which converts the reporter substrate into a detectable reporter in the microdroplets. The gel-forming agent is then solidified within the microdroplets to produce gel beads comprising both the polynucleotide and the detectable reporter produced by the product. The aqueous microdroplets are then de-emulsified and re-suspended in aqueous detection solution and the reporter detected in one or more beads the population. These methods allow high throughput and are particularly useful for biochemical processes such as high-throughput screening, directed evolution and genotyping methods.

Gel Beads in Microfluidic Droplets

This invention relates to the use of gel beads in microfluidic droplets in compartmentalised *in vitro* methods, such as screening.

Compartmentalisation of individual samples in aqueous droplets dispersed in an oil phase is a powerful method for high-throughput assays in chemistry and biology. Here the droplet is the equivalent of the test tube. The droplet contains everything needed to assess and decode a particular experiment or profile of a library member. Droplets produced with bulk emulsion techniques are not uniform in size and complications arise in experiments where a quantitative readout is required. Microfluidic devices allow the production of highly monodisperse aqueous droplets, in frequencies up to several (ten-) thousands per second. They are typically 10-200 microns in diameter, corresponding to volumes between 0.5 pL and 4 nL. In addition to droplet formation, the microfluidic format allows a number of other unit operations such as droplet fission, fusion, incubation, analysis and sorting.

However, compatibility of different biochemical reactions to each other is a problem when working with water-in-oil droplets. Most biochemical protocols are multi-step processes: solutions are added, samples centrifuged, supernatants removed, pellets washed and so on. Transferring such protocols to droplets is difficult. Although droplet-droplet fusion for adding and/or diluting components can be performed with specialised microfluidic devices, it is practically challenging when large droplet numbers are involved and additional steps (e.g. washing, buffer exchange, addition or removal of reagents) are necessary, and difficult or impossible to implement in a continuous workflow.

The present inventors have developed techniques for performing multi-step compartmentalised reactions *in vitro* using gel beads in microfluidic droplets. These techniques are particularly useful for

biochemical processes such as high-throughput screening, directed evolution and genotyping methods.

An aspect of the invention provides a method of compartmentalised *in vitro* screening comprising:

1). emulsifying an aqueous reporter solution which comprises a population of polynucleotides, a reporter substrate, and a gel-forming agent into microdroplets,

wherein each polynucleotide encodes a product which converts the reporter substrate into a detectable reporter in said microdroplets,

2). solidifying the gel-forming agent within the microdroplet to produce a gel bead comprising the polynucleotide and the detectable reporter produced by the product,

3). de-emulsifying the aqueous microdroplets and re-suspending the beads in aqueous detection solution and

4). detecting, determining or measuring the detectable reporter in one or more beads in said population.

One or more beads from the aqueous solution which contain the detectable reporter or which lack the detectable reporter may be identified and/or isolated. Polynucleotide from the one or more identified beads may be identified, amplified, cloned, sequenced or otherwise investigated.

The polynucleotides or nucleic acids may be isolated, for example in plasmids, viruses, or PCR products, or may be comprised in cells or viral particles. The polynucleotides are retained within the gel beads.

Preferably, the reporter solution is emulsified such that each microdroplet contains either a single polynucleotide molecule, or no polynucleotide molecules, either isolated or within a cell or viral particle. This may be achieved using suitable dilutions of the polynucleotide in the reporter solution. The polynucleotide molecule in the microdroplet is then captured in the solidified bead.

The population of polynucleotides may be a library of sequences, for example 10^3 or more, 10^4 or more, 10^5 or more, 10^6 or more, 10^7 or more, 10^8 or more or 10^9 or more different sequences.

The reporter solution provides suitable conditions and reagents for the reporter substrate to be converted into the detectable reporter by the encoded product.

The product encoded by the polynucleotide may be polypeptide, e.g. an enzyme. Suitable enzymes include hydrolases (such as lipases, proteases, phosphotriesterases, phosphate mono-, di- and triester hydrolases, sulfatase, glycosidases), cellulases, oxidases (such as laccases, glucose oxidases, NADPH oxidases, cytochrome P450 oxidases and peroxidases), reductases (such as nitroreductase), ligases, lyases, transferases and isomerases. Other enzymes in EC enzyme classifications EC1-EC6 may also be encoded by the polynucleotide.

In other embodiments, the encoded products may be catalytic polynucleotides, such as ribozymes.

In some preferred embodiments, the product may be a polypeptide which comprises an epitope tag, for example at the N or C terminal. This facilitates capture in the bead. Epitope tags are described in more detail below.

The gel forming agent is an agent, for example a polymer such as polysaccharide or polypeptide, which can be solidified from a liquid into a gel, for example by alteration of conditions, such as heating, cooling, or altering pH.

Suitable gel forming agents include alginate, gelatine and agarose and other gels having a sol phase sufficiently fluid to move through the channels of a microfluidic device. Preferably agarose, which is a linear polymer made up of the repeating units of a disaccharide (D-galactose and 3, 6-anhydro-L-galactopyranose), is employed. The gel forming agent may be solidified into a bead by any convenient method,

for example by changing the conditions. Preferably the hydrogel forming agent is solidified by altering the temperature, for example by cooling.

In some embodiments, the polynucleotide may be expressed to produce the encoded product within the microdroplet following emulsification, such that the encoded product co-localises with the polynucleotide within the same microdroplet.

In other embodiments, the aqueous reporter solution may further comprise the products encoded by the polynucleotides before emulsification. The products may be co-localised with their encoding polynucleotide in the reporter solution, such that each polynucleotide in the reporter solution is emulsified into the same aqueous microdroplet as its encoded product. Preferably, a polynucleotide is non-covalently associated with its encoded product, for example the polynucleotide and the encoded product may be contained within the same bead, cell or viral particle. The polynucleotide and its encoded product may be linked directly or indirectly through a non-covalent attachment.

In some preferred embodiments, each polynucleotide and its encoded product are contained in a gel bead. In other embodiments, each polynucleotide and its encoded product are not contained in a gel bead. For example, the gel-forming agent may solidify to form a bead in the microdroplet which contains the polynucleotide, its encoded product and the detectable reporter.

For example, the polynucleotide may be contained in a cell, viral particle or extract thereof and the hydrogel-forming agent may be solidified to form a bead in the microdroplets which contains the cell, or viral particle or extract thereof, along with the detectable reporter. The polynucleotide is contained in the gel bead when it solidifies and is retained within the bead. This allows the identification, isolation and/or further investigation of the

polynucleotide contained in the one or more of the beads identified as containing the detectable reporter.

Suitable cells include bacterial cells, such as E. coli, or eukaryotic cells, such as yeast or mammalian cells. The encoded product, for example an encoded polypeptide, may be secreted from the cell, expressed on the cell surface or expressed intracellularly. The cell may be lysed during or after emulsification. For example, the reporter solution may include a lysis reagent which lyses the cell.

Suitable reagents include lysozyme and detergent based lysis agents, such as triton X-100, Bugbuster™ (Novagen Inc USA) or Cellytic B (Sigma-Aldrich).

Suitable viral particles include filamentous phage, such as M13 and fd phage, T4, T7, baculovirus and lambda phage.

In some embodiments, the reporter solution may comprise a population of cells, such as *E. coli* or yeast, or viral particles which contain a library of polynucleotides, each cell or particle containing one polynucleotide sequence. In some embodiments, the polynucleotide in the cells or particles may be expressed to produce the encoded polypeptide, for example an intracellular or surface-bound polypeptide, and the cells or particles of the population containing both the polynucleotide and its encoded product may then be emulsified into separate microdroplets. The cells or particles co-localise the polynucleotide with the encoded product, such that each microdroplet contains both polynucleotide and encoded product. In other embodiments, the cells or particles of the population may be emulsified into separate microdroplets and the polynucleotide then expressed after emulsification to produce the encoded product in the microdroplet.

Alternatively, the polynucleotides may be contained in viral particles, for example, M13, fd, T4, T7 or lambda phage particles, and the reporter solution may further comprise a population of cells. The

cells may be transfected with the viral particles before, during or after emulsification, such that the cells express the polynucleotide to produce the encoded product and/or produce viral particles containing the polynucleotide and the encoded product.

In other embodiments, the polynucleotide and product may be contained in a gel bead. For example, the reporter solution may comprise a population of gel beads, each bead containing a polynucleotide and its encoded product. Preferably, each microdroplet contains a single bead following emulsification. When the microdroplet contains a gel bead, the hydrogel-forming agent may be solidified around the existing bead to form an outer layer.

The reporter solution which contains the liquid gel forming agent and the polynucleotide may be emulsified into microdroplets by any convenient technique.

The aqueous microdroplets may be microfluidic droplets. Microroplets may be produced by emulsifying the aqueous reaction mixture in a non-aqueous liquid, for example oil, comprising a surfactant. Techniques for the production of microfluidic aqueous droplets are well known in the art (Anna, S. L., et al (2003) *Appl. Phys. Lett.*, 82, 364-366; Xu, S. et al. (2005). *Angew. Chem. Int. Ed. Engl.*, 44, 724-728; Shah, R. K et al (2008) *Mater. Today* 11, 18-27; Shah, R. K., et al (2008) *Soft Matter*, 4, 2303-2309; Zhang, H., et al (2007). *Macromol. Rapid Commun.*, 28, 527-538; Oh, J. K., et al (2008) *Prog. Polym. Sci.*, 33, 448 - 477; Choi C.-H., et al (2007) *Biomed. Microdevices*, 9, 855-862).

Aqueous microdroplets may be monodisperse i.e. have a uniform size. Suitable microdroplets may be 1-300 microns in diameter, for example 10 to 200 microns.

A method may comprise providing a population of gel beads, each bead containing a polynucleotide and its encoded product. Various methods may be employed. For example, a method may comprise, before step (1) above:

i). emulsifying an aqueous expression solution comprising a polynucleotide and a gel-forming agent into a microdroplet,

ii). allowing the aqueous expression solution to express the polynucleotide in the microdroplets to produce a product encoded by the polynucleotide and

iii) solidifying the gel-forming agent to produce a gel bead comprising the polynucleotide and the product within the microdroplet;

wherein the product is produced before, at the same time as or after the hydrogel-forming agent is solidified,

iv). de-emulsifying the aqueous microdroplet and isolating the bead.

In some embodiments, a method may comprise:

i). emulsifying an aqueous expression solution comprising a diverse population of polynucleotides and a gel-forming agent into a microdroplets,

ii). allowing the aqueous expression solution to express the polynucleotide in the microdroplets to produce a product encoded by the polynucleotide and

iii) solidifying the gel-forming agent to produce a gel bead comprising the polynucleotide and the product within the microdroplet;

wherein the product is produced before, at the same time as or after the hydrogel-forming agent is solidified,

iv). de-emulsifying the aqueous microdroplet and isolating the bead.

The solution may be emulsified such that each microdroplet contains none or one polynucleotide molecule i.e. the polynucleotides are compartmentalised in the emulsion.

In some embodiments, the polynucleotide may be amplified before the encoded product is produced. This may be useful for example, when the polynucleotide is comprised within a plasmid in the microdroplet and may facilitate recovery and retrieval of the polynucleotide after screening. Before step (1), a method may comprise:

i) emulsifying an aqueous amplification solution comprising a polynucleotide and a hydrogel-forming agent into microdroplets,

ii) allowing the amplification reaction solution to amplify the polynucleotide in the microdroplets to produce amplified copies of the polynucleotide,

iii) solidifying the hydrogel-forming agent within the microdroplet to produce a core gel bead comprising the amplified copies of the polynucleotide;

wherein the polynucleotide is amplified before, at the same time as or after the hydrogel-forming agent is solidified,

iv) de-emulsifying the aqueous microdroplets and isolating the beads, and;

v) contacting the beads with an aqueous expression solution to react with the amplified copies in the microdroplets to produce a product encoded by the polynucleotide,

wherein the encoded product is contained in the solidified bead and thus co-localised with the polynucleotide.

The solution may be emulsified such that each microdroplet contains none or one polynucleotide molecule i.e. the polynucleotides are compartmentalised in the emulsion.

Amplification may be helpful in facilitating isolation and recovery of polynucleotides from beads identified by the screening methods described herein.

The amplification reaction solution may comprise amplification reagents, including enzymes and primers and nucleotides, to produce amplified polynucleotide copies of the polynucleotide. Methods of nucleic amplification in microdroplets are well known in the art (see for example, Mak, W. C., Cheung, K. Y., and Trau, D. (2008) *Adv. Funct. Mater.* 18, 2930-2937; Walser, M. *et al* (2009) *Polynucleotides Res.* 37 e57.) Preferably, the amplification reagents are isothermal amplification reagents. Techniques for isothermal amplification in agarose gels are well known in the art and include multiply-primed RCA with the Phi29 DNA polymerase, which produces a high molecular weight (>40 kb) and hyperbranched products containing amplified copies of the polynucleotide. (Michikawa, Y.et al. (2008). *Anal. Biochem.*, 383, 151-

158). Amplified DNA, especially hyperbranched amplified DNA, is unable diffuse out of the bead matrix.

When the polynucleotide and the encoded product remained co-localised without compartmentalisation, the beads may be contacted with the aqueous expression solution without emulsification. The ability of the polynucleotide and the encoded product to remain co-localised depends on the concentrations and biophysical properties (e.g. size) of the encoded product.

In other embodiments, for example where the polynucleotide and the encoded product do not remain co-localised, the beads and the aqueous expression solution may be emulsified such that the beads contact the expression solution in aqueous microdroplets. For example, step v) set out above may be performed with the beads contained within a microdroplet containing the aqueous expression solution. Step v) may comprise;

(i) emulsifying the beads comprising the amplified copies in microdroplets which comprise the aqueous expression solution,

(ii) allowing the aqueous expression solution to express the amplified copies of the polynucleotide in each said bead to produce a product encoded by polynucleotide in the bead, and

(iii) de-emulsifying the microdroplets.

The solution may be emulsified such that each microdroplet contains none or one bead i.e. the beads are compartmentalised in the emulsion.

The expression solution may comprise *in vitro* transcription/translation reagents which produce encoded polypeptide from the amplified polynucleotide copies. Suitable in vitro transcription/translation reagents are well known in the art (e.g. Isalan, M. et al (2005) *PLoS Biol*. 3 e64)

The hydrogel-forming agent may be solidified by any convenient technique. For example, when the agent is agarose, it may be solidified by reducing the temperature, for example below 25°C, below

20°C or below 15°C. The precise gelling temperature is dependent on the type of agarose and its concentration and may be easily determined by the skilled person. For example, 0.5% to 2% of ultra-low melting point agarose Type IX-A(Sigma) has a gelling point of about 17°C.

Solidification of the agent within the microdroplet moulds the solidified gel into a bead. The population of solidified beads may be monodisperse.

The encoded product may be retained in the beads by any convenient method. For example, the product may be retained in the beads through entrapment within the gel matrix or through covalent or non-covalent binding to a retention agent or the gel matrix itself.

Molecules, such as polypeptides and polynucleotides, may be retained in the beads by virtue of their size. For example, molecules greater than a threshold size may be unable to diffuse out of the bead through pores in the gel and may therefore be trapped within the gel matrix of the bead. For example, polynucleotides, such as plasmids and amplified copies of a polynucleotide, may be retained in the bead. In some embodiments, the gel may retain particles having a diameter of 50nm or more, although the precise threshold depends on the type of gel and the concentration.

In some embodiments, an aqueous solution in which a gel bead is solidified following emulsification, for example a solution comprising a hydrogel-forming agent, such as the aqueous expression, amplification and/or reporter solution described above, may further comprise one or more retention agents to reduce or prevent the diffusion of polynucleotide or encoded products from the bead, for example by binding to polynucleotides or encoded products.

Suitable retention agents for capture of molecules include particles, for example non-synthetic particles such as viral particles, or synthetic particles, such as nano-or micro-particles, or polymers including cyclodextrins or dextrans, which are sufficiently large to

be entrapped by the gel and unable to diffuse out of the bead. The retention agent may bind to the encoded product and/or the detectable reporter and retain it within the gel. For example, a retention agent may comprise an antibody coated particle which binds to an expressed polypeptide. Retention agents are described in more detail below.

Suitable nano- or micro-particles are well-known in the art. For example, a particle may be coated with an agent, such as an antibody, which binds to the encoded product and/or the detectable reporter. In some embodiments, the encoded product may be a polypeptide comprising an epitope tag. A particle may be coated with an antibody or other binding member which binds to the epitope tag. Suitable epitope tags are well-known in the art including, for example, HA (Hemagglutinin), MRGS$(H)_6$, DYKDDDDK (FLAG™), T7-, S-(KETAAAKFERQHMDS), poly-Arg (R5-6), poly-His (H2-10), poly-Cys (C4) poly-Phe(F11) poly-Asp(D5-16), Strept-tag, Strept-tag II (WSHPQFEK), c-myc (EQKLISEEDL), Influenza-HA tag (Murray, P. J. et al (1995) Anal Biochem 229, 170-9), Glu-Glu-Phe tag (Stammers, D. K. et al (1991) FEBS Lett 283, 298-302), Tag.100 (Qiagen; 12 aa tag derived from mammalian MAP kinase 2), Cruz tag 09™ (MKAEFRRQESDR, Santa Cruz Biotechnology Inc.) and Cruz tag 22™ (MRDALDRLDRLA, Santa Cruz Biotechnology Inc.). Known tag sequences are reviewed in Terpe (2003) Appl. Microbiol. Biotechnol. 60 523-533. Strept-tag may be used in some preferred embodiments.

Suitable particles may be produced by attaching antibodies or other binding members, such as streptavidin, to a particle, such as a magnetic bead. Antibodies or other binding members may be be directly covalently attached to the particle or may be biotinylated and may be attached to a streptavidin-coated particle through a biotin-streptavidin linkage using standard methods.

In other embodiments, microdroplet components such as substrates and encoded product may be retained by direct binding the hydrogel scaffold. For example, the scaffold may be engineered to contain one or more binding sites which bind to the droplet components and retain

them in the bead. For example, the scaffold may comprise covalently attached strepavidin or antibody for protein immobilisation. Other methods of protein immobilisation are well known in the art.

In other embodiments, polynucleotides and/or encoded products may be sufficiently retained in the bead without the need for binding to retention agents or the gel scaffold.

Following emulsification and production of a gel bead, the aqueous microdroplets may be de-emulsified.

In some embodiments, a deemulsification agent, for example a weak surfactant, may be added to the emulsion to separate the phases and the aqueous phase containing the beads removed. The deemulsification agent competes with the surfactant at the oil/water interface and causes it to collapse. Suitable weak surfactants include perfluorooctanol (PFO) and other fluorous compounds with a small hydrophilic group, if fluorinated oil is used, or a buffer containing SDS and Triton and other compounds with a carbon chain on one side and a small hydrophilic group on the other, if mineral oil is used. The deemulsification agent may be added to the emulsion and the mixture agitated, for example with a pipette.

In other embodiments, the emulsion may be centrifuged to separate the phases and the aqueous phase containing the beads removed.

Suitable techniques for the re-emulsification of gel beads are known in the art (Abate, A. R. et al (2009) *Lab Chip,* **9**, 2628-2631).

Following de-emulsification, the beads may be isolated and/or washed to remove buffers and other reagents. Beads may be isolated and/or washed by centrifugation or filtering using standard techniques.

Following washing, the beads may be immediately subjected to further steps in the methods described herein or may be stored, for example at room temperature or by refrigeration or freezing (preferably in the

presence of glycerol). In embodiments, in which the beads contain viable cells, the beads may be treated with a preservative such as glycerol before freezing in accordance with known techniques.

The presence of the encoded product in the beads causes the conversion of a reporter substrate into a detectable reporter. The presence of the detectable reporter is therefore indicative of the presence of encoded product in the bead. Furthermore, the amount of detectable reporter contained in a bead may be indicative of the amount or activity of encoded product in the bead.

A detectable reporter is a molecule, atom, ion or group which is detectable by standard detection methodologies. For example, the reporter may be capable of producing a detectable signal in response to a stimulus, such as a contact with a chromogenic substrate or light at an appropriate excitation wavelength.
The presence or amount of detectable reporter may be determined by detecting or measuring the signal produced by the reporter.

Suitable detectable labels may include fluorescent reporters, chromogenic reporters, Raman-active reporters, such as 4-mercaptopyridine, thiophenol (TP), mercaptobenzoic acid (MBA), and dithiobis succinimidyl nitrobenzoate (DNBA), mass-spectrometry reporters, and particles that can be identified by their shape by image analysis.

Suitable fluorescence reporters include fluorescein and fluorescein derivatives such as O-methyl-fluorescein or fluorescein isothiocyanate (FITC), phycoerythrin, Europium, TruRed, Allophycocyanin (APC), PerCP, Lissamine, Rhodamine, B X-Rhodamine, TRITC, BODIPY-FL, FluorX, Red 613, R-Phycoerythrin (PE), NBD, Lucifer yellow, Cascade Blue, Methoxycoumarin, Aminocoumarin, Texas Red, Hydroxycoumarin, Alexa Fluor™ dyes (Molecular Probes) such as Alexa Fluor™ 350, Alexa Fluor™ 488, Alexa Fluor™ 546, Alexa Fluor™ 568, Alexa Fluor™ 633, Alexa Fluor™ 647, Alexa Fluor™ 660 and Alexa Fluor™ 700, sulfonate cyanine dyes (AP Biotech), such as Cy2, Cy3, Cy3.5, Cy5, Cy5.5 and Cy7, IRD41

IRD700 (Li-Cor, Inc.), NIR-1 (Dejindom, Japan), La Jolla Blue (Diatron), DyLight™ 405, 488, 549, 633, 649, 680 and 800 Reactive Dyes (Pierce/Thermo Fisher Scientific Inc) or LI-COR™ dyes, such as IRDye™ (LI-COR™ Biosciences).

The reporter solution comprises a reporter substrate which is inactive or which is less active than the detectable reporter and is converted into a detectable reporter by the presence of encoded product in the bead.

Suitable reporter substrates contain a bond which is cleavable by the encoded product to liberate the detectable reporter. The reporter substrate produces no detectable signal or reduced detectable signal compared to the detectable reporter. For example, a fluorescent reporter substrate may contain a bond which is cleavable by the encoded product to liberate a fluorescent reporter which has increased fluorescence at an emission wavelength, compared to the fluorescent reporter substrate.

Suitable reporter substrates which may be converted into detectable reporters by the action of a specific enzyme are well known in the art.

For example, the reporter substrate fluorescein disulfate may be converted by an arylsulfatase into the detectable reporter fluorescein. Similarly, fluorescein phosphate, and fluorescein acetate may be used with phosphatases and acetylases respectively.

Numerous reporter substrates are commercially available (for example, Molecular Probes Inc) or may be synthesised by standard procedures.

Preferably the detectable reporter is retained in the beads.

The detectable reporter may comprise a macromolecular carrier, such as an oligonucleotide, dextran or polypeptide to facilitate retention by the bead and/or the polyionic shell. Preferably the size of the

carrier is greater than the pores in the shell, such that it does not readily diffuse out of an encapsulated bead. For example, the detectable reporter may be linked to an oligonucleotide having at least 10 bases, at least 15 bases, or at least 20 bases. In preferred embodiments, the detectable reporter may be retained in the bead through the encapsulation of the bead in a polyionic shell. Polyionic shells are described in more detail below.

In some embodiments, the reporter solution may comprise two or more reporter substrates which are converted into different detectable reporters by different encoded products. The signal from the different detectable reporters is distinguishable, such that the presence and/or activity of multiple products may be determined in the beads.

For quantitative measurements, the encoded product may be inhibited or deactivated after a predetermined incubation time. The incubation time before inactivation determines the stringency of the screen and may be adjusted according the particular requirements. Inactivation may be achieved by any convenient technique. For example, the reporter or detection solution may comprise an inhibitor which inhibits or inactivates the conversion of the reporter substrate into detectable reporter in the beads. Inhibitors may include transition-state analogues of the substrates and non fluorescent substrates that can diffuse through the shell and compete for the encoded product with the reporter substrate. Alternatively, the beads may be subjected to conditions, such as increased or reduced temperature or pH, which inhibit or inactivate the conversion of the reporter substrate into detectable reporter in the beads. Following inactivation of the product, the gel-forming agent may be solidified to form a bead or the outer layer of an existing bead and the reporter solution de-emulsified. Alternatively, the gel-forming agent may be solidified to form a bead or the outer layer of an existing bead and then the product may be inactivated.

One or more beads from the aqueous solution which contain the encoded product may be identified by identifying one or more beads which contain the detectable reporter.

For example, a method as described herein may comprise:

i). emulsifying into a microdroplets an aqueous reporter solution comprising a gel forming agent, a reporter substrate, and a population of cells, each cell comprising a polynucleotide encoding a target polypeptide and,

wherein the reporter substrate is converted into a detectable reporter by the target polypeptide,

ii). allowing the cells to express the target polypeptides in the microdroplets, and optionally lysing the cells,

iii). solidifying the hydrogel forming agent to produce a gel bead in each microdroplet, said bead containing detectable reporter produced by the target polypeptide,

iv). de-emulsifying the aqueous microdroplets into aqueous solution; optionally inactivating the target polypeptides and,

v) detecting, determining or measuring the detectable reporter in one or more beads in said population.

A method may comprise identifying and/or isolating one or more beads in the aqueous solution which contain the detectable reporter or contain levels of reporter above a threshold value.

The population of cells may comprise a diverse library of polynucleotides encoding variants of the target polypeptide.

The solution may be emulsified such that each microdroplet contains none or one cell i.e. the cells are compartmentalised in the emulsion.

The level or amount of reporter in the beads may be indicative of the activity or amount of target polypeptide in the beads.

Following production of the detectable reporter, the beads may then be de-emulsified and suspended in aqueous solution. The encoded product

may be inhibited or deactivated as described above. The aqueous solution may further comprise one or more reagents for encapsulating the bead, for example in a polyionic shell, as described below.

