

## In vitro compartmentalization by double emulsions: sorting and gene enrichment by fluorescence activated cell sorting

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### Abstract

Water-in-oil (w/o) emulsions can be used to compartmentalize and select large gene libraries for a predetermined function. The aqueous droplets of the w/o emulsion function as cell-like compartments in each of which a single gene is transcribed and translated to give multiple copies of the protein (e.g., an enzyme) it encodes. While compartmentalization ensures that the gene, the protein it encodes, and the products of the activity of this protein remain linked, it does not directly afford a way of selecting for the desired activity. Here we show that re-emulsification of w/o emulsions gives water-in-oil-in-water (w/o/w) emulsions with an external (continuous) water phase through which droplets containing fluorescent markers can be isolated by fluorescence-activated cell sorting (FACS). These w/o/w emulsions can be sorted by FACS, while the content of the aqueous droplets of the primary w/o emulsion remains intact. Consequently, genes embedded in these water droplets together with a fluorescent marker can be isolated and enriched from an excess of genes embedded in water droplets without a fluorescent marker. The ability of FACS instruments to sort up to 40,000 events per second may endow this technology a wide potential in the area of high-throughput screening and the directed evolution of enzymes.

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All high-throughput screening methodologies rely on means of compartmentalizing assay reactions in the smallest possible volume and means of rapidly screening these compartments by virtue of an easily detected signal. The ability of modern fluorescence-activated cell sorting (FACS)<sup>1</sup> instruments to analyze and sort up to 40,000 events per second has given this technology a wide potential in the area of high-throughput screening and directed evolution of enzymes [1]. FACS technology has been used to screen libraries of proteins displayed on bacterial, yeast, and mammalian cells [2–4]. While these

screening systems have yielded highly useful tailor-made proteins [5], they have certain limitations. First, they rely on living cells to compartmentalize the gene library and display the selected proteins. Second, selection is primarily through binding interactions [2], although enzymatic activity has been selected for in a particular case where the fluorescent product could associate with the cell surface [6]. FACS has also been applied in conjunction with completely in vitro systems such as in vitro compartmentalization (IVC) by sorting microbeads. However, these completely in vitro selections rely on the attachment of the enzymatic product to the gene via a microbead, and require the use of substrates modified, for example, with a linker and caged biotin [7].

Despite the above limitations, fluorescence is one of the most sensitive and versatile ways of detecting biological activities and is extremely useful in the context of high-throughput screening (HTS). Both binding interactions (of small ligands and proteins labeled with a

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<sup>1</sup> Abbreviations used: BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HTS, high-throughput screening; IVC, in vitro compartmentalization; PBS, phosphate-buffered saline; w/o, water-in-oil; w/o/w, water-in-oil-in-water.

fluorescent tag) and enzymatic activities (using fluorogenic substrates, namely, substrates that release fluorescent products) can be followed. Fluorescence energy transfer (FRET) has further widened the scope of fluorescence in HTS by enabling the detection of binding interactions (also by using fluorescent proteins, e.g., Green Fluorescent Protein) that are expressed in line with the binding pair [8,9] as well as enzymatic activities [6,10].

IVC is a newly developed technology that uses the aqueous droplets of w/o emulsions as cell-like compartments. In each of these aqueous droplets (of  $\sim 2\text{-}\mu\text{m}$  diameter), a single gene is transcribed and translated to give multiple copies of the protein it encodes. This ensures that the gene, the protein it encodes, and the products of the activity of this protein all remain within the same compartment, thus providing a linkage between the gene and its molecular phenotype (e.g., enzymatic activity). By applying an appropriate selection pressure, genes encoding proteins with the desired activity (binding or enzymatic) can be selected from large pools of genes [11]. Given the capacity of IVC ( $>10^{10}$  discrete compartments are available in 1 mL of emulsion), the direct sorting by FACS of artificial cell-like compartments (in which single genes are transcribed and translated) may provide the basis for versatile and powerful HTS systems. Using fluorogenic substrates, compartments that carry a gene encoding an enzyme with the desired activity would become fluorescent and could then be isolated by FACS. In principle, display libraries could also be compartmentalized in w/o emulsions together with fluorogenic substrates to enable their direct selection for enzymatic activities. However, the w/o emulsions that are currently used for IVC have a continuous oil phase that is not compatible with FACS. We have therefore explored compartmentalization systems based on double (w/o/w) emulsions that afford an external aqueous phase. W/o/w emulsions allow the creation of an external aqueous phase without the alteration of the aqueous droplets embedded in the primary w/o emulsion; they have been applied in numerous cases, including for the compartmentalization of drugs and proteins [12,13]. Thus, genes can be transcribed and translated in the aqueous droplets of a w/o emulsion (Fig. 1) as previously described [7,11,14]. Subsequent conversion of the primary w/o emulsions into a w/o/w (double) emulsion makes the emulsion amenable to sorting by flow cytometry without compromising the integrity of the inner aqueous droplets within the oil phase.

Here we show that w/o emulsions of the type used for IVC can be re-emulsified to yield stable w/o/w emulsions. These w/o/w emulsions can be sorted by FACS while their droplets remain intact. Subsequently, genes embedded in the aqueous droplets of the primary w/o emulsion together with a fluorescent marker can be isolated and enriched from a large excess of genes

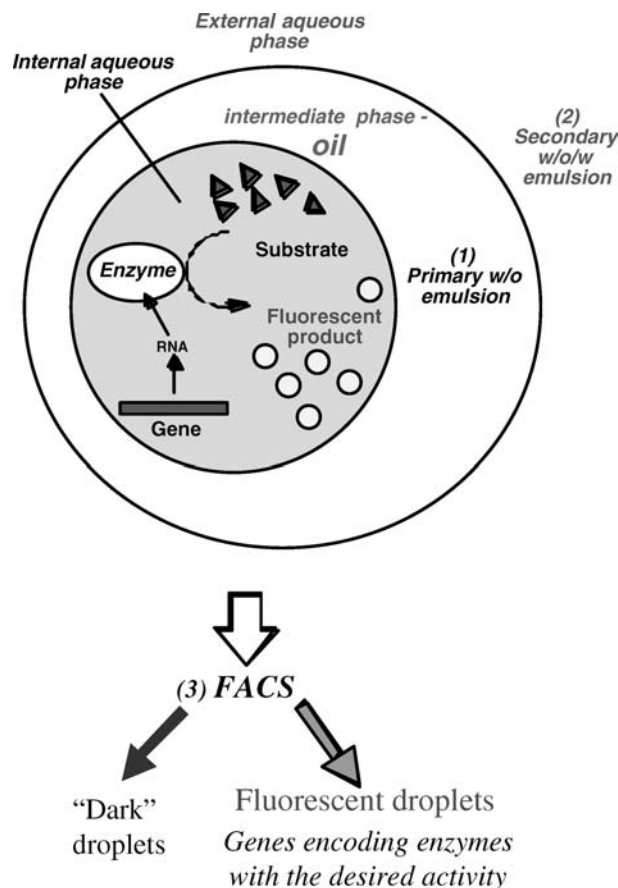


Fig. 1. Proposed scheme of selection by in vitro compartmentalization (IVC) in w/o/w emulsions. (1) Single genes are compartmentalized in a w/o emulsion, and translated in vitro in the presence of a fluorogenic substrate. Compartments in which the gene encodes an active enzyme subsequently contain a fluorescent product. (2) A w/o/w emulsion is formed from the primary w/o emulsion, thus providing an external aqueous phase. (3) Compartments containing the fluorescent product are