In some embodiments, methods described herein may comprise:

i). emulsifying an aqueous amplification solution comprising a polynucleotide and a gel forming agent into microdroplets,

ii). allowing the amplification of the polynucleotide to produce amplified copies,

iii). solidifying the gel-forming agent within the microdroplet to produce a gel bead comprising the amplified copies of the polynucleotide; wherein the polynucleotide is amplified before, at the same time as or after the gel-forming agent is solidified,

iv). de-emulsifying the aqueous microdroplets and isolating the beads therefrom,

v). contacting the beads with an aqueous expression solution which expresses the amplified copies in the microdroplets to produce a product encoded by the polynucleotide, wherein the product encoded by the polynucleotide is contained in the bead,

vi). emulsifying the beads in aqueous microdroplets in a reporter solution comprising a reporter substrate, wherein the reporter substrate is converted into a detectable reporter in the presence of the product encoded by the polynucleotide in the bead,

vii). de-emulsifying the aqueous microdroplets into aqueous solution; and,

viii). detecting, determining or measuring the detectable reporter in one or more beads in said population.

One or more beads in the aqueous solution which contain the detectable reporter may be identified and/or isolated.

As described above, step v) may be performed with the beads contained within a microdroplet containing the aqueous expression solution. Step v) may comprise;

(i) emulsifying the beads comprising the amplified copies in microdroplets which comprise the aqueous expression solution,

(ii) allowing the aqueous expression solution to express the amplified copies of the polynucleotide in the microdroplets to produce a product encoded by the copies, and

(iii) de-emulsifying the microdroplets.

As described above, a reporter solution as described herein may comprise a gel-forming agent. Following emulsification, the gel-forming agent solidifies to form a bead or solidifies around an existing bead within the microdroplet to form an outer gel layer of the existing bead.

The reporter solution may further comprise a functional reagent, such that the functional reagent is incorporated into the bead or the outer gel layer.

For example, the functional reagent may be a first charged polyion, such that, following emulsification and solidification, the reporter reaction mixture forms an outer gel layer of the bead which comprises the first polyion. This may be useful in forming a polyionic shell, as described below.

In some preferred embodiments, the beads may be encapsulated in a shell following production of the detectable reporter. The shell reduces the permeability of the beads thereby retaining macromolecules, such as detectable reporter in the beads.

Suitable shells include polyionic or polyelectrolyte shells which comprise a first and a second polyion, the first and second polyions having opposite charges.

In some preferred embodiments, the first polyion may be a polyanion and the second polyion may be a polycation. In other embodiments, the first polyion may be a polycation and the second polyion may be a polyanion.

Suitable polyanions include anionic polyelectrolytes such as alginate, polystyrene-sulfonate, noncoding nucleic acids, polyacrylic acid and PMMA.

Suitable polycation include cationic polyelectrolytes such as poly (allylamine hydrochloride) (PAH), poly-lysine, poly-arginine, and polydimethylammonium-chloride.

The first polyion may be contained in the reporter solution, such that bead comprises the first polyion when the hydrogel-forming agent solidifies. In some embodiments, the hydrogel-forming agent in the reporter solution may solidify around an existing bead, such that an outer gel layer of the bead comprises the first polyion. For example, 1 to 10mg/mL of the first and second polyions may be employed to form the shell.

Microdroplets containing beads which comprise the first polyion, either in an outer layer or throughout, may be de-emulsified in an aqueous solution which comprises a second charged polyion.

In some embodiments, the emulsion containing the beads comprising the first polyion may be mixed with an emulsion, for example a polydisperse emulsion, containing the second polyion, and then the mixture of emulsions may then be de-emulsified.
The first and second charged polyions have opposite charges.
The first polyion diffuses out of the beads and the second polyion diffuses into the beads. At the bead surface, the first and second polyions interact to form a polyionic complex.

This polyionic complex encapsulates the bead in a shell which reduces the permeability of the bead. This is useful in retaining the detectable reporter and other macromolecules in the beads.

Following de-emulsification and re-suspension aqueous detection solution, the beads may be screened for the presence and/or amount of detectable reporter.

The use of aqueous detection solution to analyse the beads allows a range of conventional techniques to be employed. Suitable techniques include standard particle sorting techniques, including flow cytometry such as FACS (e.g. Walser, M.,et al (2009) *Polynucleotides Res.*, 37, e57; Sepp, A. et al. (2002). *FEBS Lett.*, 532, 455-458; Griffiths, A. D. and Tawfik, D. S. (2003) *EMBO J.*, 22, 24-35; Gan, R.et al H. (2008). *Biotechnol. Prog.*, 24, 1107-1114).

Beads which contain the detectable reporter or display an amount of detectable reporter above a chosen or predetermined threshold, may be identified and/or isolated. In some embodiments, beads which lack the detectable reporter or display an amount of detectable reporter below the chosen or predetermined threshold, may be identified and/or isolated.

For example, the encoded products may be enzyme variants and beads containing the most active enzyme variants may be identified from the amount of detectable reporter present. These beads may be isolated to select the active enzyme variants, for example for further analysis and/or further rounds of mutagenesis and screening.

Following the identification and/or isolation of one or more beads which either contain or do not contain the detectable reporter, the shell may be removed from the beads.

Suitable methods for removing the shell include heating, freezing or subjecting the beads to high salt concentrations (e.g. 5M or more salt, such as NaCl) or pH.

Suitable pH for removing the shell may be a pH above the polycation pKa and below the polyanion pKa within the polyionic shell (e.g. pH 10-12).

Polynucleotide from the one or more beads may be isolated, amplified, cloned, sequenced or otherwise manipulated.

A method described herein may further comprise identifying and/or isolating the polynucleotide from one or more beads identified as containing the product or further product.

The polynucleotide from the one or more beads may be isolated, amplified, sequenced cloned and/or otherwise investigated. For example, the polynucleotide may be extracted using conventional techniques such as gel-extraction columns or agarose, and/or amplified isothermally (e.g. multiple-primed RCA) or by PCR or both consecutively.

The methods described above may be repeated 1 or more, 2 or more or 3 or more times, for example 5 or more, 10 or more, or 20 or more times, using polynucleotide from one or more beads identified in the previous round of screening. The methods may be repeated until a polynucleotide encoding a polypeptide or other product with the desired activity is obtained.

Iterations of the above screening methods may be performed with the same or different conditions and parameters. For example, the stringency of the screen may be progressively increased by reducing the incubation time for the conversion of reporter substrate into detectable reporter by the encoded product. In some embodiments, one or more of incubation temperature, substrate concentration, incubation time, alternative promoters to vary expression levels, presence or absence of DNA amplification, presence or absence of a competitor or other paramters, may be varied in iterations of the screening methods.

This may be useful in isolating specific polynucleotides or in methods of directed evolution. For example, a method may comprise identifying a population of polynucleotides using a method described above, introducing mutations into the population of polynucleotides and repeating the methods described above. Mutations which improve the activity of the encoded product may be identified and subjected to further rounds of mutation and assay, as described herein.

For example, a method may comprise introducing one or more sequence alterations into the polynucleotides isolated from the one or more beads to produce a population of variant polynucleotides. The methods described above may be repeated on the population of variant polynucleotides.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure.

All documents mentioned in this specification are incorporated herein by reference in their entirety.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Unless context dictates otherwise, the descriptions and definitions of the features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described below.

Figure 1 shows a schematic of aspects of the invention.

Figure 2 shows another schematic of the production of monodisperse gel-shell particles according to some aspects of the invention. In step A, the monodisperse emulsion droplets are produced with a microfluidic emulsion generator containing agarose, the polyanion alginate, E.Coli cells and a lysis agent. Incompatible components are

divided into separate aqueous flows and merged right before the flow-focusing junction using separate inlets. E.Coli cells are lysed inside the emulsion droplets releasing expressed enzyme and corresponding plasmids. In step B, after cooling of the sample to gelate agarose, the sample emulsion, mixed with emulsion containing polycation PAH, is broken. Alginate diffuses out of the agarose bead whereas PAH diffuses inside, substituting the oil/water interface of the former emulsion droplet with a polyelectrolyte complex. This shell compartmentalizes the enzyme in functional state with corresponding plasmids. In step C, small molecular weight enzyme substrates are able to diffuse through the polyelectrolyte shell and are converted into product. The graph shows an enzymatic assay with gel-shell beads equipped with E.Coli lysate containing PTE, using 1 mM paraoxon as substrate, where a is gel-shell particle and b is supernatant of the last washing step of the coating procedure. In step D, higher molecular weight substrates are retained inside gel-shell beads and converted to product when added before emulsification. Emulsion droplets equipped with single cells result in gel-shell beads displaying a phenotype linked with its encoding genotype. These composites can be sorted by flow cytometry (FACS) and used for directed enzyme evolution in high throughput.

Figure 3 shows a schematic of one round of a method of screening combining microfluidic droplets, gel beads and polyelectrolyte coating, according to an embodiment of the methods described herein.

Figure 4 shows a schematic of one round of *in vitro* directed evolution combining microfluidic droplets, gel beads, DNA amplification, display on nanoparticles or microbeads and polyelectrolyte coating, according to an embodiment of the methods described herein.

Figure 5 shows experimental data corresponding to the process described in Figure 3. Flow cytometry measurements demonstrate shell removal. Upon removal of the polyelectrolyte membrane the high light scattering of the particles return to values similar to uncoated sample (low light scattering). These data serve as an example of one round of *in vitro* directed enzyme evolution. Scale bars: A: 50um, B,

C: 20um

Figure 6 shows flow-focusing-designs used to make emulsion droplets, according to an embodiment of the methods described herein.

Figure 7 shows the formation of agarose gel bead formation. Fig 7A) shows a schematic of a microfluidic flow focusing-device used for making monodisperse emulsions. The two aqueous streams mixed at a flow-focusing geometry before droplets were formed at a second flow-focusing point. Fig 7B) shows a brightfield microscope picture of a device with flow-focusing channel widths of 20 µm and a height of 25 µm forming droplets. Scale bar: 50 µm. Fig 7C) shows the two aqueous streams coflowing together before being pinched-off by the oil phase. Fig 7D) shows an image of monodisperse emulsion. Scale bar: 20 µm. Fig 7E) shows 0.75% Agarose-beads after deemulsification. (scale bars 40 micrometer (B), 20 micrometer(D)(E)). Fig 7F) shows forward scatter plotted versus sideward scatter (ungated) of agarose gel beads measured by flow cytometer.

Figure 8 shows plasmid capture in agarose beads. 0, 15 ng/µl or 30 ng/µl of the plasmid pIVEX-HA-GFP and 0.75% agarose (in TE buffer) were encapsulated into microfluidic droplets with a flow-focusing device (25 µm high and 20 µm wide). Peak 1 - Empty beads without PicoGreen staining. Peak 2 - Empty beads with PicoGreen staining. Peak 3 - Beads containing 15 ng/µl plasmid after one week in TE buffer, stained with PicoGreen. Peak 4 -Beads containing 15 ng/µl plasmid freshly recovered from the emulsion, stained with PicoGreen. Peak 5 - Beads containing 30 ng/µl plasmid after one week in TE buffer, stained with PicoGreen. Peak 6 - Beads containing 30 ng/µl plasmid freshly recovered from the emulsion, stained with PicoGreen.

Figure 9 shows multiply-primed RCA. A plasmid was compartmentalised (at a concentration of 1.3 plasmids per droplet) into gel beads and isothermally amplified by multiply-primed RCA. The emulsion was broken, the DNA stained with PicoGreen and the beads analysed by flow cytometry. 9A) shows forward scatter versus fluorescence. The region

used to gate for single beads is indicated with a black square. 6B) shows fluorescence of the gated beads plotted against their numbers.9C) as in B), but including the negative control (red, amplification reaction without DNA). The positive beads were shaded in green colour.

Figure 10 shows IVTT of GFP. pIVEX-HA-GFP was encapsulated in agarose beads and amplified with multiply-primed RCA. IVTT was added to the beads and HA-GFP was captured by anti-HA antibody coated nanoparticles. The agarose beads were analysed by flow cytometry. 7A) shows forward versus sideward scatter plot. Single beads were gated. 7B) shows green fluorescence of agarose beads. Three different ways to perform the IVTT were compared: 1) the IVTT was carried out in solution 2) the beads were together with the IVTT re-encapsulated into microfluidic droplets 3) the IVTT was performed in bulk emulsion generated by vortexing. The beads were incubated at 30°C for 6 h and at 4°C overnight. Negative control 1 (NC1): IVTT was carried out in solution, but only incubated at 4°C. NC2: No IVTT was added to the beads. Figures 7C-D show the different ways to perform IVTT and were plotted separately together with NC1. The numbering for the different ways of performing the IVTT is as in B). The populations of beads with fluorescence higher than the negative control (positive beads) are shaded.

Figure 11 shows IVTT of GFP from plasmid or from amplified plasmid. pIVEX-HA-GFP was encapsulated in agarose beads and either not amplified (8A, 8B) or amplified with multiply-primed RCA (8C, 8D). IVTT was carried out in solution and HA-GFP was captured by anti-HA antibody coated nanoparticles. The agarose beads were analysed by flow cytometry. The low-fluorescence peak is empty agarose beads and the high fluorescence peak is gfp containing beads. The fluorescence was plotted against the forward scatter (11A, 11C) and against the number of beads (11B, 1D).

Figure 12 shows PAS activity in gel beads. The plasmid pIVEX-HA-PAS was encapsulated into gel beads and amplified. Anti-HA antibody coated

nanoparticles were also included in the droplets. After reemulsification in bigger droplets containing IVTT-mix HA-tagged PAS was *in vitro* transcribed, translated and attached to the nanoparticles (here by a anti HA-tag antibody immobilized on the surface of the nanoparticles). After incubation the emulsion was broken, IVTT-mix washed away and then the gel beads were re-compartmentalised into microfluidic droplets together with fluorescein disulfate. After incubation at 30 C for 7 h the fluorescence of the droplets was detected using an EM-CCD camera coupled to an epifluorescence microscope. Bright dots show “filled” droplets containing beads from IVTT produced arylsulfatase while, less bright dots show background fluorescence substrate from “empty”, non DNA/protein containing emulsion droplets

Figure 13 shows the workflow for making gel-shell beads. Emulsion droplets harboring agarose and polyanion (alginate) were produced using an emulsion generator (a). After cooling to gelate agarose, the monodisperse emulsion was mixed with excess amount of polydisperse emulsion containing a polycation (PAH) (b). After vortexing to ensure proper mixing of the two emulsions, a weak surfactant was added (PFO, perfluorooctanol) and vortexed. The emulsions collapsed, the polyanion alginate from the inside of the droplet diffused out, whereas the polycation diffused into the agarose bead, forming a polyelectrolyte complex at the region of contact (c). The agarose provided a spherical template for the process. Short centrifugation of the sample allowed collection of the aqueous phase containing gel-shell beads. Beads were washed with centrifugation and, optionally, additional polyelectrolyte layers added using standard protocols. Image d) shows an aggregate of two agarose particles (40um in diameter) illustrating polyelectrolyte complex formation. The image, taken slightly out of focus, visualizes the polyelectrolyte shell due to its different light scattering properties. With single particles complex formation was fast enough to happen on the surface of the agarose bead, substituting the former oil/water interface. With particle aggregates, a freestanding complex was formed in the space between agarose cores, pointing to shell formation being based on diffusion of the polyions.

Figure 14 shows encapsulation of a fluorescein labelled oligonucleotide. 10 µM, 5 µM, 1 µM, 0.5 µM, 0.1 µM and 0 µM concentrations of a fluorescent oligonucleotide were compartmentalised in microfluidic droplets together with 0.75% agarose and 0.5% alginate. The emulsion was broken into a PAH solution to coat the beads with a polyelectrolyte complex. Single beads were gated and the geometric mean and the standard deviation of these populations were plotted against the concentration of compartmentalised fluorescein-oligonucleotide on a double logarithmic scale.

Figure 15 shows automated gel-shell bead production with microfluidic chips: Gel-shell particles can be produced continuously by coupling microfluidic chips in series. Monodisperse emulsion droplets containing agarose and alginate (e.g. 0.5% alginate and 0.75% agarose) were produced in the first emulsion generator (a), the tubing of the outlet is cooled on ice to gelate agarose before entering the inlet of the second chip. There the emulsion sample is mixed with polydisperse emulsion containing the polycation (e.g.PAH) (b) and subsequently added to a stream of PFO (c). At the outlet (d) the two phases are separated; gel-shell particles with a diameter of about 40 µm were harvested from the aqueous phase of the collection tube. The beads were covered with a shell of alginate/PAH. Scale bars: (a), (d): 50um; (b), (c): 150um.

Figure 16 addresses layer thickness and permeability of polyelectrolyte shells and shows the results of flow cytometry experiments with gel-shell particles.

Figure 16A shows flow cytometric analysis of particles (diameter: 40um) with fluorescence-labelled polyelectrolyte (orange: FITC-labelled PAH; black: rhodamine-labelled PAH). Fluorescence values were normalized to samples coated with 4 layers (a standard layer made of PAH/PSS/labeled PAH/PSS). The amount of absorbed labelled PAH in the initial layer corresponds to 40-70% of the amount of PAH deposited in later layers and its thickness can therefore estimated to a similar

percentage of the regular layer. Samples coated with 6 layers in total, 3rd and 5th layer coated with FITC-labelled PAH confirmed the expected linear growth regime of the multilayer coating.

Figure 16B shows flow cytometric analysis of particles (diameter: 20 um, showing that particle fluorescence corresponds linearly to the concentration of encapsulated labelled oligonucleotides for the indicated concentration range of at least two orders of magnitude (panel a). Figure 16B also shows flow cytometric analysis of particles (diameter: 40 um) containing agarose, alginate and FITC-labelled oligonucleotide (10uM), that were deemulsified by mixing with various reagents:(1) mixed with buffer, (2) mixed with $CaCl_2$ and (3) mixed with PAH (panel; b). Samples have been washed twice in buffer before measurements. The results of the the fluorescence of single particle populations (geometric mean) is plotted for several samples. Whereas gel-shell particles are able to retain the fluorescent primers, they diffuse from unmodified agarose/alginate particles as well as from crosslinked alginate-agarose particles. Sample (3a) represents particles that were frozen with liquid nitrogen, stored at -80°C and thawed. No significant loss of encapsulated FITC-labelled primer was observed. Similar freeze-thaw experiments were performed with particles harboring single-cell lysate assays. No significant difference in fluorescence signal between frozen and non-frozen samples could be detected, providing indication that their integrity is maintained and freezing is a suitable storage method.

Figure 16C shows agarose particles filled with two concentrations of a FITC-labelled peptide (HA-tag). Particles with a shell (3) are able to retain the cargo whereas in unmodified agarose/alginate particles (1) and particles containing crosslinked alginate (2), the labeled peptides diffuse. These data providing indication that retention of specific molecules is conferred by the shell.

Figure 17 shows a phosphotriesterase (PTE) substrate coupling. In an EDC-mediated coupling the PTE substrate *O,O*-diethyl *O*-(5(6)-carboxyfluorescein) phosphorothioate was attached via an amide bond to

a 24 nt long oligonucleotide containing a primary amine at the end of a C12 linker.

Figure 18 shows the encapsulation of product. The oligonucleotide coupled PTE substrate was turned over by PTE. 0.5 µM of the fluorescent product was encapsulated in gel beads. The beads were analysed by flow cytometry. Single beads were gated and their fluorescence was plotted against their number. As comparison the fluorescence of beads without product was measured.

Figure 19 shows the reaction of substrates and enzymes in an agarose matrix. Figure 19A shows the chemical structure of paraoxon and the fluorescein-based oligo-coupled substrate for PTE (Oligo-linked-tetraethyl O-(Fluorescein 5(6)-isothiocyanate) diphosphate. Figure 19B shows a comparison of substrate-turnover in agarose/alginate (a), buffer with enzyme (b) and agarose/alginate without enzyme as control (c).

Figure 20 shows the recovery of DNA. Approximately 280 beads containing pIVEX-PTE(S6)-HA were added to 20 µl PCR reactions. 15 µl aliquots were resolved by a 1% agarose gel. The plasmid in the beads was either left at a concentration of one plasmid per bead (lanes 2 and 4) or amplified by multiply-primed RCA (lanes 3 and 5). The beads were either treated with polyelectrolytes (lanes 4 and 5) or not (lanes 2 and 3). For lane 1 the PCR did not contain beads, but 1 ng of the pIVEX-PTE(S6)-HA plasmid. For lane 6 the PCR also contained 1 ng of the pIVEX-PTE(S6)-HA plasmid plus empty beads treated with polyelectrolytes. A) PCR reaction with Taq polymerase and 2 µM primers. B) PCR reaction with Taq polymerase, 4 µM primers and 1 mg/ml BSA.

Figure 21 shows an overlay of fluorescence and brightfield images of an emulsion after an enzymatic assay for phosphotriesterase activity). Bright, fluorescent droplets: contain lysed *E.Coli*; non fluorescent droplets: empty

Figure 22 shows brightfield image of gel beads coated with a polyelectrolyte shell comprising PAH and alginate.

Figure 23 shows FACS sorting of gel-shell beads harboring E.coli cells containing PTE-wt in a dilution of one cell per five droplets. Image (A) shows a dotplot with forward- and sidescatter. The population of single particles is gated and shown as fluorescence histograms in Image B. Events scattering at lower values can be assigned to small satellite droplets originating from emulsion generation. These are produced when the emulsion generator is operated close to the jetting regime to maximize throughput. They do not affect monodispersity of the emulsion sample. Image B(a) shows two sorting gates applied to the fluorescence histogram, the first on the left sorting empty gel-shell beads, the second gate sorting beads equipped with lysed single cells (therefore with accumulated fluorescent product of the enzyme reaction). Reanalysis of the sorted samples confirmed successful sorts of empty (b) and filled (c) gel-shell beads

Figure 24 shows the freeze/thaw of gel-shell beads for storage. In the presence of glycerol (Figure 24A panels (a-c)) freeze-thaw cycles do not destroy gel-shell bead particles. In the absence of glycerol gel shell bead particles are damaged or destroyed (Figure 24A panel (d)). Figure 24B panel (a) shows a corresponding bead crosslinked with CaCl2 in 0.1M NaCl. The particle lacks a visible shell compared to the gel-shell beads shown in Figure 24A. These crosslinked particles are not deformed in the presence of glycerol (panel b) and can be freeze-thawed (penal c). Freezing in the absence of glycerol results in broken and deformed beads (panel d). Figure 24C shows freezing-experiments with particles exclusively made up of agarose (0.75%, diameter: 18 um). Agarose beads before (panel a) and after freezing (panel b) in glycerol show similar morphology. Freezing without glycerol distorts their morphology (panel c).Scale bars: A, B: 25um, C: 20um.

Figure 25 shows the disassembly of the shell with high-pH treatment. From left to right: panel 1 shows forward scatter vs side-scatter

plots of agarose/alginate particles, deemulsified without assembling a polyelectrolyte complex. Panel 2 shows forward-scatter vs side-scatter plots of agarose/alginate particles, with a polyelectrolyte shell assembled. Panel 3 shows forward-scatter vs Side-scatter plots of agarose/alginate particles with the polyelectrolyte shell assembled but subsequently disassembled with high-pH treatment. The shell, due to its optical properties, makes the particles scatter much higher, in forward scatter as well as in side scatter (panel (2)).

Figure 26 shows FACS data of beads containing phosphotriesterase library. The enzymatic assay was stopped at two different timepoints (after 9h and 3h incubation at 30°C) by heating for 10' in 95°C. After cooling the emulsion for gelling the agarose the samples have been coated with the polyelectrolyte shell for FACS sorting. From left to right: 'Side-scatter vs pulse width' -plot used for gating single particles; side-scatter vs green fluorescence plot for the sample incubated for 9h; side-scatter vs green fluorescence plot for the sample incubated for 3h. The sorting gate is indicated. Histogram-overlays (green fluorescence vs bead count) of the two samples: due to the longer incubation time of the 9h-sample the percentage of beads in the sorting gate is higher reflecting less stringent sorting conditions. 2nd row: histograms (green fluorescence vs bead count) for the respective dotplots above.

Figure 27 shows the results of screening and sorting 400,000 enzyme members of a phosphotriesterase library which narrowed down to 100-200 library members in two subsequent rounds of selection. The higher average activity of a library sorted after shorter reaction time (3 vs 9 hours of incubation) suggests that the stringency of selections can be adjusted at will by varying the incubation ties.

Figure 28 shows the sorting and analysis of a neutral drift library based on the mutant PTE-G5. Fig 27A shows a dotplot (forward scatter vs fluorescence) of gel-shell beads equipped with the initial library in a dilution of one cell per five droplets. Fig 27B shows a comparison of the overall activity of samples between the initial

library (a), the subset obtained after the first sorting step incubated for 3h (b) and the result of the second round of screening with an incubation time of 1.5h (c). Fig 27C shows monoclonal lysate assays were carried out with 250 uM paraoxon as substrate on 172 clones from the second screening cycle picked randomly and grown on plate. 74% of the clones showed 0.2-1.4 fold activity relative to the parent sequence.

Figure 29 shows selection experiments with gel-shell beads and FACS. Figure 29A shows the rescue of PTE-wt diluted 1:10000 in acylphosphatase-encoding plasmids. Plasmids before and after selection have been digested with restriction enzymes and applied to gel electrophoresis. Before selection only the Acp-gene (500bp) can be detected, after the first sort both genes are present, whereas the second selection results in PTE-wt genes (1000bp) only. Figure 29C shows FACS-histograms of two emulsion aliquots of a sample harboring a library based on PTE-G9 representing half a million variants. The aliquots have been incubated for 15h (1) and 1.5h (2) before heat inactivation. Sample (2) allows for enrichment of beneficial mutants. After sorting the best 2% of the population, 50 of the 10000 selected beads (0.5%) have been randomly selected and subjected to lysate assays on plate using the substrate paraoxon. The inset shows lysate assays of the best clone found (a) together with the parent enzyme PTE-G9 (b) as control.

Experiments

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Materials & Methods

Microfluidic Chips

Microfluidic droplet generators were fabricated from Poly(dimethyl)siloxane (PDMS) using standard soft lithography. CAD-files of chip designs (see sFigure1) were prepared using the free software Draftsight (Dassault Systems) and printed on acetate film in

high resolution (Micro Lithography Services Ltd.). Clean 3" silicon wafers were spin coated with SU-8 2025 photoresist. Spinning conditions were varied to achieve different layer heights (initial spinning: 500rpm for 5s at an acceleration of 300rpm/s): for droplet generators 16um in width 4000 rpm for 40s at 300 rpm/s was used resulting in a channel height of 20um (1). for droplet generators 40um in width a protocol for 50um channel height was applied (1650 rpm, 40s, 300 rpm/s), (2). Devices 150um in width were equipped with two subsequent layers of photoresist 50um in height (baked in- between) resulting in a channel height of about 100um. After baking ((1): 1min 65°C, 3min 95°C, 1min 65°C; (2): 2min 65°C, 6min 95°C, 2min 65°C) the patterns were exposed onto wafers using a mask aligner (MJB4, Suess MicroTec), baked again and developed with propylene glycol methyl ether acetate (PGMEA). The patterned wafers were used as mold for PDMS-chips (PDMS:curing agent 10:1 (Sylgard 184, Dow Corning)); cured at 70°C > 4h). Chips were cut out using a scalpel, inlets/outlets punched with a biopsy punch (Harris Uni-Core – 0.75) and the device was sealed onto a glass slide using oxygen plasma treatment.

After incubation at 90°C for 10-20min, the channel surface was fluorinated using Duxback^{tm} and flushed with air or fluorous oil (HFE 7500, 3M). For operating droplet generators, Portex Fine Bore Polyethylene Tubing ((0.38mm ID, 1.09mm OD), Smiths Medical International Ltd) was used to connect the droplet generators with glass syringes (SGE analytical science; 100uL for aqueous phases, 2.5mL for oil phase).

Microfluidic Setup

Images showing droplet production have been made using a Phantom Miro3 high speed camera (Vision Research) for better image quality. For experimental work a more minimalistic rig was used consisting of a Pike F-032B camera (Allied Vision Technologies; low shutter times allow to monitor monodisperse droplet formation at high frequencies), Navitar 12X Zoom body tube, white light LED array (Thorlabs) and three PHD 2000 infusion pumps (Harvard Apparatus).

Brightfield and fluorescence microscopy images of emulsions and gel-shell beads were prepared using a Olympus BX51 microscope equipped with a 10X objective. Emulsions have either been imaged using Kova glasstic slides or standard glass slides. To stabilize emulsions when imaging with glass slides, emulsion samples were embedded in a drop of mineral oil on the glass slide before adding the coverslip. Images were adjusted in brightness/contrast using ImageJ software.

Production of monodisperse emulsions and gel-shell beads (GSBs)

Polydisperse emulsions were used for method development. Aqueous solutions can be applied on prewarmed (30-37°C) fluorous phase with surfactant (solution:oil ratio should be about 1:10) and emulsified by vortexing or other means and processed further. This polydisperse emulsion and resulting polydisperse gel-shell beads allow to derive qualitative data on functioning of protocols and assays while production being simple and fast enough to explore protocol variations in parallel.

Fluorous oil and surfactants:

3M Novec HFE-7500 fluorous oil with 0.5% (w/w) surfactant was used as carrier phase for all experiments. As surfactant, EA-surfactant (Raindance Technologies, United States Patent Application 20100022414) was applied. Due to the limited availability of EA-surfactant, most experiments have also been either conducted in parallel or repeated using a krytox-jeffamine triblock copolymer prepared using standard techniques. This surfactant was used without further purification. Additional removal of diblock-copolymers (for example by using aminopropyl-functionalized silica-gels) may be beneficial to reduce the presence of aminogroups at the oil/water interface of emulsions.

Agarose/alginate emulsions

Precursors for gel-shell beads are emulsions containing agarose and alginate. Gelated agarose provides a spherical template for subsequent shell fabrication. Alginate was chosen as polyanion due to its good compatibility with biological building blocks and reactions. Other hydrogel/polyelectrolyte systems can be used for GSB-fabrication. For

simplicity, aqueous-phase components of assays were divided in two syringes depending on sensitivity towards temperature (and assay compatibility). A temperature-sensitive solution can be conveniently cooled (if necessary) with an ice-filled glove covering syringe and tubing, whereas the second syringe containing agarose can be kept at elevated temperature the same way using inexpensive heated hot/cold compresses (Boots Pharmaceuticals).

Droplet generators were operated at high throughput. Presence of small satellite droplets in samples did not diminish quality of assays and library screens. Due to the viscosity of the aqueous phase when using hydrogels, throughput of droplets is decreased compared to emulsions with buffer only and applicable flowrates change depending on assay components and their concentrations. Fluorous-oil containing surfactant can be conveniently recycled for a number of times when filtered (Acrodisc syringe filter, 0.45 um PTFE membrane) without loss of function. Emulsions were harvested off-chip in Eppendorf™ tubes with an overlay of mineral oil (Sigma). This protects the emulsion from shrinkage and allows for extended storage without losing monodispersity. Additionally, small sample volumes of emulsions can be pipetted accurately without loss.

Emulsion generation

For emulsions 40um in diameter, a droplet generator with dimensions of 40um width/50um height in the flowfocusing geometry was applied (typical flowrates: 2*100uL/h aqueous phase, 1500 uL/h oil; 1.7kHz); for emulsions 18um in diameter, a chip with smaller geometry (16um width/20um height) was used and operated with 2*15uL/h aqueous phase, 500uL/h oil (2.7 kHz). Agarose in sol (1.5% (w/w), in Tris-HCl (50mM pH 7.5, 100mM NaCl)) and sodium-alginate (1.5% (w/w) in Tris-HCl (50mM pH 7.5, 100mM NaCl)) was applied on chip from two separate syringes (see sFigure 1), yielding emulsion droplets containing 0.75% (w/w) alginate and agarose respectively. The alginate-filled syringe was used to apply components for shell-permeability experiments (see sFigure 3). Emulsions were harvested in tubes with a mineral oil

overlay and cooled on ice for about 10min before fabrication of gel-shell beads.

Gel-shell-bead fabrication

Polydisperse emulsion in HFE 7500 (0.05%(w/w) surfactant) containing the polycation PAH (5-10mg/mL in 0.5M NaCl) was prepared either by vigorous vortexing or using a homogenizer (omni international TH 220). 1mL of this polydisperse emulsion was mixed with 5-10uL (2-3*10E6 droplets) agarose/alginate-containing monodisperse emulsion of 18um in diameter (50uL emulsion for samples 40um in diameter) and vortexed to thoroughly mix the two constituents. Then, 300-500uL PFO was added and vortexed. After centrifuging the sample for 10s, the aqueous supernatant containing the gel-shell beads was harvested and washed 2-3 times by centrifugation (2500g, 3-5min) in 100mM NaCl. Additional polyelectrolyte layers were applied using standard protocols (alternate layers of polycation (PAH) and polyanion (PSS); added gel-shell beads to polyelectrolyte solution (1-5mg/mL, in 0.5M NaCl), vortex, incubated for 10min on shaker (RT), washed 2-3 times with 0.1M NaCl in centrifuge, iterated.)

Ultra-low melting point agarose Type IX-A (Sigma) with a gelling point of 17°C was used as the gel forming agent for these studies.

Continuous gel-shell-bead fabrication

Agarose-alginate containing emulsions (40um in diameter) were produced on chip. The emulsion was cooled in the outlet tubing to gelate agarose (2m in length, wrapped around an ice-filled 50mL tube) and directly connected to two subsequent devices (100um height, 150um width), were the sample was first mixed with polydisperse PAH-containing emulsion (1000uL/h) following mixing with PFO (2000uL/h). Gel-shell particles were collected from the aqueous phase of the tube used for harvesting.

Production of encapsulated Microspheres

A microfluidic droplet generator (Fig. 2) produced monodisperse water-in-oil emulsion droplets (with volumes of 3pL at a frequency of 2700 s^{-}

[1]) from an aqueous stream harboring agarose and the polyanion alginate. Temperature reduction from 37 to 4° C solidified the droplet contents, to yield an agarose gel core within the droplet. Breaking this emulsion sample rapidly in the presence of the polycation poly(allylamine-hydrochloride) (PAH) surrounded the gel particle with a polyelectrolyte shell: alginate and PAH diffused and formed a polyelectrolyte complex surrounding the agarose core. The thickness of this shell was only nanometers and serves as general template for polyelectrolyte multilayer technology. The coating resembled a semipermeable membrane: retention experiments with fluorescent small molecules, fluorescein-labeled oligonucleotides and peptides provided indication of a molecular-weight cutoff at or below 2 kD: bigger molecules were retained inside the shells, small compounds diffused between the bead and its surrounding

Cloning and library preparations

Phosphotiesterase PTE and PTE-variants (G5, G9) were derived from a directed evolution project in progress with the aim to evolve the enzyme from a phosphotriesterase to an esterase (N.T., manuscript in preparation). The constructs (N-terminally tagged with streptag) were cloned from a pET-vector into a modified pRSFDuet vector (Novagen) with the second multiple cloning site removed. Additionally, an acylphosphatase (ACP) was cloned in parallel to serve as inactive control and as source for cut vector backbone for library preparations. PTE and PTE-variants were randomly mutated by error-prone PCR (Mutazyme II low fidelity polymerase, Stratagene) following standard protocols. PCR-product was ligated into pRSFDuet using HindIII/NcoI (Fastdigest, Fermentas) and transformed into electrocompetent E.coli cells (E.cloni 10G Supreme, Lucigen). Background of vector without insert was below 5%. Cells were plated on agar plates and counted to derive library size. Approximate mutation rates were determined by sequencing 10 randomly picked clones per library. Plasmid preparations of the libraries were transformed into electrocompetent E.coli BL21 (DE3), small aliquots plated on agar plates to derive numbers of transformants and the rest of the sample grown o/n in 10mL liquid culture. After dilution into fresh medium and

culturing to OD 0.6, samples were induced with IPTG and either incubated for 2-3h at 37°C or o/n at RT. Induced cultures with added glycerol (25% v/v) were aliquoted into 2mL eppendorf tubes, frozen with liquid nitrogen and stored for later use.

Single cell enzyme assays and library screens in gel-shell beads

Aliquots of induced E.coli harboring PTE, PTE variants or libraries were thawed on ice and washed 4-5 times with centrifugation (2min, 2000g) with Tris-HCl buffer (pH 7.5, 50mM, 100mM NaCl). OD(600) of the concentrated aliquot was measured and cell concentration was calculated estimating 1mL OD1 being 2*10E8 cells. Two mixes 100uL each were prepared to being later filled into glass syringes. The first containing the desired number of cells (for example a dilution corresponding to one cell every 5/10/50 droplets; approximate percentage of doublet/triplet cells in single droplets can be estimated using poisson statistics), along with 1.5% (w/w) alginate solution in Tris-HCl (pH 7.5, 50mM, 100mM NaCl). Alginate not only served as polyanion for shell assembly but also as density matching reagent to prevent cells from sedimenting in the syringe to ensure constant cell concentrations in droplets throughout emulsion preparation. The second mix consisted of melted agarose (1.5% (w/w) in Tris-HCl buffer), CellyticB Cell Lysis Reagent (10X conc, Sigma) in a final concentration of 0.1X (2uL), 2uL rLysozyme solution (Novagen, 1200kU) and substrate (Oligo-linked- tetraethyl O-(Fluorescein 5(6)-isothiocyanate) diphosphate) ZnCl2. This mix was kept in sol state using heated hot/cold compresses. Cells and substrate solution were applied in rather concentrated solutions to minimize dilution of agarose and alginate. Libraries were assayed using 10-30 uM substrate concentration, therefore all screens were performed under kcat/km conditions. Droplet generators (20um channel height, 16um width at flow focussing junction) were used and operated at 2.7 kHz with flowrates of 2*15uL/h (aqueous phase) and 500uL/h oil, yielding droplets of about 3pL in volume (18um diameter). The two aqueous solutions are mixed immediately after droplet generation and cell lysis takes place allowing the enzyme to react with substrate.

Results

To circumvent compatibility problems of biochemical reactions (e.g. for *in vitro* directed evolution) in microfluidic droplets, we maintained the compartmentalisation established inside the droplet in the form of a gel bead (Figures 1 to 3). Agarose gel beads were formed inside droplets as the temperature of the droplets was lowered below the gelling point of the agarose. The beads were then recovered in the aqueous phase after de-emulsification. Some droplet components (e.g. DNA and nanoparticles) remained in the bead due to their size and other components (e.g. buffer and proteins of the IVTT) were exchanged. Expressed enzymes were trapped by binding to enclosed nanoparticles to preserve genotype-phenotype linkage. This made it possible to perform sequentially a set of incompatible biochemical reactions while maintaining the compartmentalisation. The principle of combining microfluidic droplets and gel beads also minimised the complexity and number of microfluidic devices. Simple re-encapsulation of gel beads into droplets replaced the need for a fusion device and the coating of the beads with a polyelectrolyte complex allowed the gel beads to be further sealed. The compartmentalisation of enzymatic assay products such as fluorescein was maintained, especially when coupled to a higher molecular weight complex. Gel beads were handled in the aqueous phase and could therefore be analysed and sorted by flow cytometry, without the need for a specialised sorting device. The result is a flexible platform that involves only robust and easy to use microfluidic devices and will prove of value for multi-step biochemical reactions *in vitro*.

A summary of the workflow for one round of directed *in vitro* evolution is depicted in Figure 4. First, a library of plasmids was compartmentalised in droplets together with liquid (sol phase) agarose (1). Nanoparticles (diameter of 50 nm) displaying an antibody against the HA (Hemagglutinin) epitope tag were also included into the droplets. They were later used to capture the expressed protein. A simple flow-focusing device was used to form droplets. The droplets were collected on ice, thus inducing gel solidification. Second, in some experiments, an optional isothermal DNA amplification step by

multiply-primed rolling circle amplification (RCA) was performed (2). When the DNA amplification was performed, the necessary amplification reagents (Phi29 DNA polymerase, primers, dNTPs and buffer) were also included in the droplets. Next, the emulsion was broken and the DNA amplification reaction components were washed away (3). The (amplified) DNA and the nanoparticles remained entrapped in the gel beads. Then the enzyme was expressed by IVTT (4). Because it was fused to an HA tag, the enzyme was bound by the antibodies on the nanoparticles and thus stayed in the gel bead, even after washing away all the other IVTT components. The IVTT was performed in solution without cross-contamination between the different agarose beads. However, for a quantitative enzymatic assay re-encapsulation with the substrate into droplets was performed (5). A layer of agarose-containing alginate was added to the gel beads. The anionic alginate is later used for the encapsulation of the product with a shell of polyelectrolytes. In step 6, the droplets were incubated for the enzymatic turnover of the substrate to a fluorescent product by the immobilised enzyme. The substrate was coupled to an oligonucleotide to enable product encapsulation: the emulsion was broken and simultaneously the alginate complexed with the cationic polyelectrolyte poly(allylamine hydrochloride) (PAH) (7). The beads were thus coated with a layer of alginate and PAH to prevent the diffusion of the fluorescent product (which was coupled to an oligonucleotide) out of the gel beads. During the coating procedure, the enzyme was also inactivated (e.g. by low pH) to stop the reaction. The beads were sorted by flow cytometry according to their fluorescence (8). Finally, the coding sequences contained in beads identified as positive were recovered by PCR (9) and new diversity was introduced into the library. The coding sequences were then ligated or recombined back into the plasmid backbone for the next round.

An example of one round of directed *in vitro* evolution is shown in figure 5. For clarity, the samples shown were made using *E.coli* cells harboring only PTE-wt instead of a library of clones. Single *E.coli* cells with expressed enzymes were introduced into monodisperse droplets following a poisson distribution (A). After droplet

formation, cell lysis took place and the enzyme started to turn over the substrate, allowing for the fluorescent product to accumulate. After incubation, emulsion samples were heated to 95C to inactivate the reaction. Two emulsion droplets containing lysed E.Coli cells are shown (B) after enzymatic reaction and heat inactivation. Neighboring droplets are empty. The emulsion sample was converted into gel-shell beads (C). The image shows a brightfield microscopy image of a bead together with a fluorescence image highlighting the polyelectrolyte shell using a fluorescein-labelled polycation. The shell is located at the former oil/water interface forming a thin shell around the particle. Flow cytometry measurement and sorting of gel-shell beads was performed (D). Single beads were gated using forward- and side-scatter values. The population split into two peaks with different fluorescence and reflected the occupancy of cells in droplets. In the case of a library screen under stringent conditions, only a small number of particles reach the assay endpoint. After sorting, encoding plasmids were extracted from gel-shell beads, genes amplified and recloned for another round of mutation and/or selection (F). Flow cytometry measurements characterizing shell removal were then performed (E). Agarose-alginate particles exhibit only weak light scattering properties compared to gel-shell beads. Upon removal of the polyelectrolyte membrane the scattering values of the particles return to values similar to uncoated sample

Figures 6 and 7A show the device that was used to form droplets for agarose gel beads. All emulsion generators used in this study were based on the geometries depicted in figure 6, using either one or two inlets for aqueous phases. Flow rates were adjusted as such that emulsion droplets exhibited the smallest diameter possible for the respective chip used, with the highest aqueous flowrate that allowed reliable monodisperse emulsion generation.

The device shown in Figure 7A contained two aqueous inlets - one for the agarose and the second one for the remaining aqueous phase (e.g. containing nanoparticles). Microfluidic chips with channel width of 16um (20um channel height) were used. Applying flowrates of 500uL/h

for oil and 2*15uL/h for the aqueous phases resulted in droplets of about 18um diameter (3pL volume) and a throughput of 1*10E7/h. The two aqueous streams mixed at a flow-focusing geometry just before droplets were formed at a second flow-focusing point (Figures 7B and 7C).

Devices with the same basic design (Figure 7A), but with different channel widths (20µm, 30µm, 40µm and 50µm) at the flow-focusing droplet formation point were used to produce droplets of different diameters. Devices of 25µm or 50µm height were made out of PDMS and flushed with a hydrophobic surface coating agent (Duxback). The oil phase was fluorinated (HFE-7500) and stabilised with 0.5% EA surfactant (RainDance). The device shown in Figure 7A had a flow-focusing channel width of 20µm and a height of 25µm. Devices with these dimensions were usually used to encapsulate the DNA into the gel beads (Figure 4, step 1). When only the plasmid and the nanoparticles were encapsulated, these devices were typically operated with a total aqueous flow rate of 100 µl/h and an oil flow rate of 1000 µl/h at frequencies of 5 kHz or 10 kHz. When the DNA amplification mixture was also encapsulated, the aqueous solution became more viscous. Therefore, the flow-rates had to be reduced to a total aqueous flow rate of 50 µl/h and a oil flow rate of 900 µl/h in order to prevent jetting and maintain monodisperse droplet formation. This resulted in frequencies of approximately 10 kHz. Figure 7D shows droplets on a glass slide generated under these conditions. They have a diameter of 20µm.

The agarose was dissolved in the chosen buffer typically for an end concentration of 0.75% by heating to 90°C and kept in its liquid form by keeping the syringe and the tubing warm with hot sports hold/cold compresses. Droplets were collected on ice. If required, the emulsion was incubated and then broken by the addition of perfluorooctanol. Perfluorooctanol is a surfactant and can be used to form droplets in fluorinated oil, however it does not provide good droplet stability. When added in excess, it out competed the EA surfactant at the droplet interface and the droplets easily coalesced. Beads could then be recovered in the aqueous phase. Figure 7E shows the 0.75% agarose

beads recovered from the droplets shown in Figure 7D. They had approximately the same diameter as the droplets in which they were formed. Flow cytometry analysis (Figure 7F showed forward scatter (related to size) and sideward scatter (internal granularity) were similar to scatters obtained from polystyrene or latex beads of much smaller size (1 µm). Despite the preparation of highly monodisperse beads in size, as confirmed with microscopy, a rather high dispersion in the forward scatter was often observed. As the absolute value of forward scatter is very low, small differences in forward scatter might cause this dispersion.

Beads were concentrated and/or washed by centrifugation in a spin column. The beads did not pass through the membrane and could be re-suspended in a new buffer of choice.

Two concentrations (15 ng/µl and 30 ng/µl) of a plasmid (pIVEX-HA-GFP) were encapsulated in 0.75% agarose beads following the procedure outlined in the last section. Beads were either freshly recovered from the emulsion into the aqueous phase (TE buffer) or left in the buffer for one week at 4°C. PicoGreen – a sensitive fluorescent dsDNA stain – was added to the beads and their fluorescence was measured by flow cytometry (Figure 8). The fluorescence of the gel beads was identical after a week of incubation in TE buffer to a sample freshly recovered from the emulsion at both DNA concentrations tested. This observation indicates that the plasmid can be retained inside those beads for long periods of time in a quantitative manner.

As with plasmids (Figure 8), DNA amplified by isothermal amplification did not diffuse out of the agarose gel beads.

The illustra TempliPhi™ DNA amplification kit (GE Healthcare) was used to perform multiply-primed RCA. The kit components were compartmentalised in droplets together with the plasmid, BSA and the agarose dissolved in Phi29 DNA polymerase reaction buffer (NEB). The amplified DNA inside the gel beads was stained with PicoGreen. The stained beads were analysed by flow cytometry. Figure 6 shows an

example of such an analysis. Two peaks with different fluorescence were obtained (Figure 9A and 9B). The peak with the lower fluorescence overlaid with the negative control (no DNA added) and corresponded therefore to gel beads without amplified DNA (negative beads) (Figure 9C). The second peak represented the beads containing the amplified DNA (positive beads). 73% of the analysed beads were positive and 27% negative.

The amplification of DNA was also confirmed by quantitative PCR (qPCR). Approximately 50 copies of pIVEX-HA-GFP were compartmentalised per gel bead and isothermally amplified or not amplified. The same amount of emulsion was broken up for both samples. The gel beads were melted (3 min at 95°C), diluted and the amount of DNA was determined by qPCR. By comparing the amount of measured DNA in both samples an amplification factor of 36,000 was calculated.

The protocol for the illustra TempliPhi™ DNA amplification kit requires an initial heating step of the plasmid (3 min at 95°C) in the presence of the sample buffer containing the random primers to facilitate their annealing. This step was performed prior to droplet encapsulation.

In order to retain the expressed protein in the agarose beads after breaking of the emulsion, it was immobilised, as the pore size of the agarose allows proteins to diffuse through the gel. We used the HA epitope tag – anti-HA antibody interaction for immobilisation. The high affinity anti-HA antibody was biotinylated and could therefore be used to coat streptavidin-labelled latex particles with an average diameter of 50 nm. These nanoparticles were retained in the 0.75% agarose beads.

The plasmid encoding HA-GFP was encapsulated into agarose beads at a concentration of approximately 1.4 plasmids per droplet together with anti-HA antibody coated nanoparticles and amplified by multiply-primed RCA. The emulsion was broken and the beads were washed. Next, the amplified DNA in the beads was *in vitro* transcribed and translated and

the expressed GFP was captured on the enclosed nanoparticles. Three different ways to perform the IVTT were compared: 1) the IVTT was carried out in solution 2) the beads were together with the IVTT re-encapsulated into microfluidic droplets 3) the IVTT was performed in bulk emulsion generated by vortexing. After incubation at 30 C for 6 h and at 4 C overnight for chromophore maturation the beads were washed (TE buffer) and analysed by flow cytometry.

The population of single beads was identified and gated in the forward versus sideward scatter plot. An example is shown in Figure 10A. Figure 10B shows the green fluorescence (from GFP) for the different samples. Each of the samples 1-3 (corresponding to the different ways of performing IVTT) contained two bead populations of different fluorescence. The peaks with the lower fluorescence coincided with the two negative controls: beads to which no IVTT was added (NC2) and beads to which IVTT was added, but the incubation temperature was 4oC (NC1). Beads with low fluorescence (i.e. background fluorescence) lacked GFP (negative beads), because they did not contain any DNA. Peaks with a mean fluorescence higher than the negative controls contained GFP (positive beads).

Performing the IVTT in solution provided the highest proportion of negative beads. The value of 24% empty beads also corresponds well to the expected 24.6% at a loading of 1.4 plasmids per droplet. Individual agarose beads were too far away from each other within the 65µl reaction volume for significant contamination of agarose beads containing no DNA with GFP.

Next, the yield of IVTT from the unamplified plasmid was compared to IVTT from the product of multiply-primed RCA (Figure 11). pIVEX-HA-GFP and anti-HA antibody coated nanoparticles were encapsulated into agarose beads either with or without the DNA amplification mixture. The average concentration of plasmid was approximately 0.1 per gel bead. After incubation at 30oC for 10 h, the beads were washed and IVTT was carried out in solution, followed by flow cytometry analysis. The IVTT from single plasmids resulted in fluorescent agarose beads

that were clearly distinguishable from the beads without plasmid (Figure 11A and B) with a geometric mean of 19 for the positive beads. Detection of IVTT expression from single plasmids was also confirmed with pIVEX-HA-RFP.

By comparison the expression from amplified plasmid (Figure 11C and D) yielded a 2-fold higher signal for the positive beads (geometric mean 38).

An enzyme activity can be detected by fluorescence when the enzyme converts a non-fluorescent substrate into a fluorescent product. Fluorescein-derivatives are commonly used as substrates to assay hydrolytic enzymes. To perform compartmentalised and quantitative enzymatic assays, agarose beads were re-enclosed into monodisperse microfluidic droplets. The fluorescence was then measured and the droplets sorted.

The *Pseudomonas aeruginosa* arylsulfatase (PAS) and the substrate fluorescein disulfate were used to detect enzymatic activity in droplets that contained a re-encapsulated agarose bead. The plasmid pIVEX-HA-PAS was encapsulated into agarose gel beads together with anti-HA antibody coated particles. The concentration of the plasmid was approximately 10,000 copies per droplet - a concentration that may also be achieved with multiply-primed RCA. HA-PAS was *in vitro* expressed and the protein was immobilised on the anti-HA antibody coated particles. The gel beads were washed and re-encapsulated into microfluidic droplets together with the substrate fluorescein disulfate. After incubation at 30°C for 7 h the fluorescence of the droplets was detected using an EM-CCD camera coupled to an epifluorescence microscope (Figure 12). Bright fluorescent droplets indicate that the enzymatic hydrolysis of the substrate had occurred. Droplets without gel beads showed a low fluorescence and were a negative control for the background hydrolysis of the substrate. This experiment confirmed that enzyme activities can be detected from enzymes immobilised on particles in gel beads. Coupling of the substrate to a compound of higher molecular weight, such as dextran or

an oligonucleotide, renders encapsulation. The substrate (and consequently also the product was immobilised on an oligonucleotide (24 nt).

In order to achieve fast covering of the uncharged agarose beads with a complex of positive and negative polyelectrolytes, one polyelectrolyte was already added to the sample before making the emulsion and the other one was constituent of the aqueous phase into which the emulsion was broken up (Figure 13). Alginate was mixed with agarose to end concentrations of 0.5% and 0.75% respectively. The gelled agarose formed a scaffold for the polyelectrolyte complexation at the bead interface during de-emulsification. The agarose/alginate mixture was compartmentalised into the microfluidic droplets together with the agarose beads during droplet formation for the enzymatic assay. A device with a 40µm wide flow-focusing channel and 50µm channel height was used. The device was run with a total aqueous flow rate of 300 µl/h and 2000 µl/h for the oil flow rate. The resulting 40 µm diameter droplets were generated at 2.25 kHz. The droplets containing the negatively charged alginate were then broken into the positively charged polyelectrolyte PAH (10 mg/ml in 0.5 M NaCl). In that way the alginate (diffusing out of the bead) formed a complex with the PAH (diffusing into the bead) at the bead interface.

The sample was mixed with polydisperse vortexed emulsion containing PAH prior to de-emulsification. When the droplets were broken up, the PAH was released in immediate vicinity to the agarose beads and was forming a complex with alginate around individual beads preventing aggregation.

10 µM, 5 µM, 1 µM, 0.5 µM, 0.1 µM and 0 µM concentrations of a fluorescein labelled oligonucleotide were compartmentalised in microfluidic droplets together with 0.75% agarose and 0.5% alginate. The emulsions were broken and the beads were coated as described above. The fluorescence of the coated beads was measured by flow cytometry. Single beads were gated and the geometric mean and the standard deviation of these populations were plotted against the

concentration of compartmentalised fluorescein-oligonucleotide (Figure 14). The input concentration of fluorescent oligonucleotide correlated linearly with the measured fluorescence. This observation suggested that this method can be used to measure (end) points of enzymatic reactions that produce a fluorescence product.

For short kinetic measurements with incubation times of less than an hour, a protocol for on-chip de-emulsification and polyelectrolyte complex formation was developed (Figure 15). Three flow-focusing devices were connected to each other for continuous automated de-emulsification and polyelectrolyte complex formation. As described previously, 0.75% agarose and 0.5% alginate emulsion droplets were formed in a flow-focusing device (40 µm width, 50 µm height) resulting in droplets of 40 µm diameter (Figure 15). Instead of collecting the droplets in a tube, the droplets were routed into a tubing of 380 µm internal diameter. The length of the tubing determined the residence time of the droplets and therefore the incubation time for the reaction. The end of the tubing was connected to another simple flow-focusing device (150 µm width, 75 µm height) with one inlet connected to the outlet of the flow-focusing device producing the gel beads and the second inlet streaming in a vortexed bulk emulsion (1000 µl/h) containing the PAH solution (Figure 15b). The purpose of this device was to mix the two types of droplets so that they would be close to each other at the de-emulsification step to prevent aggregation. The emulsion entered a third flow-focusing device (150 µm wide, 75 µm high) were it was mixed with PFO (2000 µl/h) for de-emulsification (Figure 15C). Beginning droplet coalescence could already be observed on the device and was completed when they were collected in an Eppendorf tube containing more PAH and PFO. The recovered gel beads were not aggregated (Figure 15d) and were coated with an alginate/PAH complex of approximately 3 µm thickness.

In order to measure the amount of product produced after a specific timepoint, the enzyme is inactivated at this timepoint during the de-emulsification step. Depending on the enzyme this might be achieved by changing the pH, adding chelators for the metal cofactor or an

inhibitor. The PAH solution may be adjusted to the required conditions leading to inactivation of the enzyme in the course of de-emulsification and complexation. The state of the gel bead at the timepoint of de-emulsification is thus preserved and analysis can be performed later.

Flow cytometry experiments were performed with gel-shell particles 40um in diameter with fluorescence-labelled polyelectrolyte (orange: FITC-labelled PAH; black: rhodamine-labelled PAH) (Figure 16A). Single particle populations were gated and the geometric mean of the population plotted against the number of polyelectrolyte layers. Fluorescence values were normalized to samples coated with 4 layers (PAH/PSS/PAHlabelled/PSS) which served as example for a standard layer corresponding to the well-known PAH/PSS model system. The amount of absorbed labelled PAH in the initial layer corresponds to 0.4-0.7 times the amount of PAH deposited in later layers and its thickness was estimated to be about half of a regular layer. Samples coated with 6 layers in total, 3rd and 5th layer coated with FITC-labelled PAH confirmed the expected linear growth regime of the multilayer coating.

Flow cytometry data of gel-shell particles 20um in diameter, coated with one single layer of PAH is shown in Figure 16B.The samples originated from emulsions equipped with FITC-labelled primers (24bp in length, MW approx. 8kD) of increasing concentration (a). Single particle populations were gated and the geometric mean of the particles plotted against primer concentration. The inset to figure 16B shows fluorescence microscopy image of a bead. The fluorescence signal appeared to be dispersed rather homogeneously throughout the entire inner volume. The particle fluorescence corresponded linearly to the concentration of encapsulated labelled primers for a concentration range of at least two orders of magnitude. Figure 16B(b) shows emulsion droplets containing agarose, alginate and 10uM FITC-labelled primer, 40um in diameter, were deemulsified in mix with emulsion containing buffer only (1), emulsion containing CaCl2 (2) and emulsion containing PAH (3). Samples have been washed twice in buffer before measurements. Geometric mean fluorescence of single particle

populations are plotted. Whereas gel-shell particles are able to retain the fluorescent primers, they diffuse from unmodified agarose/alginate particles as well as from crosslinked alginate-agarose particles. Sample (3a) corresponds to gel-shell particles which were frozen with liquid nitrogen, stored at -80C and thawed. No significant loss of encapsulated FITC-labelled primer was observed. Similar freeze-thaw experiments have also been performed with gel-shell beads harboring single-cell lysate assays. No significant differences in fluorescence signal between frozen and nonfrozen samples could be detected. Figure 16B(c) shows similar experiments as in 16B(d) but with particles filled with two concentrations of a FITC-labelled peptide (HA-TAG, MW approx. 2kD?). Again, gel-shell particles (3) are able to retain the cargo whereas in unmodified agarose/alginate particles (1) and particles containing crosslinked alginate (2) the molecules diffuse. Experiments with fluorescein alone resulted in almost complete loss of fluorescence in all samples.

Gel-shell beads (GSBs) were equipped with reagents to enable enzyme assays. For example, the phosphotriesterase from *Pseudomonas Diminuta* (PTE) can be compartmentalized and assayed in situ. While the small molecule substrate is mobile, enzyme molecules do not pass the shell. The substrate, the pesticide paraoxon (Figure 19A), is small enough to enter the polyelectrolyte shell by diffusion and is converted into product there. PTE in a bead without gel-shell is degraded by proteinase K, but is protected when the shell layer is added.

Phosphotriesterase (PTE) catalyses the hydrolysis of organophosphate triesters, thioesters and fluorophosphonate compounds. The synthesis of *O,O*-diethyl *O*-(5(6)-carboxyfluorescein) phosphorothioate as substrate for PTE is shown in Figure 17. Hydrolysis of the monosubstituted fluorescein to fluorescein causes a 28-fold increase in fluorescence. The carboxylgroup of the substrate was then coupled to an oligonucleotide containing a 5′ primary amine at the end of a C12 linker to form an amide bond (Figure 17). The coupling was mediated with EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride). The PTE substrate coupled to the oligonucleotide was

hydrolysed by PTE in solution. A 0.5µM solution of this fluorescein coupled to the oligonucleotide (i.e. 5µM of the oligonucleotide) was encapsulated in gel beads by covering with a polyelectrolyte complex. The fluorescence of these beads measured by flow cytometry (Figure 18) corresponded well to the fluorescence of the fluorescein labelled oligonucleotide previously measured at this concentration (Figure 18). 10%-15% of the oligonucleotide was coupled to the fluorescein thiophosphatediester, which remained a substrate for PTE in the coupled form.

The results of another enzymatic assay in gel-shell particles are shown in Figure 19B. Samples with E.*coli* cells containing PTE-wt were prepared corresponding to protocols for droplet formation. Solution containing cells and alginate was mixed with solution containing agarose, lysis agent, lysozyme, fluorescein-based oligo-coupled substrate and agarose (everything kept on 30C to prevent gelation of the agarose), applied to a well in a 96-well plate and the enzymatic reaction measured at 30C (line a figure 19B). Sample contained buffer instead of agarose/alginate (line b figure 19B), and a sample lacking enzyme (cells) as control (line c figure 19B). Agarose/alginate-containing samples show similar kinetics to reactions without hydrogel component. Interestingly, the hydrogel matrix seems to slightly accelerate substrate turnover. This behaviour was confirmed in experiments using other enzyme variants of PTE.

After establishing compartmentalisation of enzyme, retention of reaction product by the polyelectrolyte shell was established. To this end, we co-compartmentalised substrate at the point of emulsion formation together with enzyme. The phosphotriester substrate tetraethyl O-(Fluorescein 5(6)-isothiocyanate) diphosphate was coupled to a 24 bp DNA oligonucleotide (Fig 19A). This tag prevents diffusion of substrate as well as of the fluorescent reaction product out of the GSBs. High-quality miniaturized assays in GSBs may be used for high-throughput screening experiments (Figures 4 and 5). In addition to the agarose and the polyanion alginate for gel-shell formation, the microdroplets are filled with single E.coli cells harboring the

expressed enzyme and the encoding plasmid DNA, a lysis agent to liberate the catalyst and the substrate. Single occupancy of cells in droplets follows a Poisson distribution and can be controlled by cell concentration. In the droplet, the two aqueous phases from the separate inlets are mixed, the cells are lysed and the enzyme is released and thus able to react with substrate. The reaction is conducted in emulsion with agarose in its liquid (sol) form. Reaction time is controlled by heat-inactivation of the emulsion sample. After inactivation, the sample is cooled on ice to gelate the agarose. The agarose core serves as template for shell fabrication which preserves the genotype-phenotype linkage and allows subsequent sorting of improved enzyme variants by flow cytometry. The genotype of a sorted particle is represented by a high copy number plasmid used for expression of the enzyme in the bacterial cell. Recovery of plasmids is achieved by disassembly of the polyelectrolyte shell upon short treatment with a solution exceeding the pK_a of the polycation (Figure 5E). The polyelectrolyte complex disintegrates and plasmids can be extracted by standard gel-extraction. After amplification and cloning, the selected variants are ready for another round of screening and/or mutation, or sequencing.

For directed evolution, the coding polynucleotide sequences of the selected clones may be retrieved. pIVEX-PTE (S6)-HA was compartmentalised into droplets at a concentration of one copy per droplet together with 0.75% agarose or with 0.75% agarose and 0.5% alginate. The DNA was either not amplified or amplified by multiply-primed RCA. The beads containing alginate were complexed with PAH during de-emulsification. Encapsulated beads without DNA were also prepared. All five types of beads were washed and counted to estimate their concentration. Approximately 280 beads were added to 20 µl PCR reactions for amplifying the PTE gene. 15 µl of the product were resolved by an 1% agarose gel (Figure 20). As positive control a PCR reaction containing 1 ng of purified plasmid was run. In the first attempt, the primers were used at a concentration of 2 M (Figure 20A). Only a very faint band resulted from the beads without complexation containing the unamplified plasmid (lane 2). In contrast, a strong

signal was detected from the beads without complexation containing the isothermally amplified DNA (lane 3). No signal was detected from beads that contained the polyelectrolyte shell (lanes 4 and 5). One or both of following possible explanations were thought to be the reason for the lack of bands: 1) The bead capsule did not release the DNA and the polymerase could not enter the bead. 2) The polyelectrolytes inhibit the PCR. To test the second hypothesis 1 ng of pIVEX-PTE(S6)-HA plasmid and empty beads with the alginate/PAS shell were added to a PCR reaction. No band could be detected (lane 6), indicating the polyelectrolytes were inhibiting the PCR. The positively charged PAH might have sequestered the primers. Therefore the primer concentration was increased to 4µM and BSA at a concentration of 1 mg/ml was also added to the PCR reaction (Figure 20B). An additional band appeared: The PCR amplified DNA from the complexed beads containing the isothermally amplified plasmid (lane 5). These observations showed that DNA was recovered from the gel beads and that isothermally amplifying the DNA inside the gel beads facilitated recovery.

E.Coli cells with expressed phosphotriesterase from *pseudomonas diminuta* were emulsified on chip (in an aproximate dilution of 1 cell every 10 emulsion droplets) together with agarose, alginate, cell-lysis agent and a primer-coupled phosphotriesterase-substrate. After incubation at 30C the enzymatic activity inside the droplets was killed by heat treatment for 5min at 95C and chilled on ice for 10 minutes to gelate the agarose. Then 2µL of the emulsion was embedded with mineral oil on a microscope slide and imaged (Figure 21).

After the enzymatic assay was conducted, the monodisperse emulsion was mixed with emulsion containing the cationic polyelectrolyte PAH (polyallylaminehydrochloride) (Figure 22). Polydisperse PAH-emulsion was used since it is quick to prepare by vortexing or other means. Then, the emulsion-mix was pipetted into a tube containing a weak surfactant (perfluorooctanol), and mixed and vortexed. The weak surfactant competed with the strong surfactant used for making the emulsion and destabilized the emulsion. Upon collapse of the emulsion the alginate diffusing out of the agarose beads and PAH, trying to

diffuse into the beads, formed a polyelectrolyte complex, the “shell”, along the surface of the agarose beads. This shell was used to entrap the substrate (and product) of the enzymatic reaction leading to a particle in aqueous solution that still retained the co-localization from enzyme geno- and phenotype. Unlike emulsions, these particles were able to be FACS-sorted.

FACS sorting of gel-shell beads harboring E.coli cells containing PTE-wt in a dilution of one cell per five droplets was performed (Figure 23). A dotplot with forward- and sidescatter is shown in figure 23A. The population of single particles is gated and shown as fluorescence histograms in Image B. Events scattering at lower values can be assigned to small satellite droplets originating from emulsion generation. These are produced when the emulsion generator is operated close to the jetting regime to maximize throughput. They do not affect monodispersity of the emulsion sample. Two sorting gates were applied to the fluorescence histogram (Figure 23B panel a), the first on the left sorting empty gel-shell beads, the second gate sorting beads equipped with lysed single cells (therefore with accumulated fluorescent product of the enzyme reaction). Reanalysis of the sorted samples confirmed successful sorts of empty (Figure 23B panel b), and filled (Figure 23B panel c), gel-shell beads.

Monodisperse emulsions, 40um in diameter, containing agarose and alginate were produced and deemulsified in mix with PAH-containing emulsion (Fig 24A) and CaCl2 containing emulsion (Fig 24B). A resulting gel-shell particle in 0.1M NaCl with a diameter of 40um is shown in Fig 24A panel a. If glycerol is added to a concentration of 25% (v/v), particle shrinkage is observed of about 15-20% of its former diameter (Figure 24Ab). The polyelectrolyte shell is deformed due to osmotic effects of the water/glycerol exchange. Particles in glycerol may be frozen and thawed without losing cargo. Upon washing and resuspension in 0.1M NaCl, or simple dilution of glycerol to 2.5% the particle is reconstituted to its original shape. With some gel-shell particles the agarose core stays slighly deformed (Fig 24A panel c). Freeze-thawing gel-shell particles in 0.1M NaCl lacking glycerol

results in deformed and broken gel-shell beads (Fig 24A panel d). A corresponding bead crosslinked with CaCl2 in 0.1M NaCl is shown in Fig 24B panel a. The particle lacks a visible shell as with gel-shell beads from Fig 24A panel a. These crosslinked particles were not deformed in the presence of glycerol (Fig 24B panel b)and could be freeze-thawed (Fig 24B panel c). As with gel-shell particles, freezing lacking glycerol resulted in broken and deformed beads (Fig 24B panel d). Freezing-experiments with agarose-only particles (0.75%), 18um in diameter, are shown in Fig 24C. Agarose beads before (Fig 24C panel a) and after freezing (Fig 24C panel b) in glycerol show similar morphology. Freezing without glycerol distorts their morphology.

The polyelectrolyte-shell may be disassembled by switching the pH in solution above/below the pKa of one of its constituents. In this case the beads were immersed in a solution of pH 12, washed and then analyzed with flow cytometry. In this conditions, PAH becomes uncharged and the polyelectrolyte-shell falls apart (Figure 25). Similar results can be obtained when incubating the sample in 5 M NaCl solutions and breaking down electrostatic interactions. After removal of the shell, the samples scatter similar to non-coated beads, proving successful disassembly.

Phosphotriesterase assays of gel-shell beads containing a phosphotriesterase library in emulsion were performed and stopped at two different timepoints (after 9h and 3h incubation at 30°C) by heating for 10' in 95°C. After cooling the emulsion to gel the agarose, the samples were coated with the polyelectrolyte shell for FACS sorting. FACS analysis of beads containing the phosphotriesterase library was performed (Fig 26).

After DNA-recovery from sorted beads, DNA-amplification, recloning and expression of the sorted pool of enzymes in E.Coli (or respective beads treated with IVTT) the samples were tested for a successful sort with an assay in 96-well plates. Every sample (library, sorted library after 9h incubation, sorted library after 3h incubation) consisted of an equal number of IVTT-treated beads or lysed *E.coli* respectively and

was tested with a substrate for overall enzymatic activity in one well of the plate. Figure 27 shows the results for a phosphotriesterase assay with paraoxon, absorption measurement at 405nm. Compared to the original library (figure 26), the samples show a 50 fold (for the 9h incubation sample) and a 100 fold (for the 3h incubation sample) increase of overall activity. The latter was subjected to another round of flow-cytometry sorting, this time with 1.5h incubation. This added, after recloning of the sample to another 2 fold overall increase of phosphotriesterase activity. In this example, a library of 400000 enzyme members was narrowed down to 100-200 library members in two subsequent rounds of selection. The remaining mutants were either tested clonally or this pool directly be subjected to another round of mutation following FACS-screening.

Further directed enzyme evolution of PTE in gel-shell beads was performed. First, enrichment was characterised by separating PTE from an enzyme without triesterase activity (acylphosphatase, ACP). PTE-encoding E. coli were diluted 1:10,000 in cells containing ACP. This mixture was then compartmentalized and two rounds of emulsion-based assays were carried out where GSBs were sorted with FACS. Figure 28A shows the enrichment analysis that reverses the 1:10,000 undershoot of PTE to exclusively observed PTE, giving an enrichment of > 100,000 fold in two rounds.

Next the system was used to select active PTE variants in a highly mutated error-prone PCR derived library with 7.5 mutations/gene on average ($3.8*10^5$members). Such selections are often performed first when evolving an enzyme towards new functions. It provides an initial pool of enzyme mutants as starting point for further mutation/selection cycles which is highly diverse but still catalytically functional towards its native activity. However, the high mutation rate should render most library members inactive. The remaining active members will give a broad spectrum of variation in activity at or below the enzymatic activity of the libraries parent sequence. A dotplot (forward scatter vs fluorescence) of gel-shell beads equipped with the initial library is shown in Figure 28A. The

small subpopulation on the right of Fig 28A is doublets of gel-shell particles due to non-ideal shell fabrication.

The DNA library was generated starting from a PTE-variant with a k_{cat}/K_m of 10^6 $M^{-1}s^{-1}$ for paraoxon and sorting experiments were conducted. The emulsion sample was incubated for 3h at 30C before heat inactivation. 4800 gel-shell beads were sorted out of 2*10E6 single beads (approx. the best 1% of all variants), plasmids extracted and recloned. To challenge the library for higher activity, selection pressure was applied by adapting incubation time and sorting threshold to estimated 20% of the activity of the parental PTE-mutant. The enrichment of active clones was determined by comparing enzyme activity of cell lysates derived from identical numbers of *E.coli* cells from the library before and after sorting in single wells of a 96 well plate using 250 uM paraoxon as substrate. The overall enzymatic activity of the sorted library exceeded the initial library by 100-fold after the first enrichment cycle with 3 h incubation time (see Fig 28).

The second round of sorting with samples incubated for 1.5h reduced the library to approx. 200 individual clones, the fittest 0.05% of the initial library. Increasing the selection pressure in the second round by incubation for half the time (1.5 h) doubled the activity (i.e. 2-fold improvement), indicating proportionality between incubation time and further improvement. Single clones from the second screening cycle were picked randomly and monoclonal lysate assays were carried out with paraoxon as substrate. 74% of the clones showed 0.2-1.4 fold activity relative to the parent sequence and confirm the systems capability for enrichment (see Figure 28).

In contrast to highly mutated sequences, lower mutation rates yield libraries with considerable background of the native parent sequence. Screening such libraries must enrich beneficial variants efficiently enough that at least one beneficial clone can be isolated after a screen which serves as template for another round of mutation. To test this concept, a library of a PTE variant with a k_{cat}/K_m of $2.5*10^4$ $M^{-1}s^{-1}$ for paraoxon was generated containing ~1.5 mutations/gene and samples

were screened. Figure 29C shows FACS histograms of single gated beads of two sample aliquots with different incubation times. Incubating the sample emulsion for 1.5h and sorting the best 2% of the total population allowed for sufficient enrichment of the library (initially containing 5 x 10^5 clones) to isolate a beneficial variant in fifty randomly picked clones (Fig 29B). Purified, this clone showed a 9-fold improvement in k_{cat}/K_m for the substrate paraoxon and had accumulated three point mutations.

A novel platform for *in vitro* directed evolution combining microfluidic droplets and gel beads is set out herein.
The combination of microfluidic droplets and gel beads is useful for many applications, in addition to *in vitro* directed evolution. The ability to wash, perform multiple steps sequentially, the ease of use and flexibility are important advantages. After de-emulsification, the droplet compartmentalisation is maintained in a gel bead and thus making it possible to perform washing steps. The workflow may involve isothermal DNA amplification, IVTT and an enzymatic assay and uses only simple microfluidic flow-focusing devices. Application of polyelectrolyte complexation allows encapsulation the product of an enzymatic assay. Thus, the gel beads can be analysed and sorted with a flow cytometer.

High-throughput is important for multi-step biochemical reactions such as directed evolution. With a single device at least $9x10^6$ droplets/h were produced for the DNA compartmentalisation step. The re-encapsulation step had a similar throughput of at least $8.1x10^6$ droplets/h and beads can be sorted at a rate of at least $1.1x10^7$ beads/h. As the DNA does not diffuse out of the gel beads, so large batches of beads containing (amplified) DNA can be produced and stored. Similarly, if the enzyme is inactivated during the gel bead coating, the bead may be stored before analysis.

Our approach simplifies the complex engineering tasks of directing protein evolution in monodisperse droplet compartments using microfluidic devices: a simple flow focussing device is required for

droplet formation and a standard flow cytometry sorter can sort 1 x 10^8 GSBs per hour. Time control is achieved with heat-inactivation of samples. To further tune assay times towards the timescale of reactions, changes in emulsion diameter as well as transcription/ translation efficiency can be employed. Using cells to produce and deliver a macromolecule together with its DNA-encoding into composites is only the simplest form of producing "monoclonal" composites. Entirely in-vitro based workflows are possible through combining technologies, for example upon replacing the use of cells with libraries of beads displaying the catalyst together with its encoding. Assays based on the lysis of cells in general remove in vivo constraints and make selections in non-natural environments feasible. GSB-encoded protein evolution may be expanded towards evolving characteristics of biological building blocks in composite materials themselves, either by direct means or by introducing fabricated composites again into emulsion systems. The compatibility of gel-shell beads towards further engineering with (nano-) technologies might therefore lead to a class of hybrid composite materials where a given functionality of their biologically-derived parts can be tailored in-situ by evolution rather than design.

Claims:

1. A method of compartmentalised *in vitro* screening comprising:

1). emulsifying an aqueous reporter solution which comprises a population of polynucleotides, a reporter substrate, and a gel-forming agent into microdroplets,

wherein each polynucleotide encodes a product which converts the reporter substrate into a detectable reporter in said microdroplets,

2). solidifying the gel-forming agent within the microdroplet to produce a gel bead comprising the polynucleotide and the detectable reporter produced by the product, wherein the detectable reporter is produced before, during or after the solidification,

3). de-emulsifying the aqueous microdroplets and re-suspending the beads in aqueous detection solution and

4). detecting, determining or measuring the detectable reporter in one or more beads in said population.

2. A method according to claim 1 wherein the polynucleotides are expressed within the microdroplets to produce the encoded products following emulsification.

3. A method according to claim 1 wherein the reporter solution further comprises the products encoded by the polynucleotides, each said product being co-localised with its encoding polynucleotide.

4. A method according to claim 3 wherein each said product is co-localised in a cell or viral particle with its encoding polynucleotide.

5. A method according to claim 4 wherein the cell expresses the polynucleotide to produce the encoded product.

6. A method according to claim 3 wherein each said product is co-localised in a first gel bead with its encoding polynucleotide.

7. A method according to claim 6 wherein the gel forming agent is solidified around the first gel bead to produce a gel bead which comprises the detectable reporter and the polynucleotide.

8. A method according to claim 6 claim 7 comprising providing a population of gel beads, said beads comprising a diverse population of polynucleotides and encoded products.

9. A method according to claim 8 wherein the population of beads is provided by a method comprising:

i). emulsifying an aqueous expression solution comprising a diverse population of polynucleotides and a gel-forming agent into microdroplets,

ii). allowing the aqueous expression solution to express the polynucleotides in the microdroplets to produce products encoded by the polynucleotides, such that each polynucleotide and its encoded product are co-localised in a microdroplet and

iii) solidifying the gel-forming agent to produce gel beads comprising the polynucleotide and the encoded product in the microdroplets;

wherein the product is produced before, at the same time as or after the hydrogel-forming agent is solidified, and

iv). de-emulsifying the microdroplets and isolating the population of beads,

said beads comprising a diverse population of polynucleotides and encoded products.

10. A method according to any one of claims 6 to 9 comprising amplifying the polynucleotides.

11. A method according to claim 10 comprising:

i). emulsifying an aqueous amplification solution comprising a diverse population of polynucleotides and a gel-forming agent into microdroplets,

ii). allowing the amplification solution to amplify the polynucleotides in the microdroplets to produce amplified copies of the polynucleotide,

iii) solidifying the gel-forming agent in the microdroplets to produce gel beads containing the amplified copies of the polynucleotides;

wherein the polynucleotides are amplified before, at the same time as or after the gel-forming agent is solidified,

iv). de-emulsifying the microdroplets and isolating the beads, and;

v). contacting the beads with an expression solution to express the amplified copies in the microdroplets to produce the products encoded by the polynucleotides,

wherein each product is co-localised with its encoding polynucleotide in a solidified bead,

such that said beads comprising a diverse population of polynucleotides and encoded products.

12. A method according to claim 11 wherein step v) comprises:

(i) emulsifying the beads comprising the amplified copies in microdroplets which comprise the expression solution,

(ii) expressing the amplified copies of the polynucleotide in each said bead to produce a product encoded by polynucleotide in the bead, and

(iii) de-emulsifying the microdroplets.

13. A method according to any one of the preceding claims wherein the gel-forming agent is solidified in the presence of a retention agent which binds to the encoded product.

14. A method according to claim 13 wherein the retention agent is an antibody coated nanoparticle.

15. A method according to claim 14 wherein the encoded product comprises an epitope tag and the nanoparticle is coated with an antibody which binds the epitope tag.

16. A method according to any one of the preceding claims wherein the reporter solution further comprises a functional reagent, such that the functional reagent is incorporated into the bead.

17. A method according to claim 16 wherein the functional reagent is a first polyion.

18. A method according to claim 17 wherein the aqueous microdroplets are de-emulsified in a solution comprising a second polyion having opposite charge to the first polyion,

such that the first and second polyions form a polyionic shell on the surface of the bead.

19. A method according to claim 17 or claim 18 wherein the first polyion is alginate, polystyrene-sulfonate, non-coding polynucleotide, polyacrylic acid or PMMA.

20. A method according to any one of claims 17 to 19 wherein the second polyion is poly(allylamine hydrochloride) (PAH) poly-lysine, poly-arginine, or polydimethylammonium-chloride.

21. A method according to any one of claims 17 to 20 comprising removing the polyionic shell of one or more beads identified as containing the detectable reporter or an amount of detectable reporter above a threshold value.

22. A method according to any one of the preceding claims comprising inactivating the encoded product.

23. A method according to any one of the preceding claims wherein the beads are isolated and/or washed following de-emulsification.

24. A method according to claim 23 comprising storing the beads.

25. A method according to any one of the preceding claims comprising measuring the detectable reporter in one or more beads in said population by flow cytometry.

26. A method according to any one of the preceding claims comprising;

5) isolating and/or identifying one or more beads from the aqueous solution which contain the detectable reporter or which lack the detectable reporter or which contain levels of reporter above a threshold level.

27. A method according to claim 26 wherein the beads are isolated and/or identified by flow cytometry.

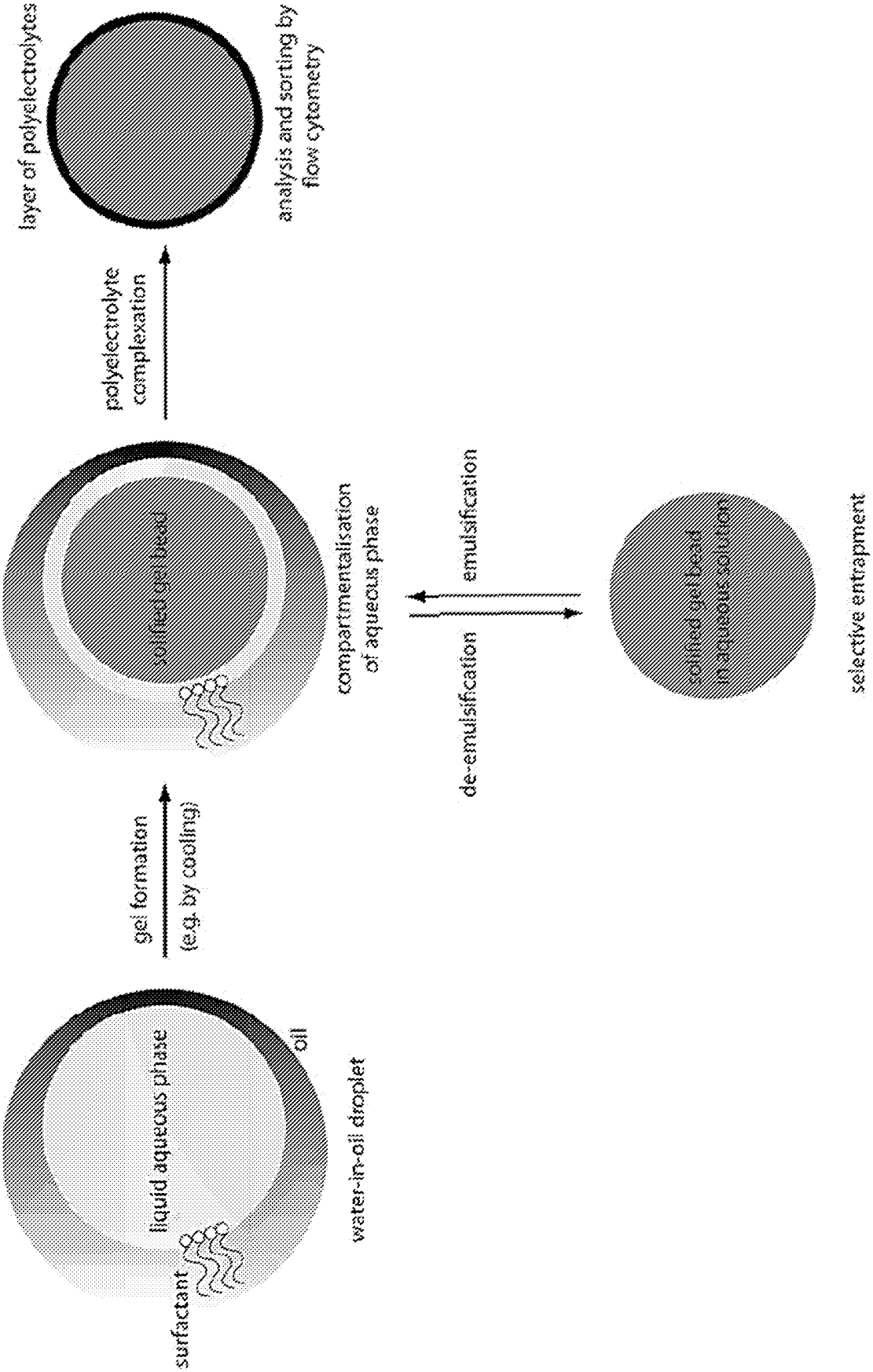
28. A according to any one of the preceding claims method comprising;

6) isolating and/or identifying the polynucleotides from one or more beads which contain the detectable reporter or which lack the detectable reporter.

29. A method according to claim 28 comprising introducing one or more sequence alterations into the polynucleotides to produce a population of variant polynucleotides.

30. A method according to any one of the preceding claims comprising repeating steps 1 to 4 and optionally steps 5 and 6, one or more times.

Figure 1


surfactant
liquid aqueous phase
oil
water-in-oil droplet
gel formation
(e.g. by cooling)
solified gel bead
compartmentalisation
of aqueous phase
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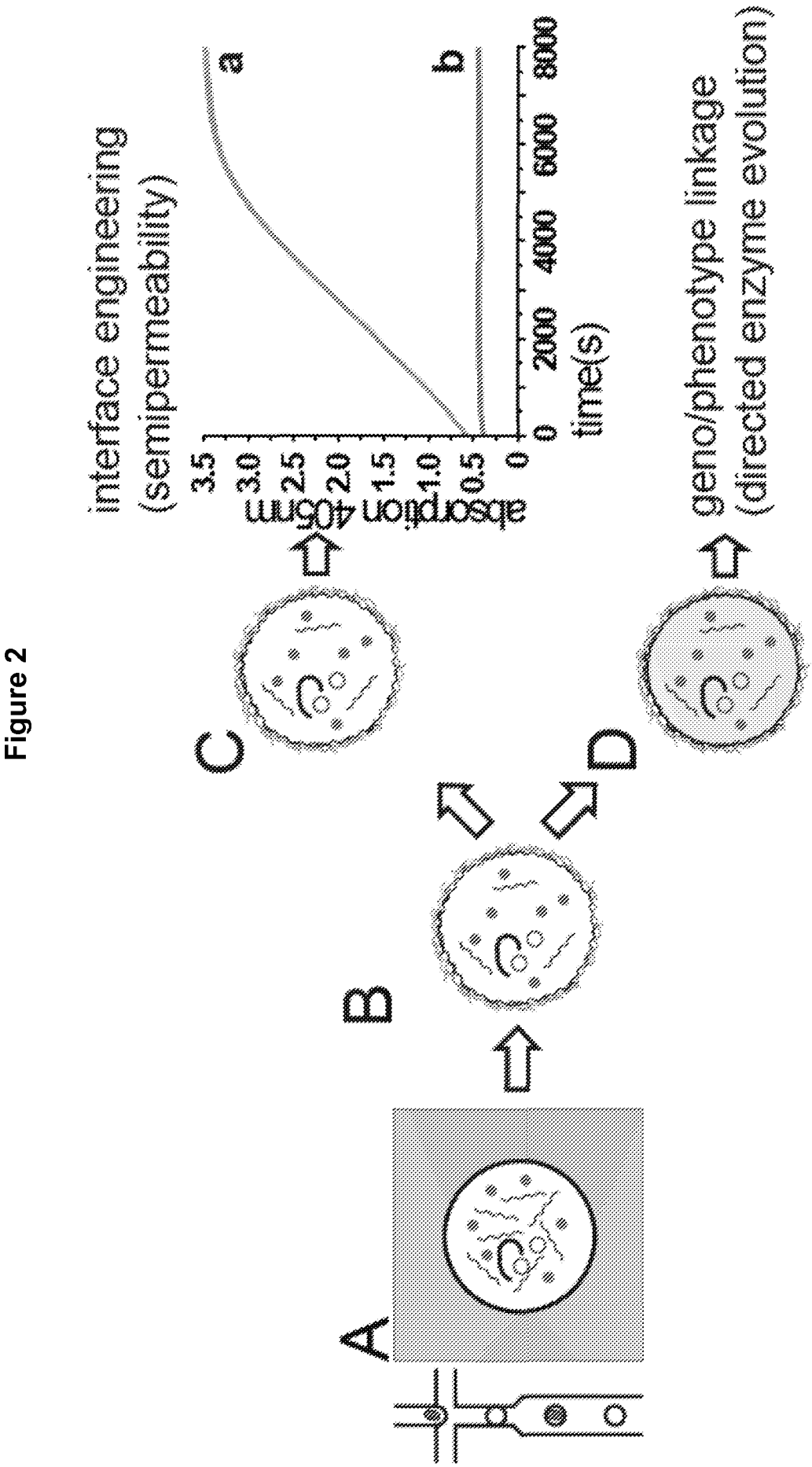
de-emulsification
solified gel bead
in aqueous solution
selective entrapment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0
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4000
6000
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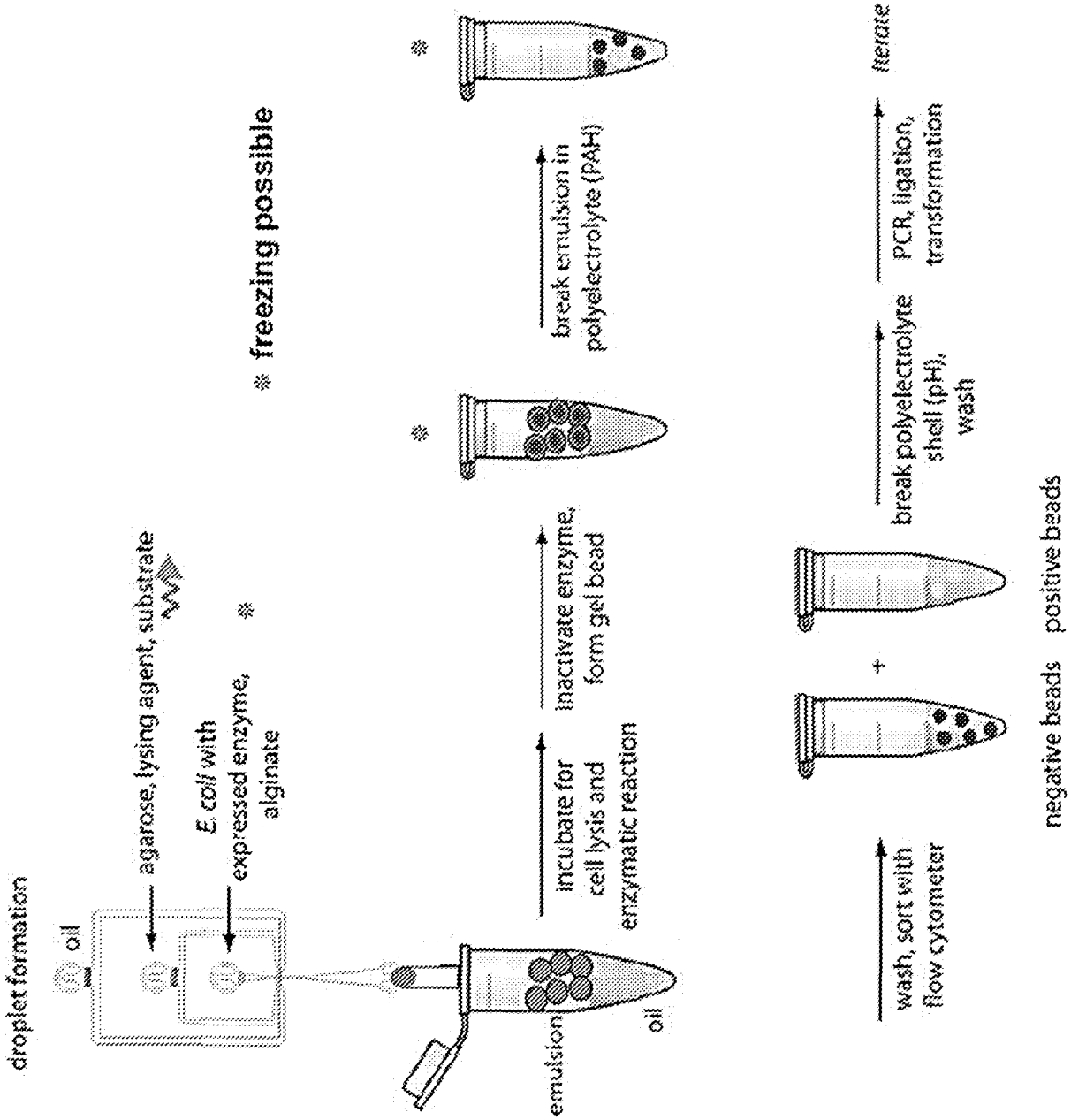
time(s)
geno/phenotype linkage
(directed enzyme evolution)

Figure 2

Figure 3


droplet formation
oil
agarose, lysing agent, substrate
E. coli with expressed enzyme, alginate
emulsion
oil
incubate for cell lysis and enzymatic reaction
inactivate enzyme, form gel bead
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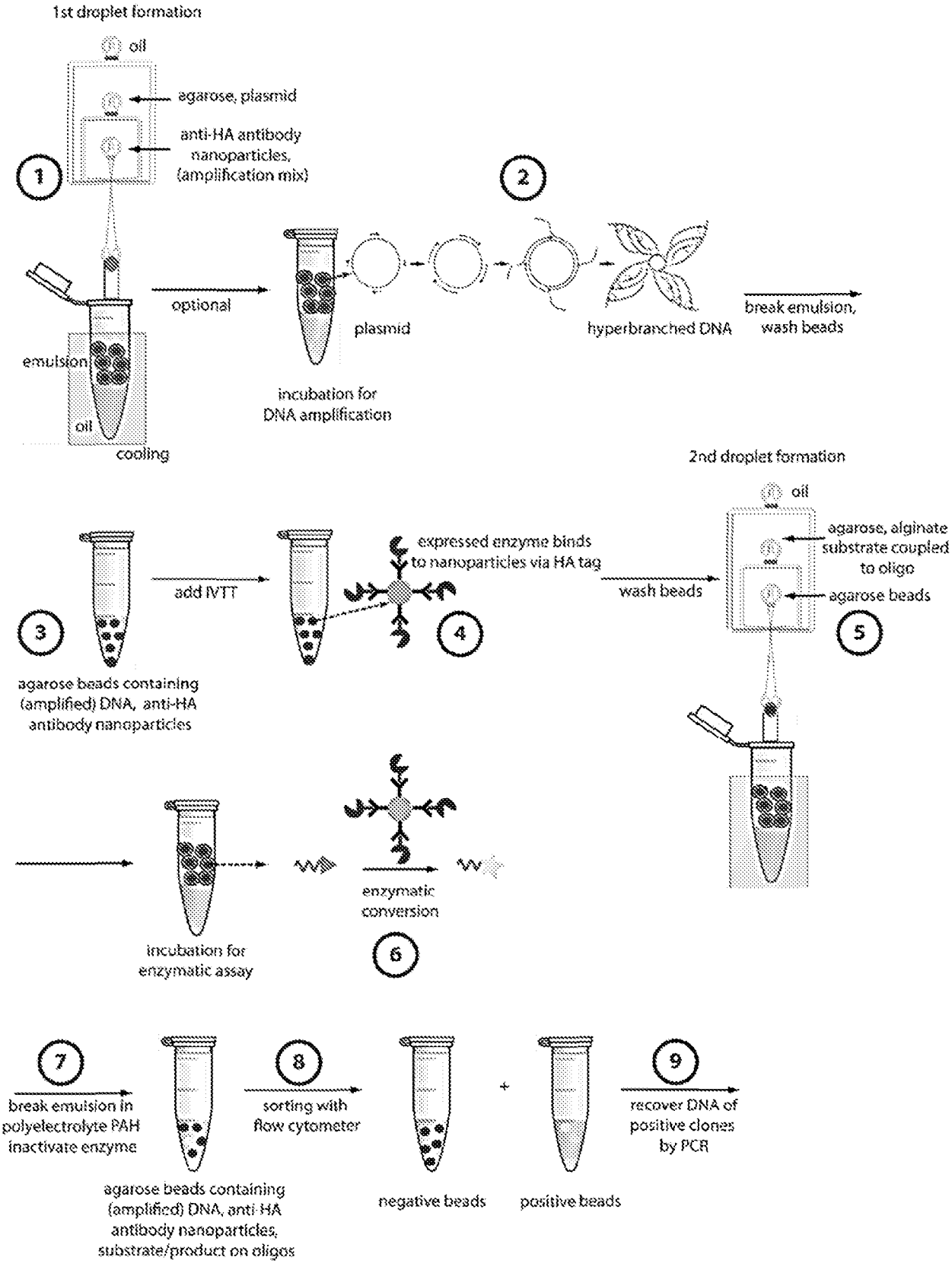
break emulsion in polyelectrolyte (PAH)
break polyelectrolyte shell (pH), wash
PCR, ligation, transformation
iterate
wash, sort with flow cytometer
negative beads
positive beads

Figure 4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agarose, plasmid
anti-HA antibody nanoparticles, (amplification mix)
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2
optional
plasmid
hyperbranched DNA
break emulsion, wash beads
emulsion
oil
cooling
incubation for DNA amplification
2nd droplet formation
oil
agarose, alginate substrate coupled to oligo
agarose beads
5
wash beads
add IVTT
expressed enzyme binds to nanoparticles via HA tag
4
3
agarose beads containing (amplified) DNA, anti-HA antibody nanoparticles
enzymatic conversion
6
incubation for enzymatic assay
7
break emulsion in polyelectrolyte PAH inactivate enzyme
8
sorting with flow cytometer
9
recover DNA of positive clones by PCR
agarose beads containing (amplified) DNA, anti-HA antibody nanoparticles, substrate/product on oligos
negative beads
positive beads

Figure 5

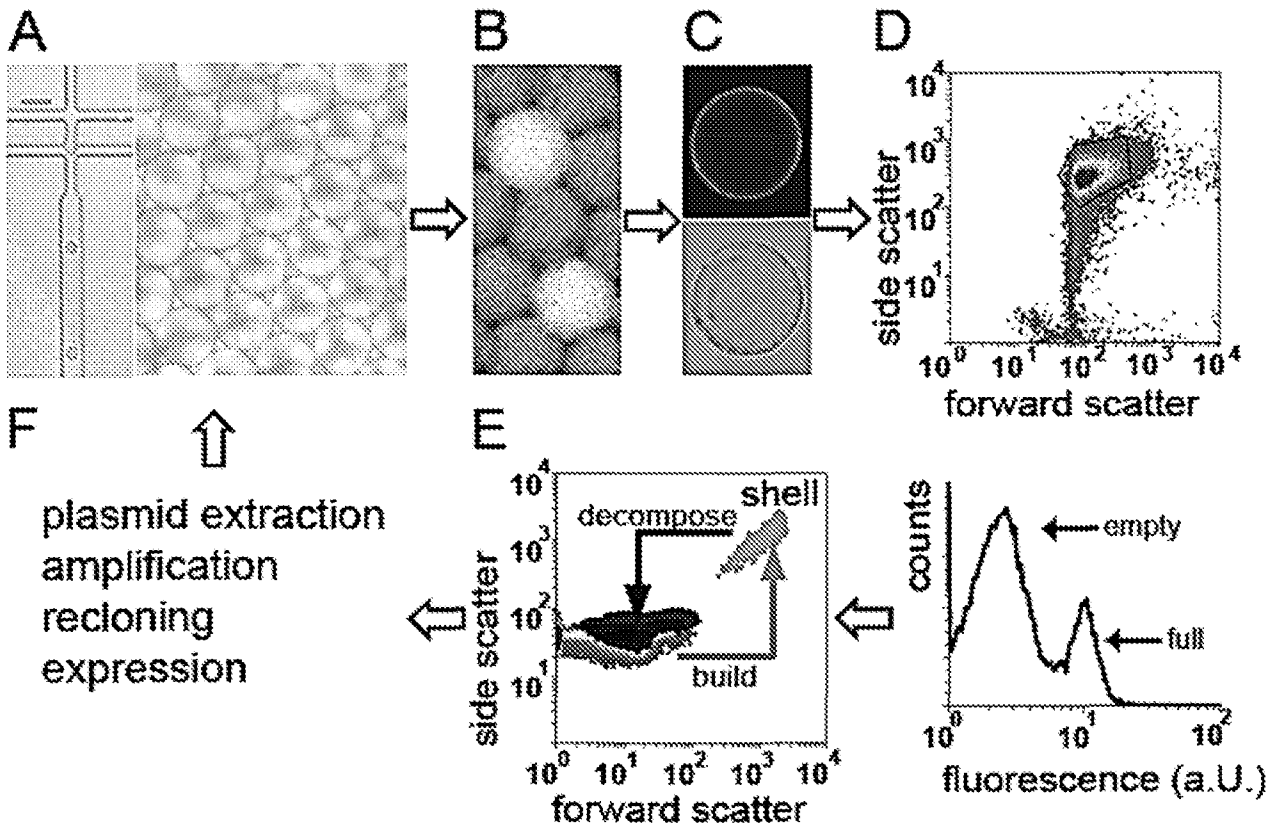


Figure 6

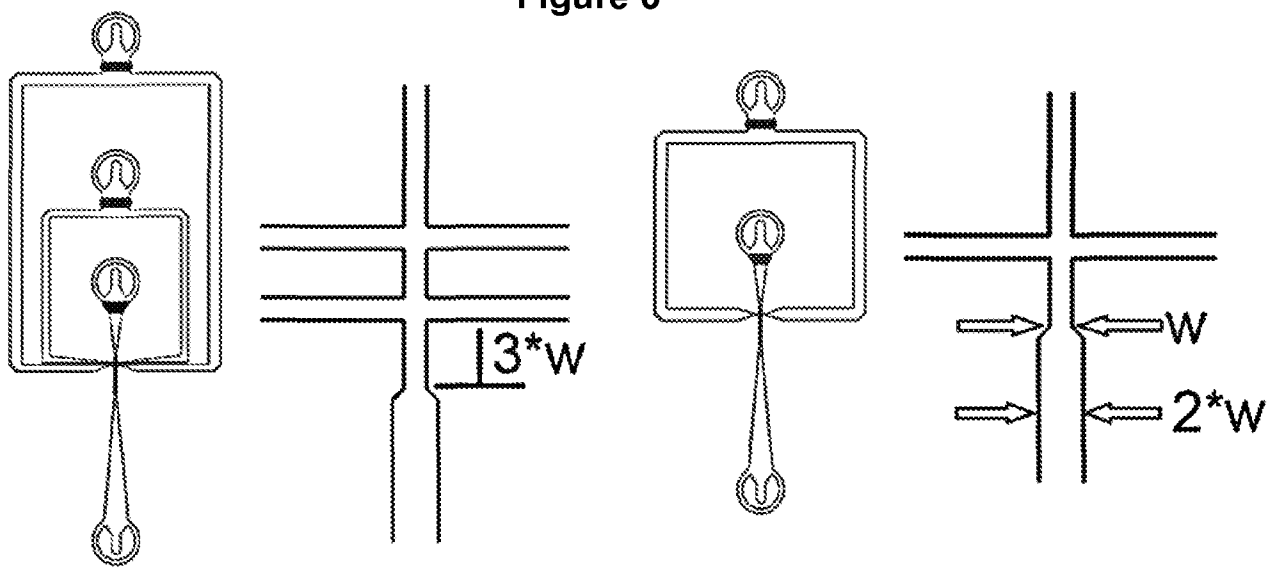


Figure 7

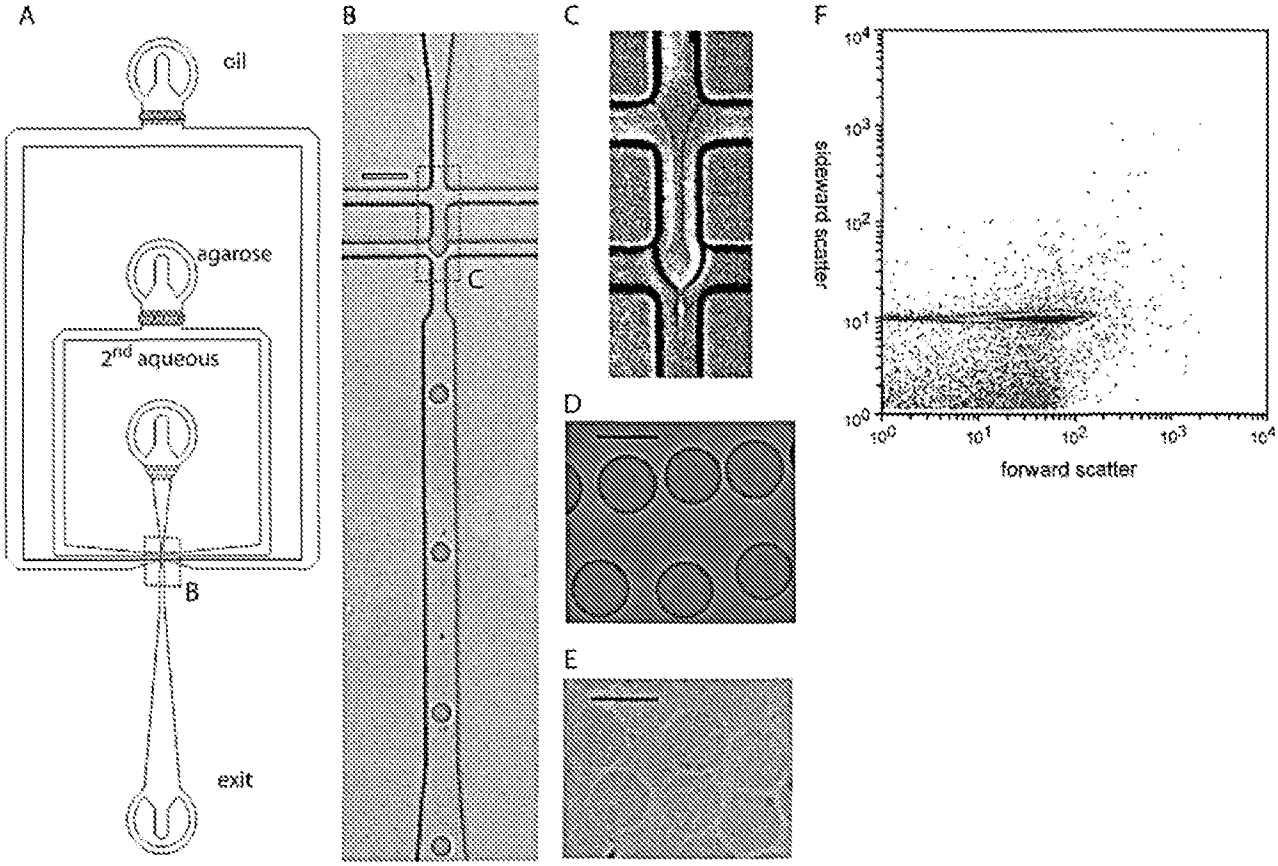


Figure 8

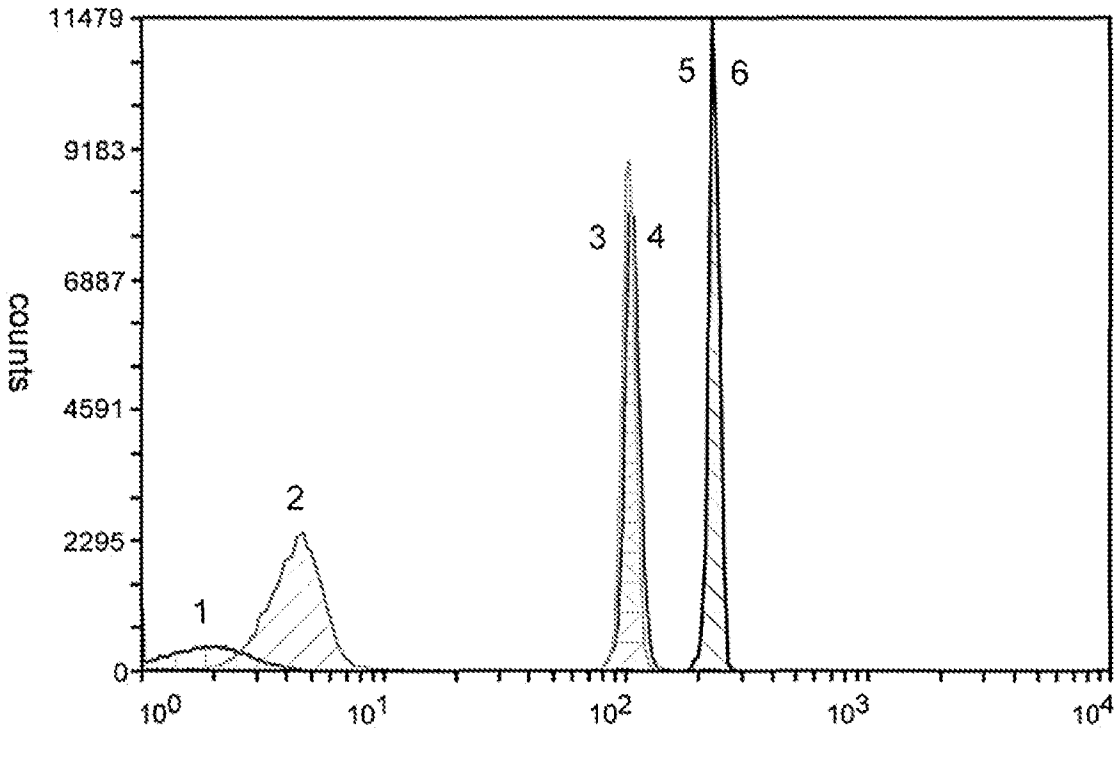
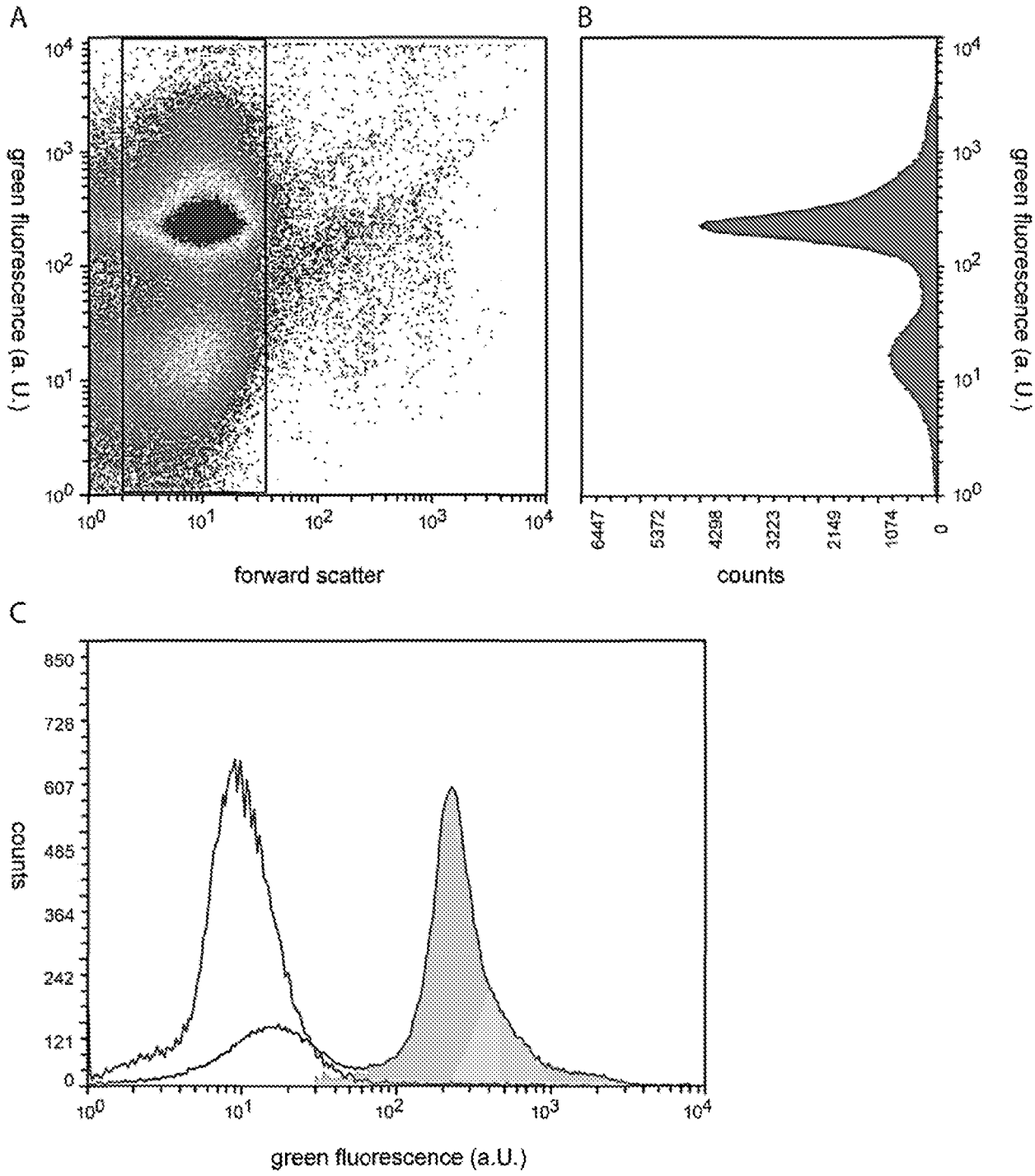


Figure 9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B
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forward scatter
counts
green fluorescence (a. U.)
green fluorescence (a.U.)
counts
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10^2
10^3
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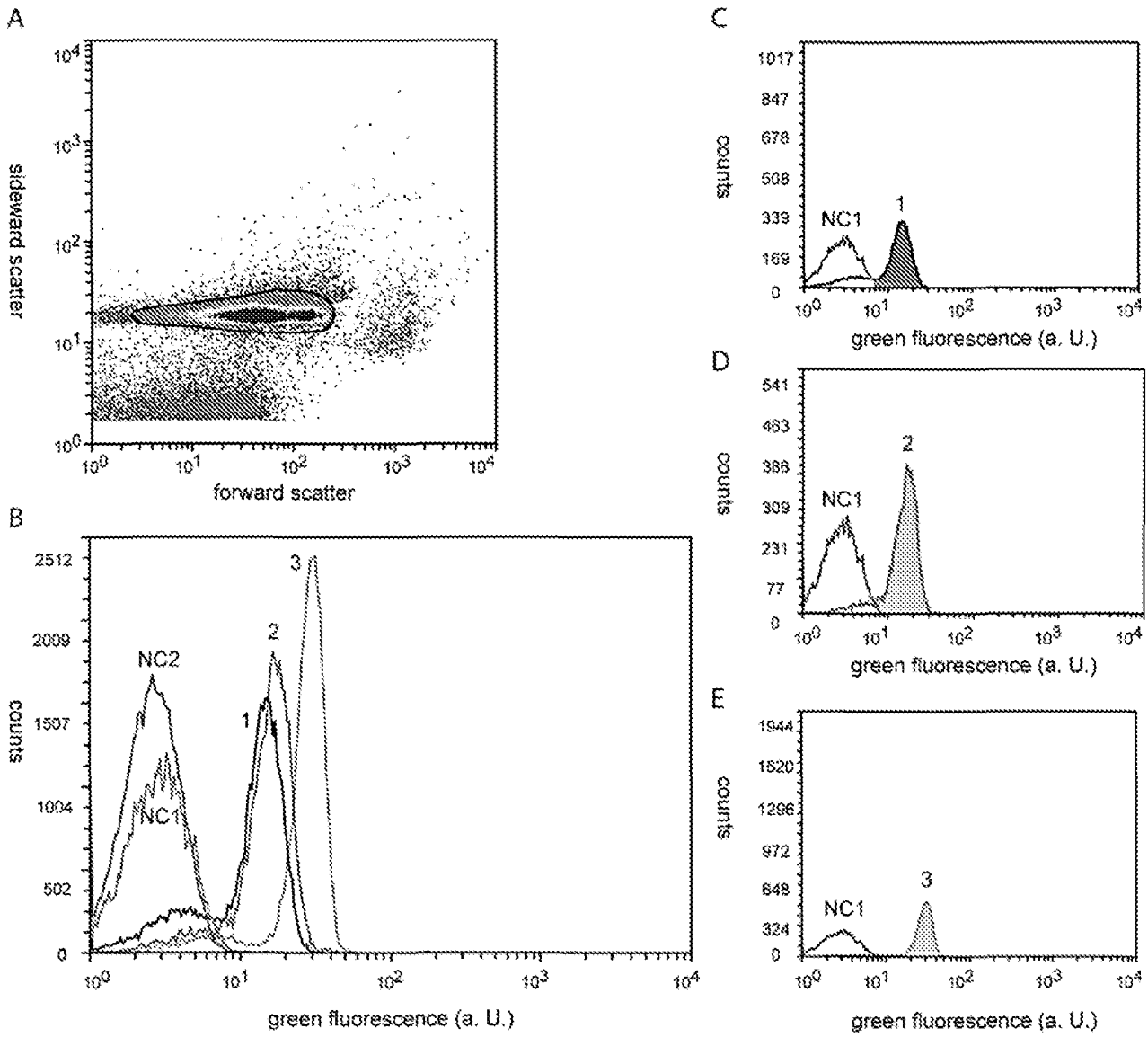
Figure 10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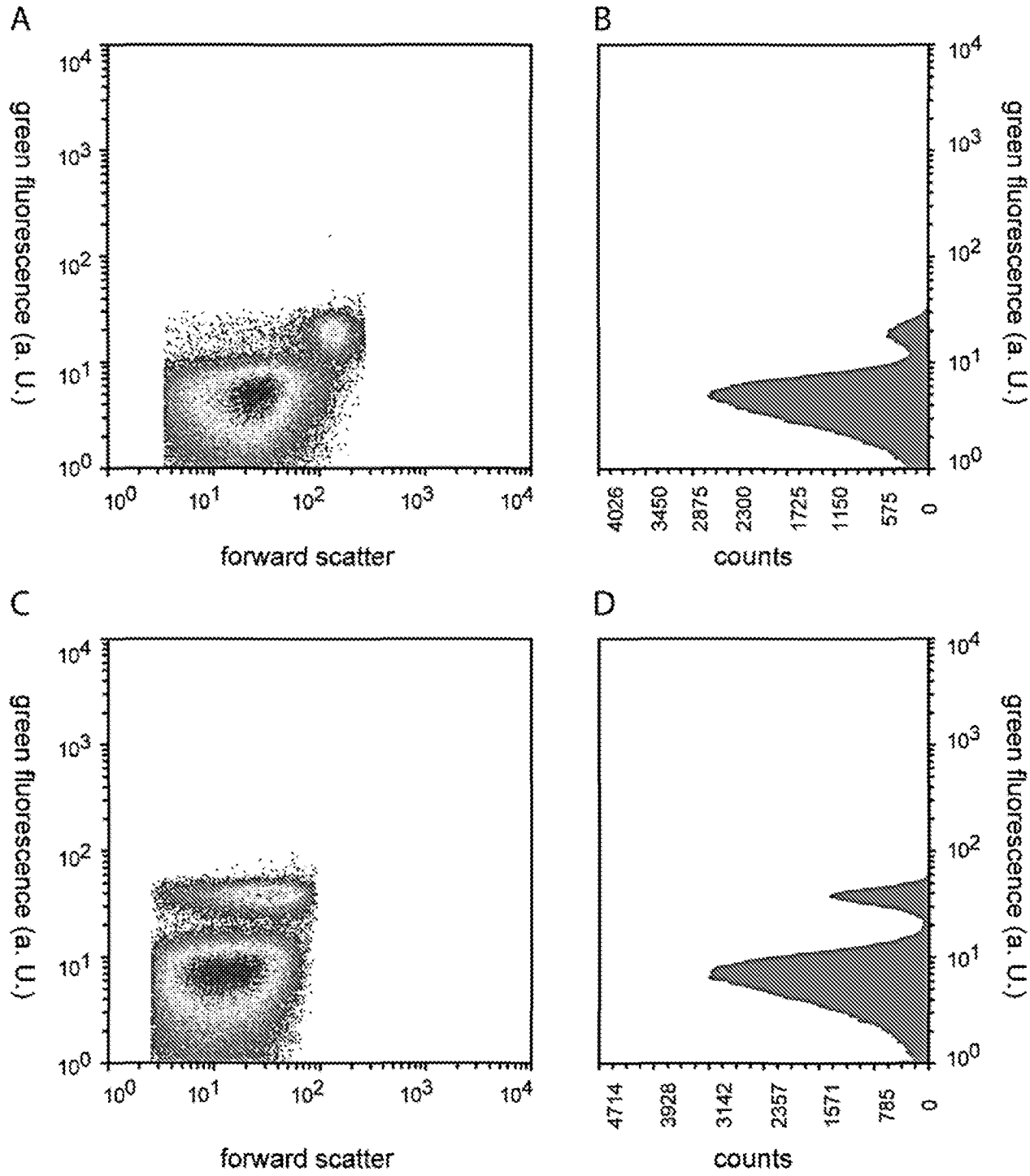
D
counts
NC1
2
green fluorescence (a. U.)
E
counts
NC1
3
green fluorescence (a. U.)

Figure 11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forward scatter
counts
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10^3
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Figure 12

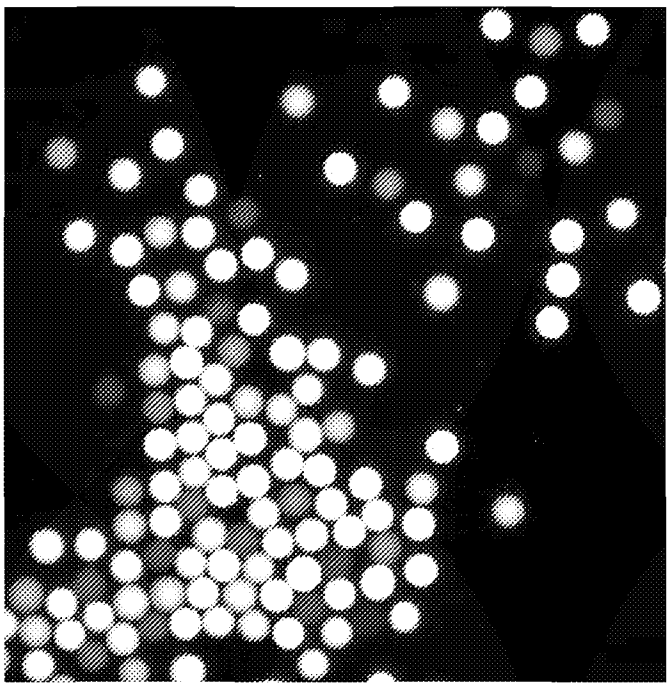
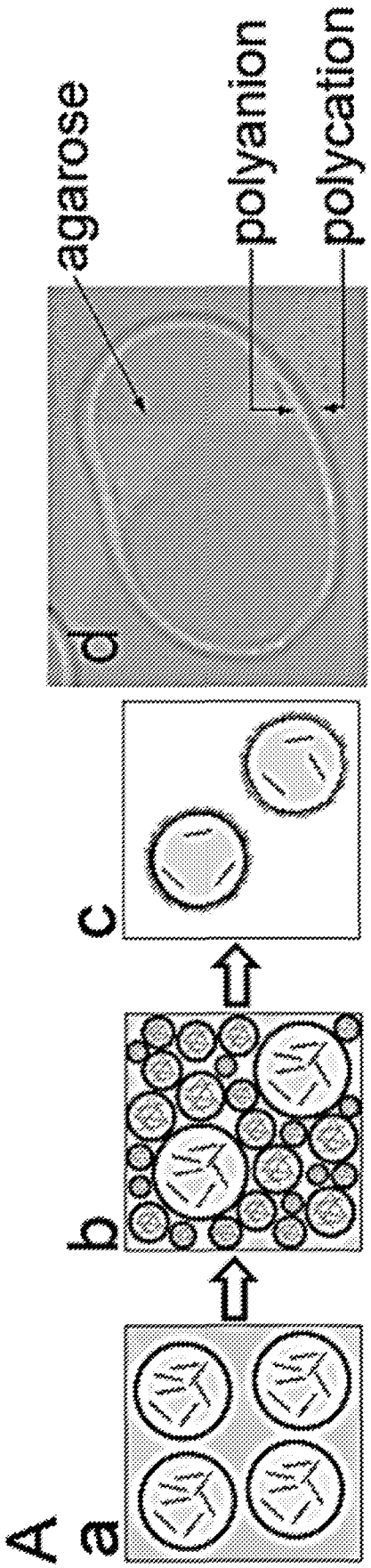


Figure 13



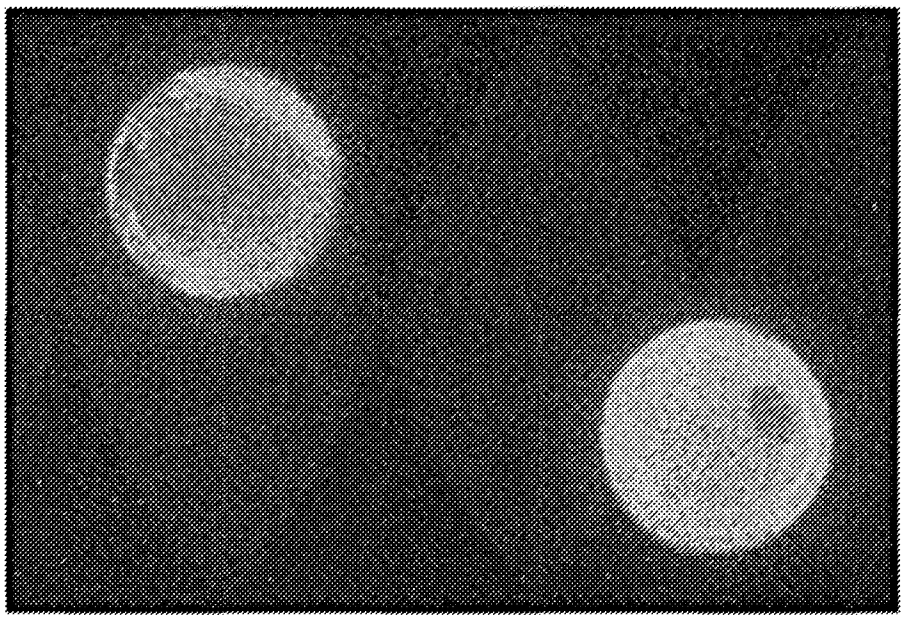
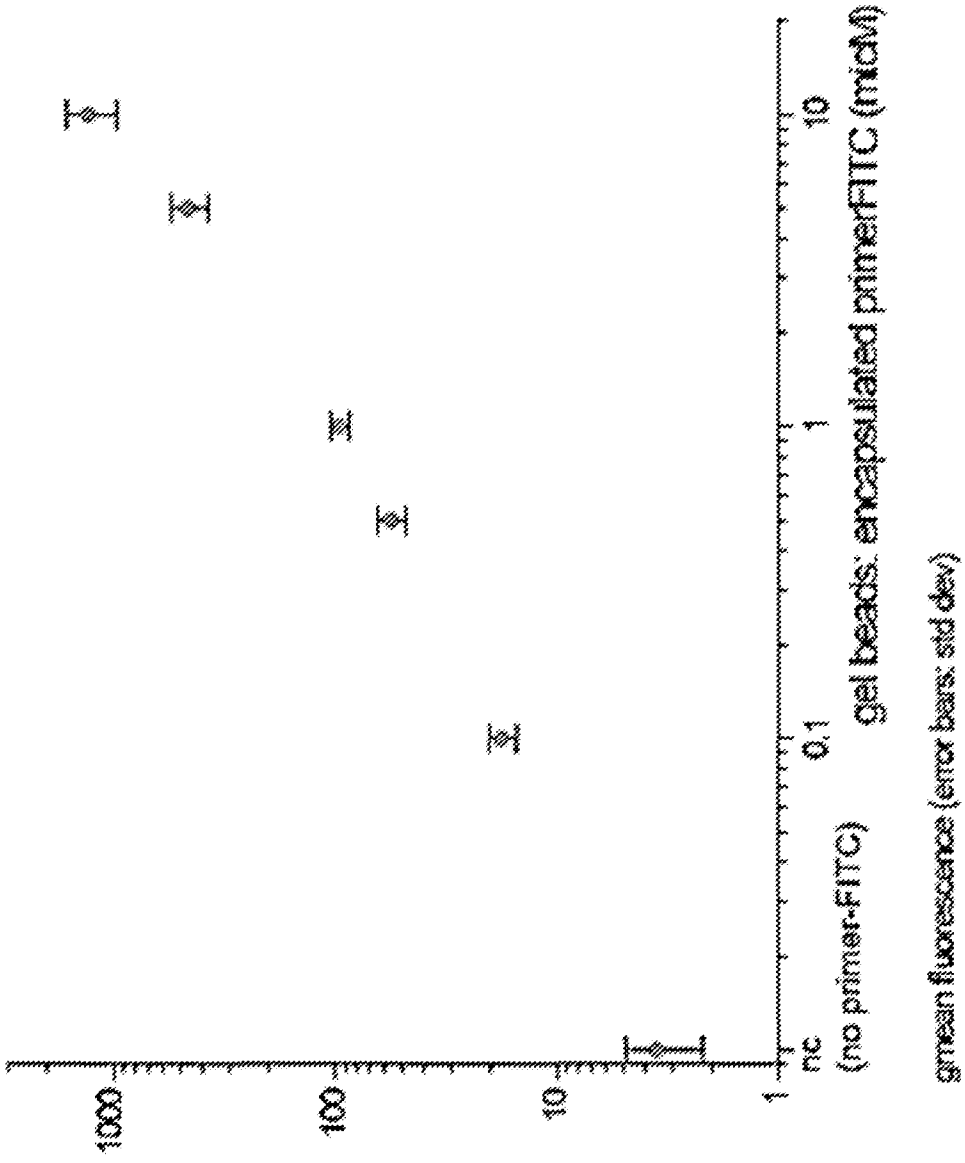


Figure 14


green fluorescence (a.u.)
1000
100
10
1
nc
(no primer-FITC)
0.1
1
10
gel beads: encapsulated primerFITC (mid/l)
gmean fluorescence (error bars: std dev)

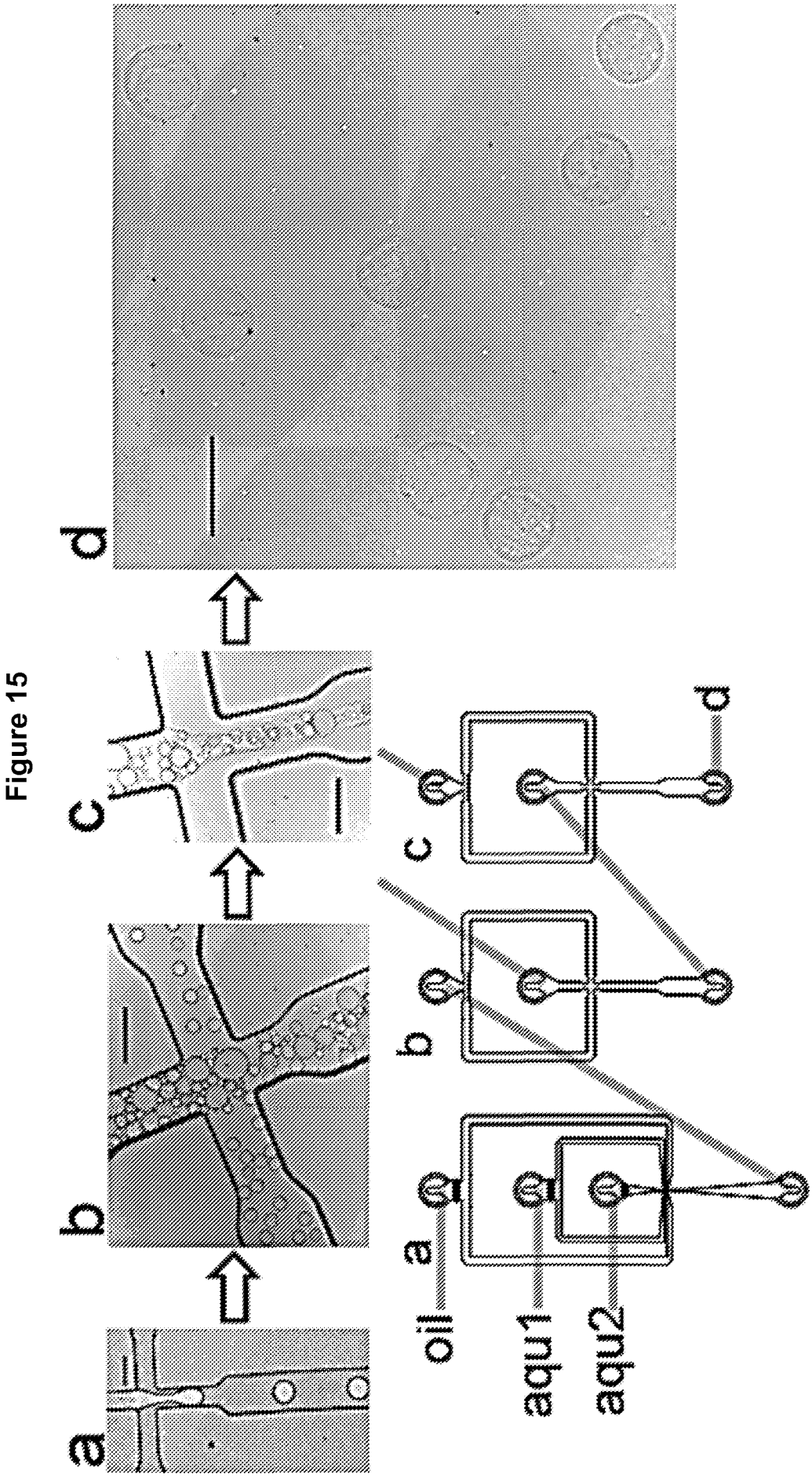


Figure 15

Figure 16

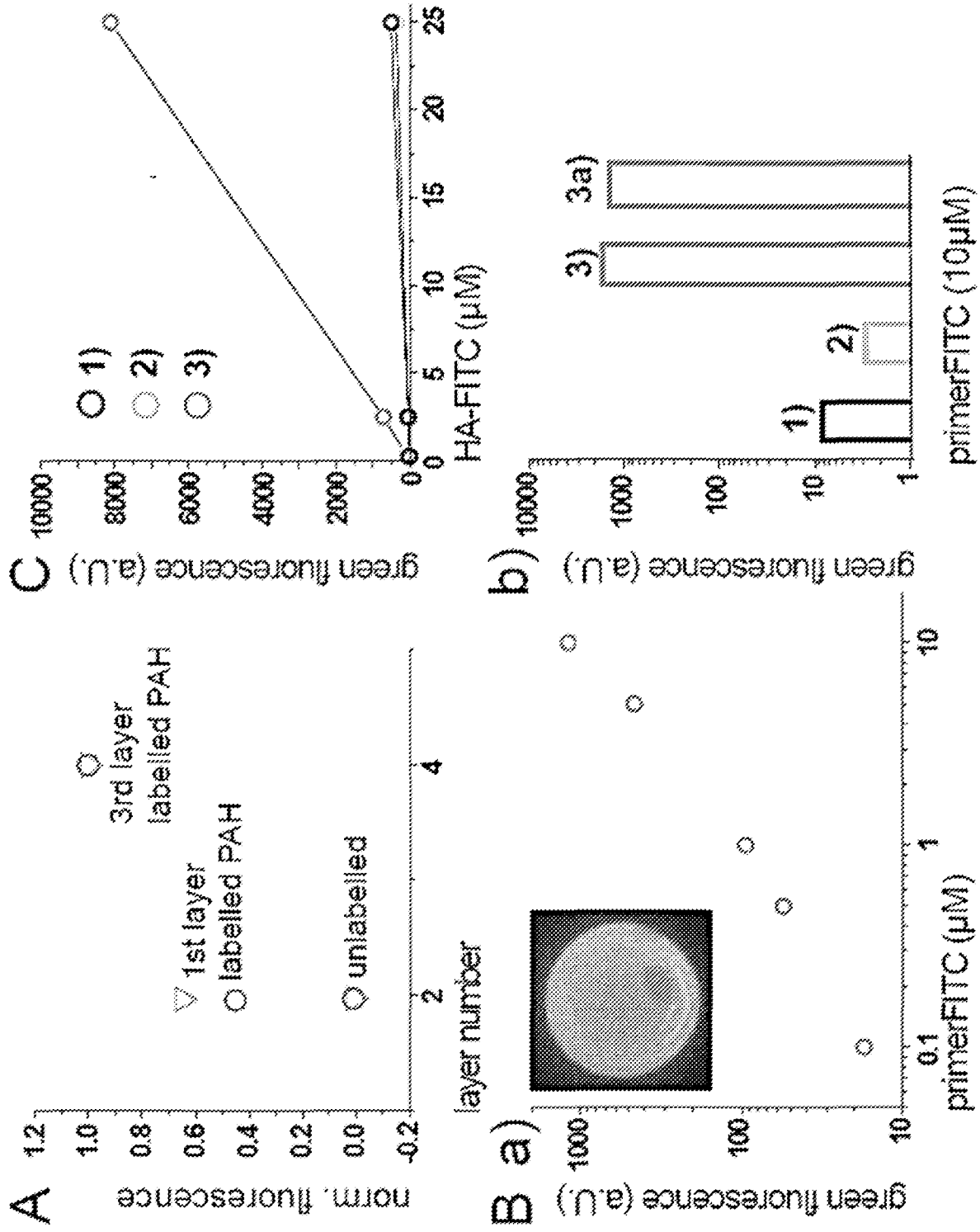

A
norm. fluorescence
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
2
4
layer number
3rd layer labelled PAH
1st layer labelled PAH
unlabelled
B a)
green fluorescence (a.U.)
1000
100
10
0.1
1
10
primerFITC (µM)
b)
green fluorescence (a.U.)
10000
1000
100
10
1
1)
2)
3)
3a)
primerFITC (10µM)
C
green fluorescence (a.U.)
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
HA-FITC (µM)
1)
2)
3)

Figure 17

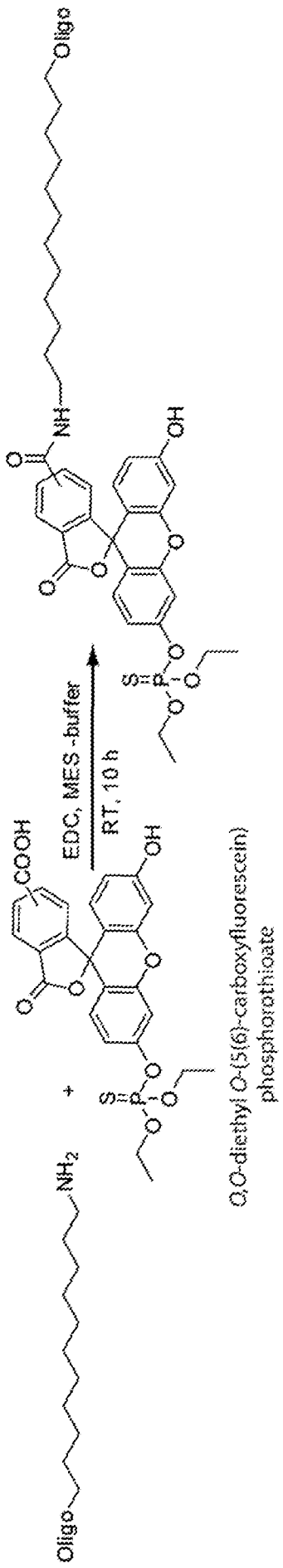

Oligo
NH
O
OH
O
O
O
S=P
O
O
EDC, MES -buffer
RT, 10 h
COOH
OH
O
O
O
S=P
O
O
+
NH2
Oligo
O,O-diethyl O-(5(6)-carboxyfluorescein) phosphorothioate

Figure 18

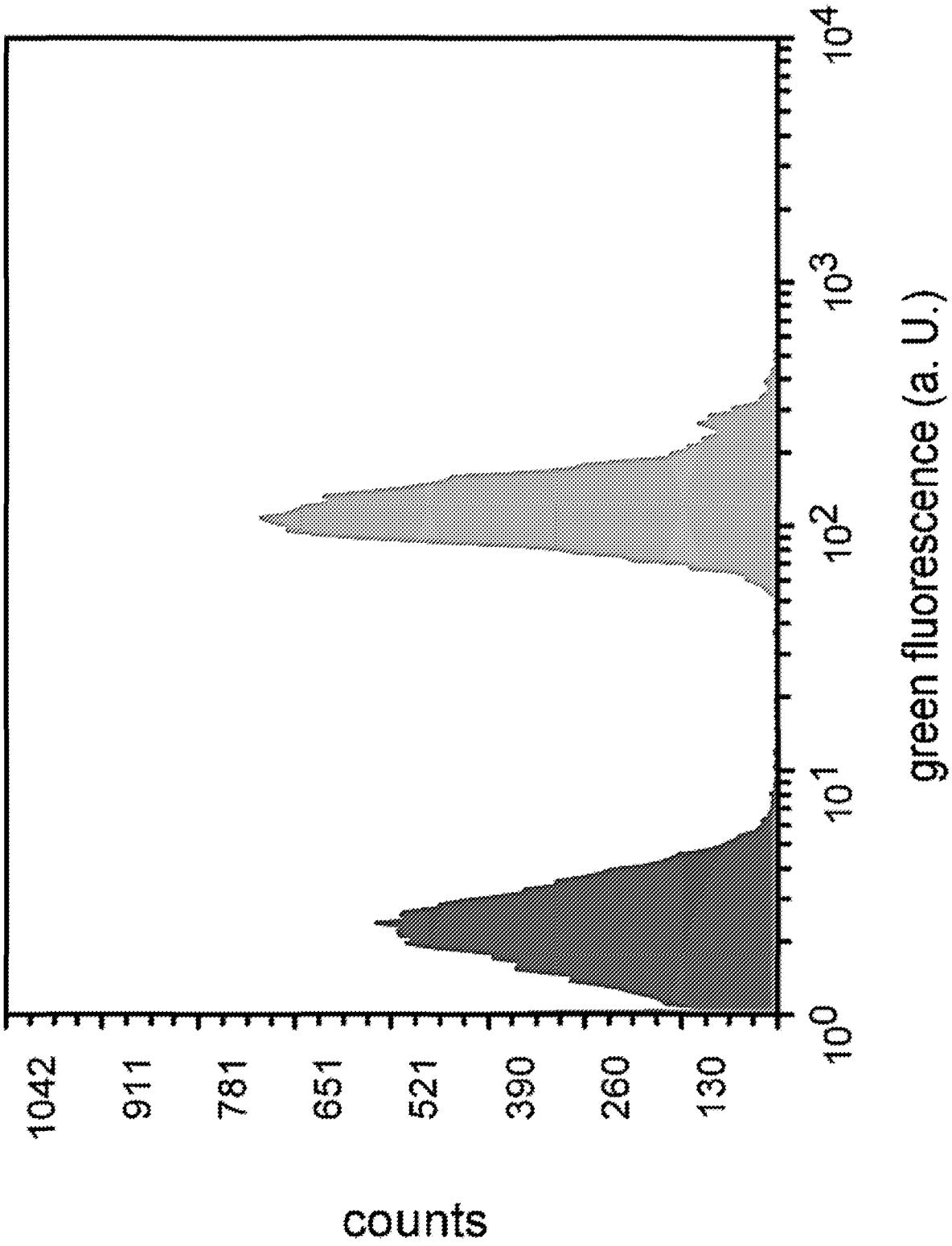

counts
1042
911
781
651
521
390
260
130
10^0
10^1
10^2
10^3
10^4
green fluorescence (a. U.)

Figure 19

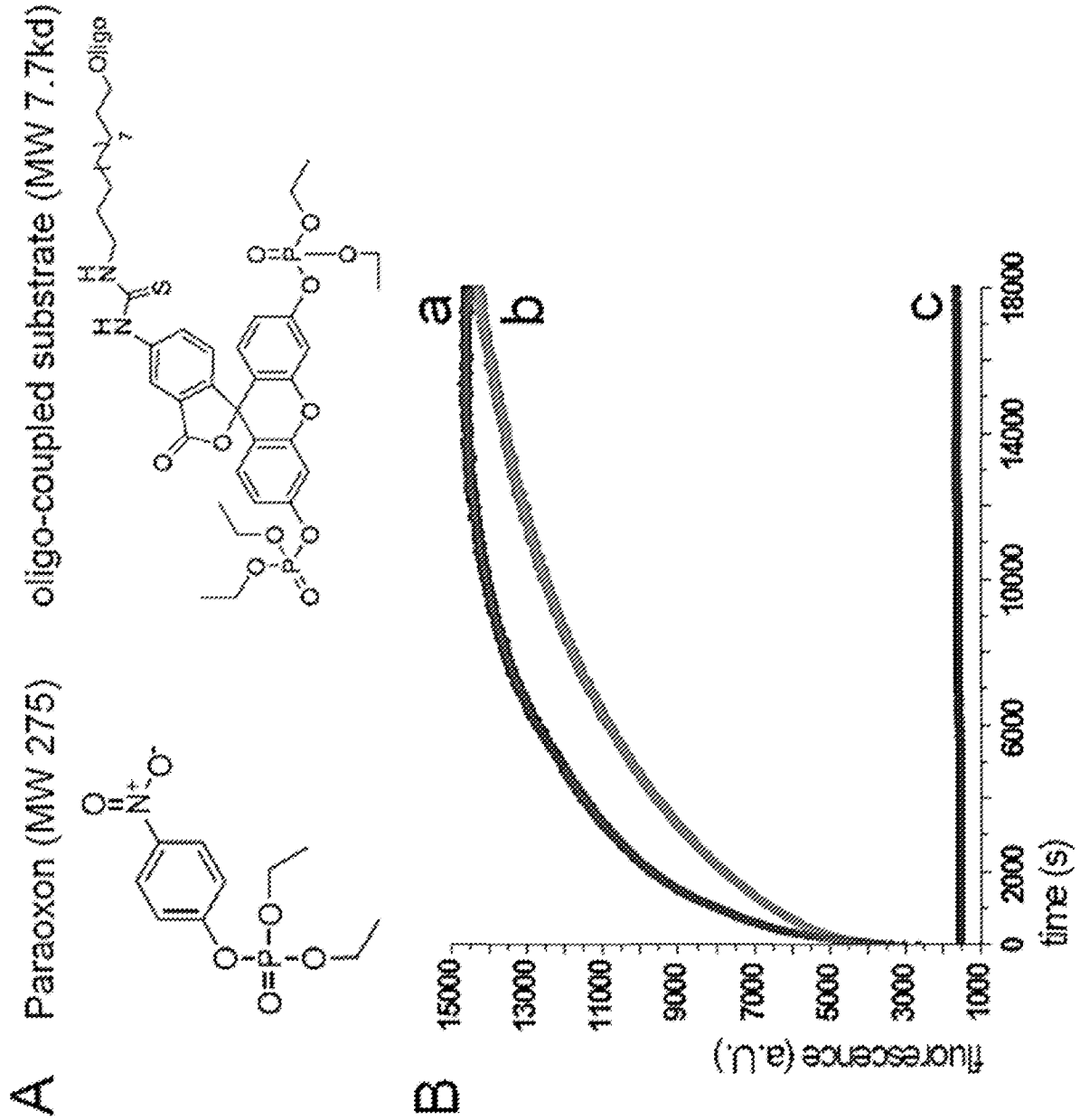

A
Paraoxon (MW 275)
oligo-coupled substrate (MW 7.7kd)
Oligo
B
a
b
c
fluorescence (a.u.)
time (s)
15000
13000
11000
9000
7000
5000
3000
1000
0
2000
6000
10000
14000
18000

Figure 20

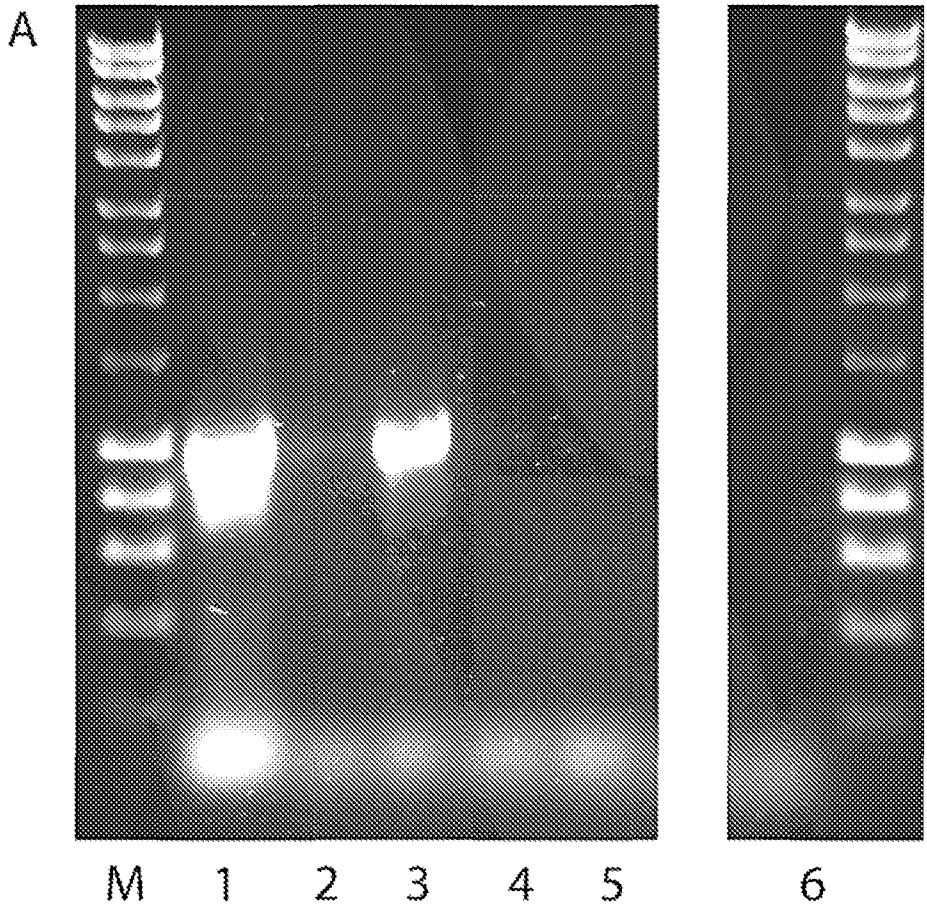


Figure 21

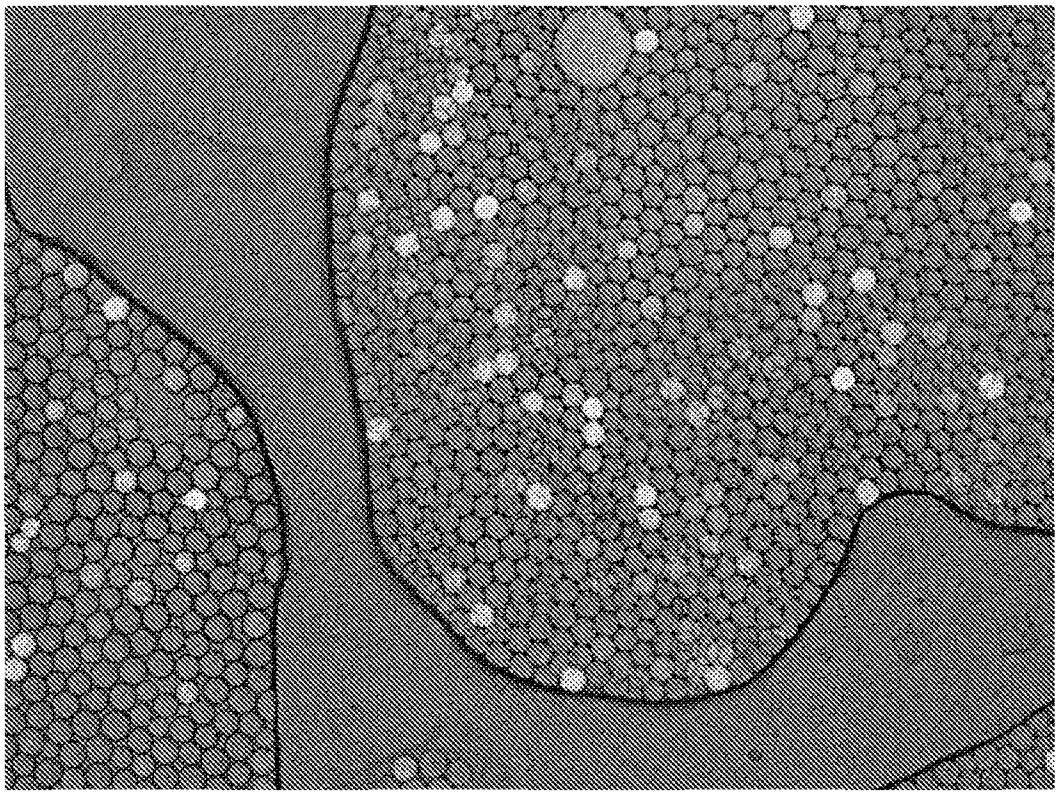
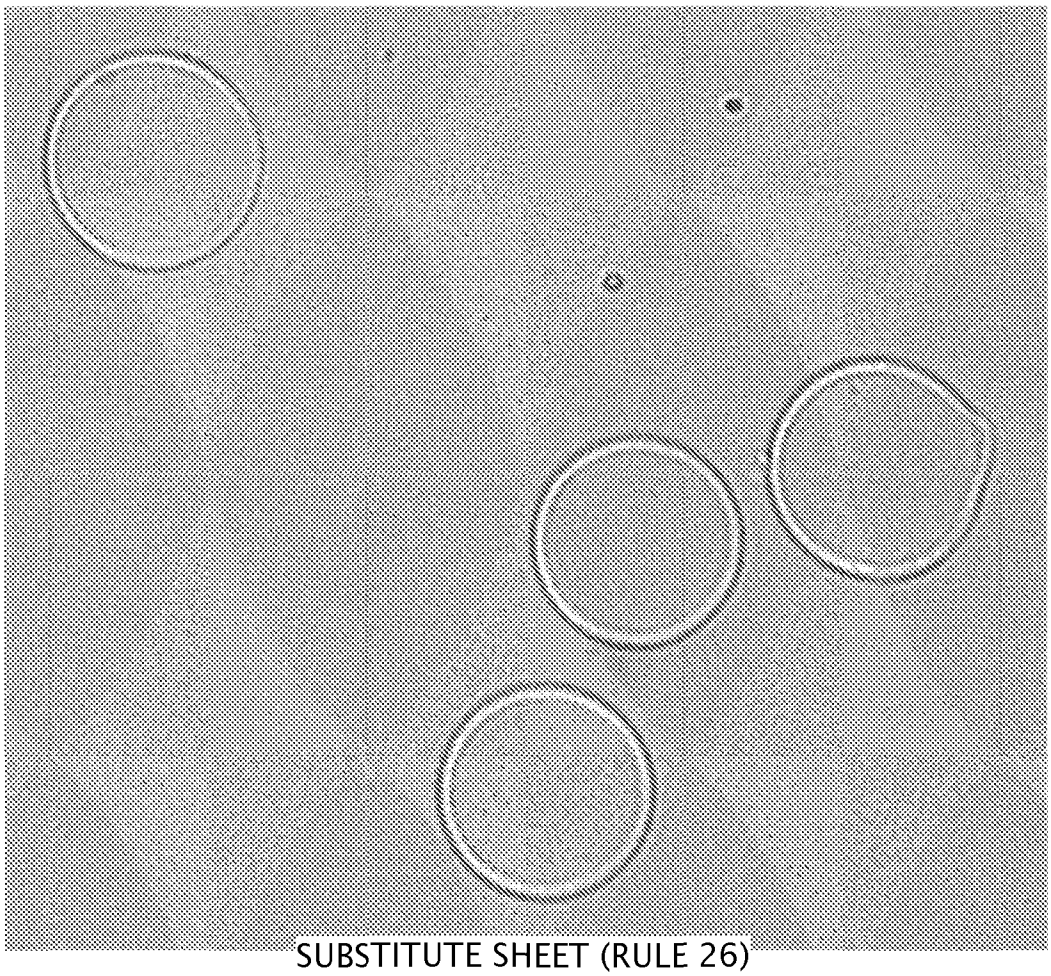


Figure 22



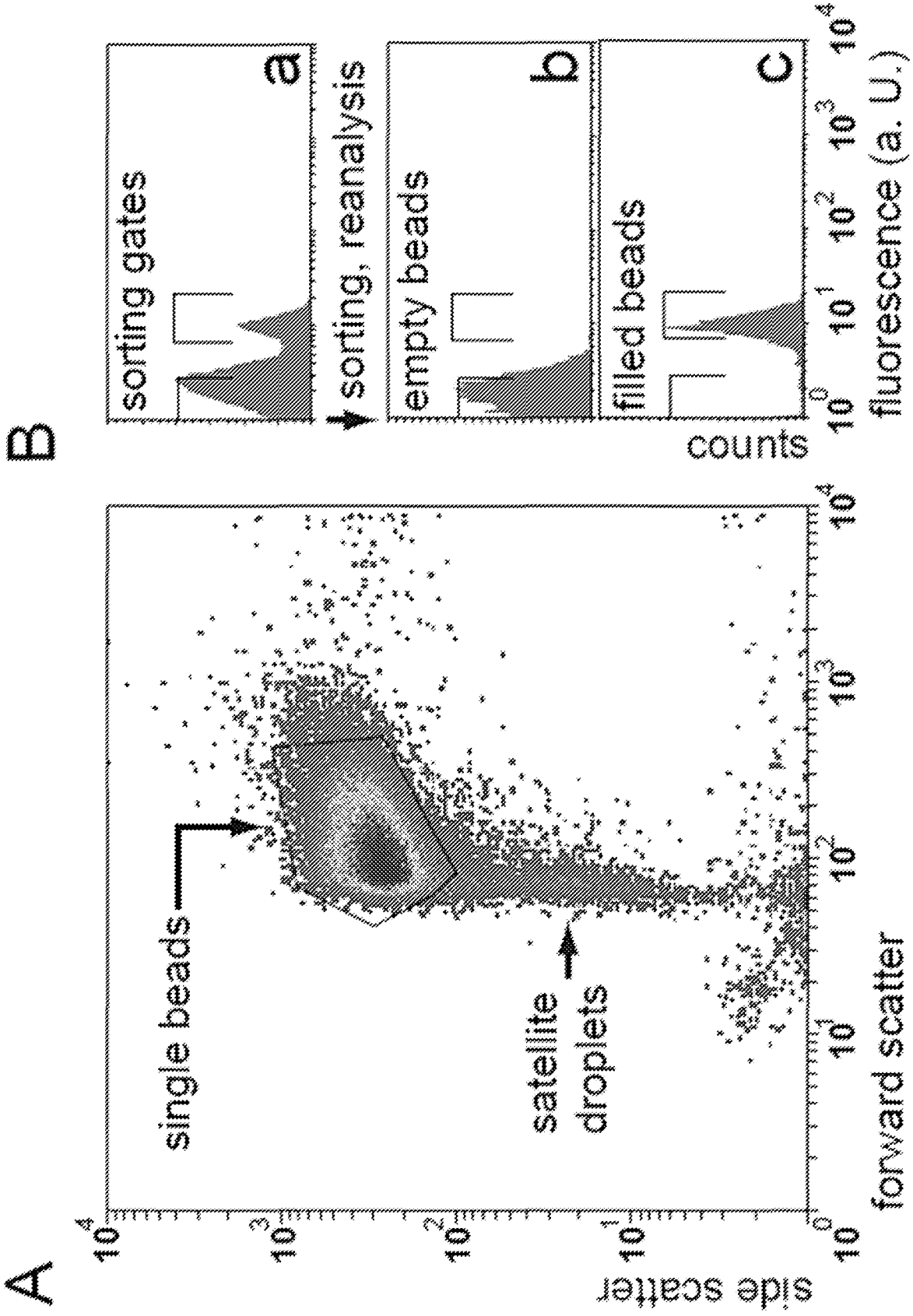
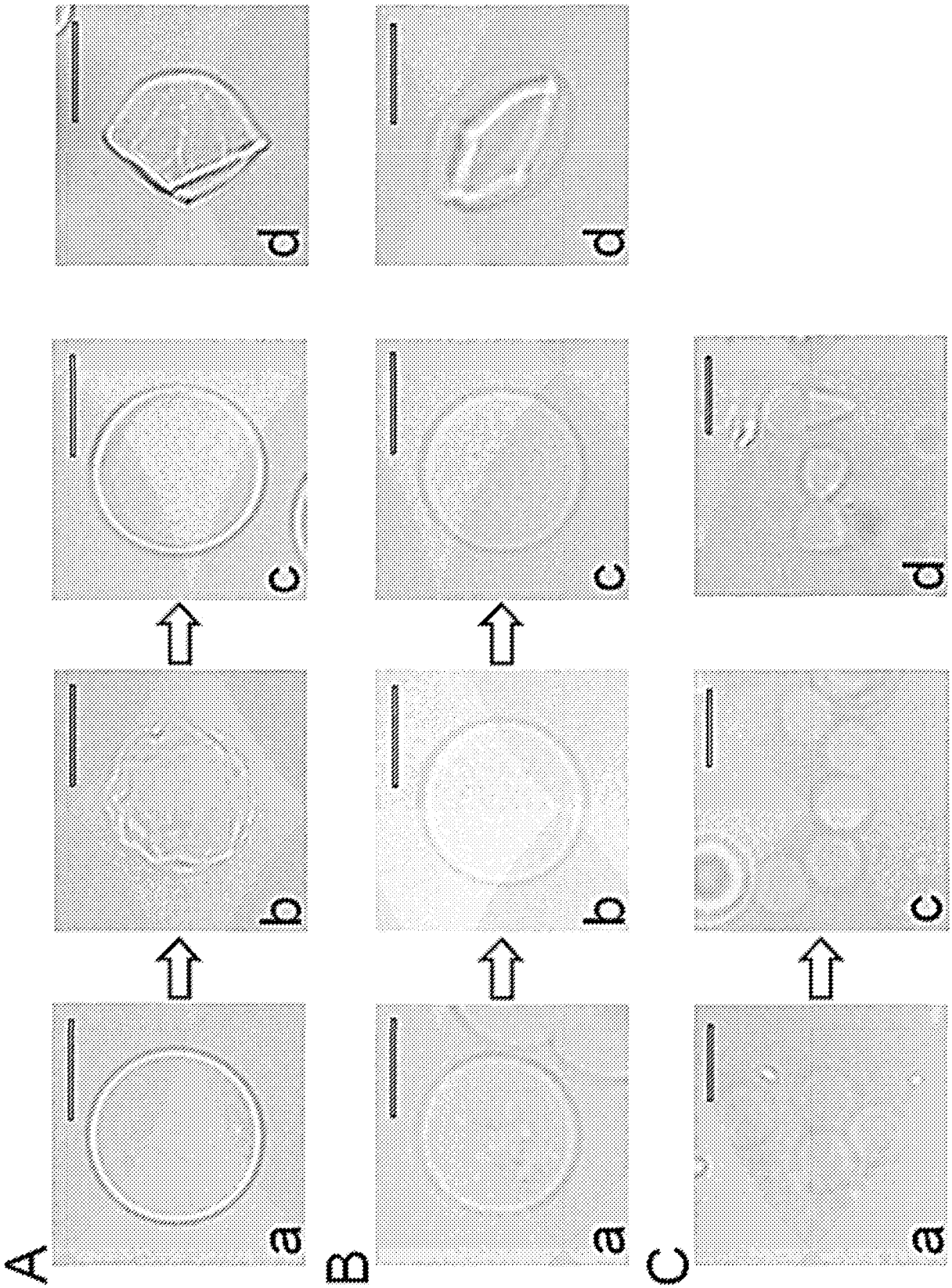

A
side scatter
forward scatter
single beads
satellite
droplets
B
sorting gates
a
sorting, reanalysis
empty beads
b
filled beads
c
counts
fluorescence (a. U.)

Figure 23

Figure 24



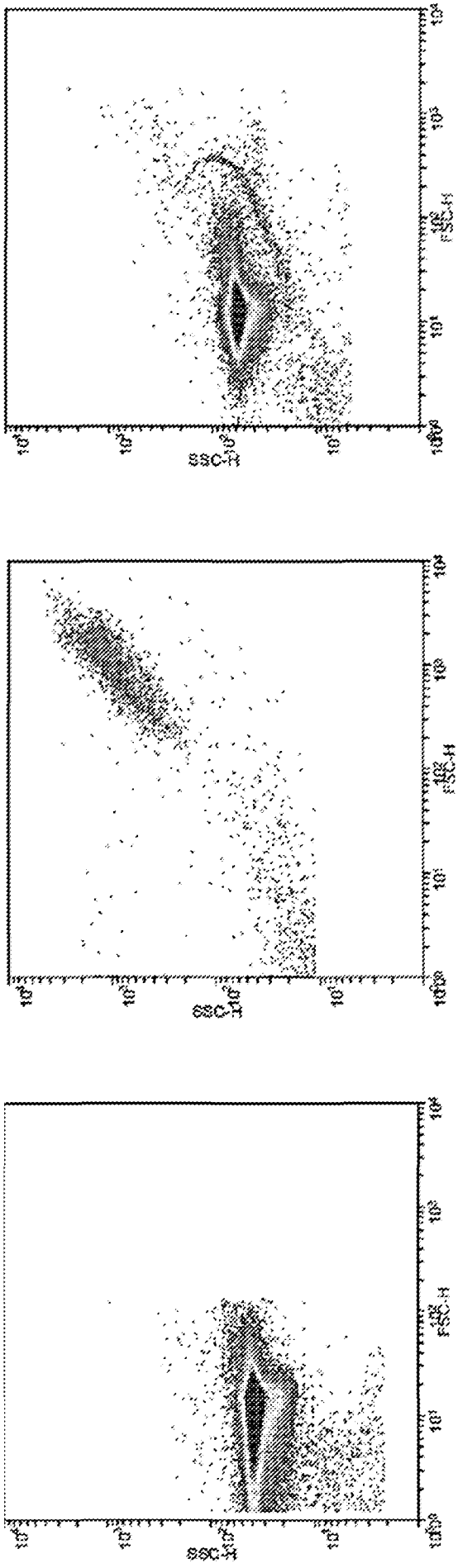


Figure 25

Figure 26

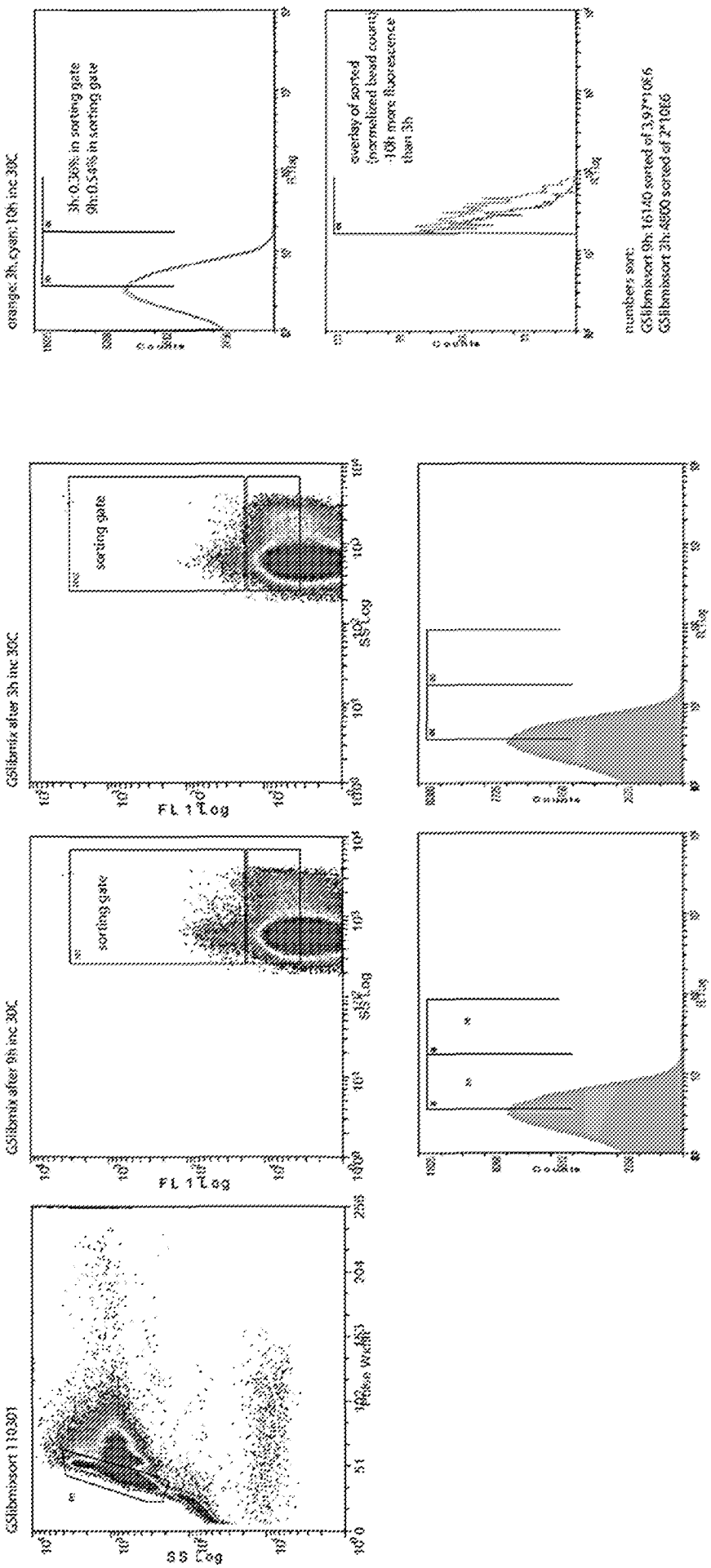
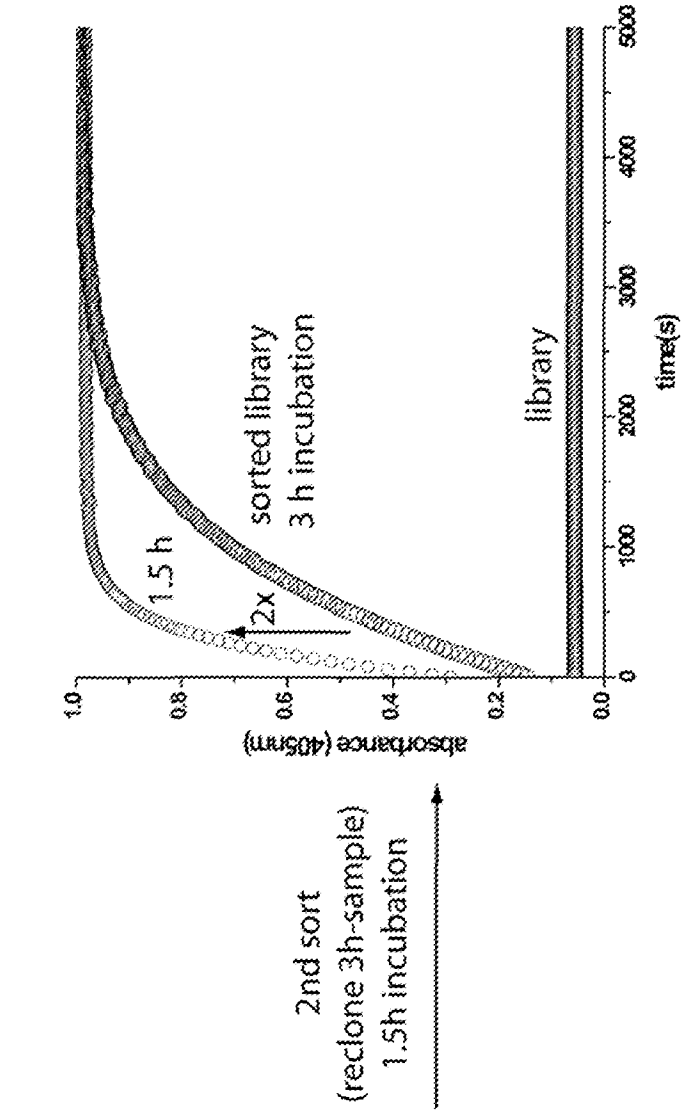

orange: 3h, cyan: 9h inc 30C
3h:0.36% in sorting gate
9h:0.54% in sorting gate
overlay of sorted (normalized head count) -10x more fluorescence than 3h
numbers sort:
GSlibmixsort 9h: 16140 sorted of 3.9*10^6
GSlibmixsort 3h: 4800 sorted of 2*10^6
GSlibmix after 3h inc 30C
sorting gate
FL1 Log
SS Log
GSlibmix after 9h inc 30C
sorting gate
FL1 Log
SS Log
GSlibmixsort 110301
SS Log

Figure 27


absorbance (405nm)
time(s)
library
sorted library
3 h incubation
1.5 h
2x
2nd sort
(reclone 3h-sample)
1.5h incubation
phosphotriesterase activity (in plate reader)
absorbance (405 nm)
time (s)
sorted library
3 h incubation
sorted library
9 h incubation
library
100x
50x

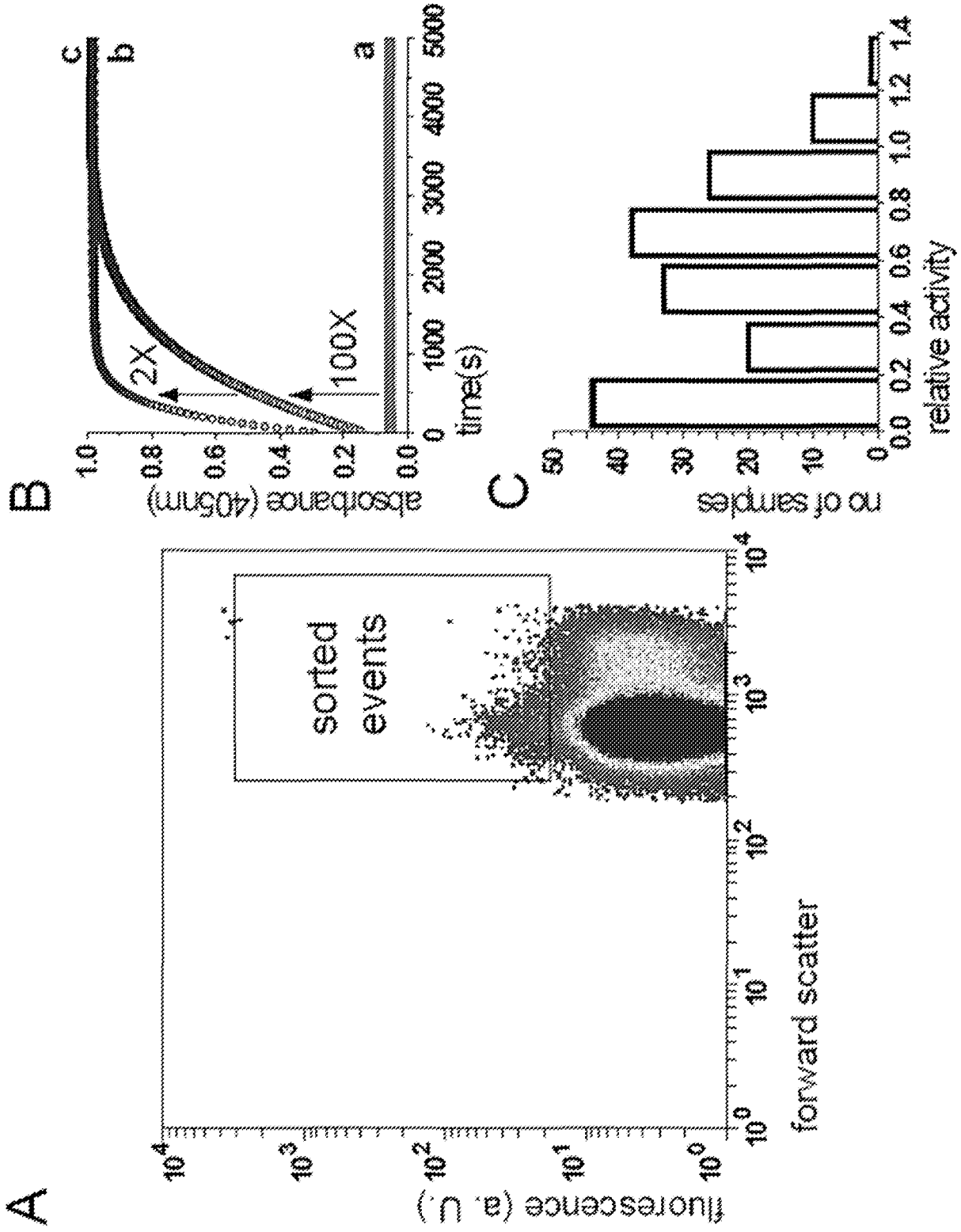
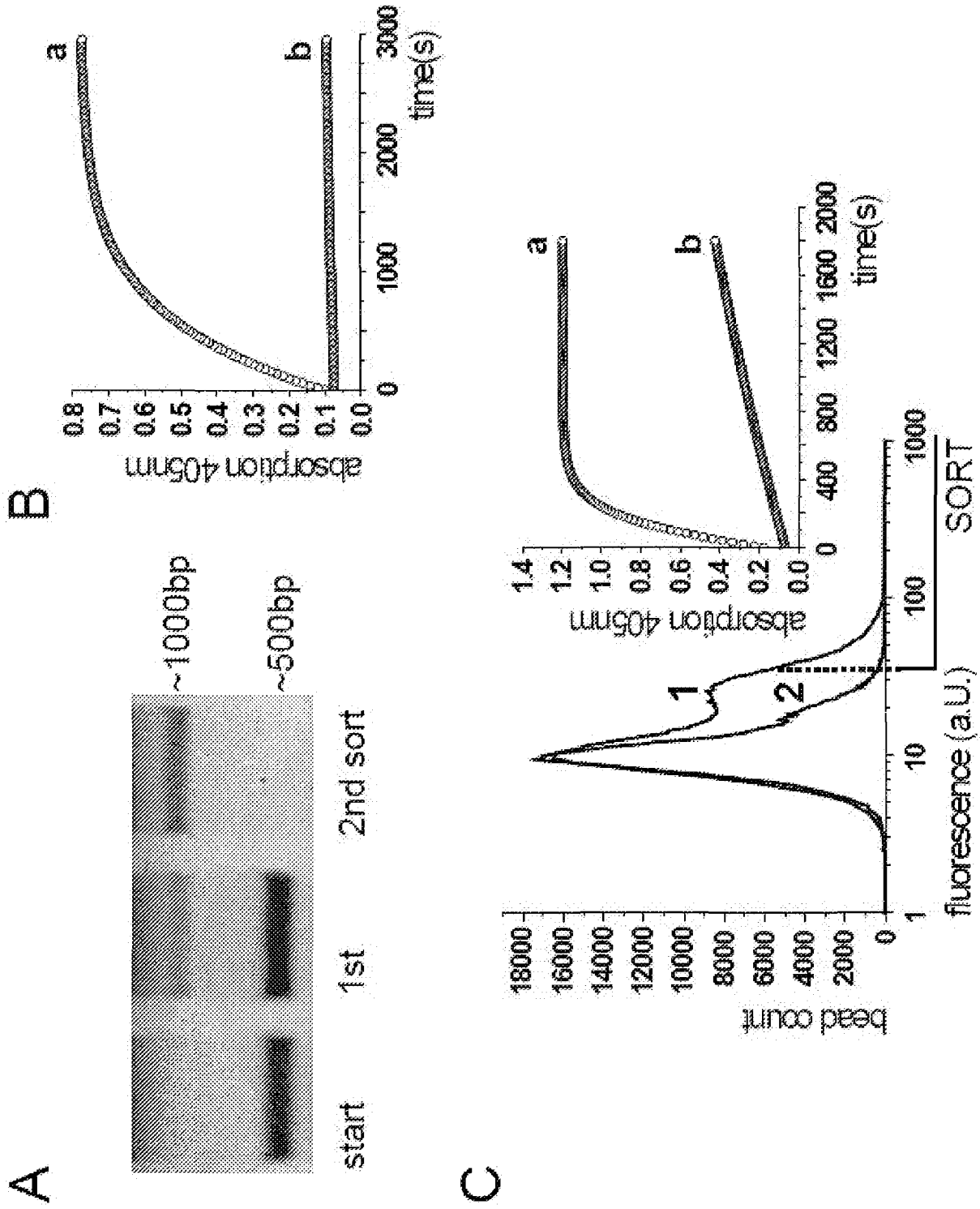

A
fluorescence (a. U.)
forward scatter
sorted events
B
absorbance (405nm)
time(s)
a
b
c
2X
100X
C
no of samples
relative activity

Figure 28

Figure 29


A
~1000bp
~500bp
start
1st
2nd sort
B
a
b
absorption 405nm
time(s)
C
a
b
absorption 405nm
time(s)
bead count
fluorescence (a.U.)
1
2
SORT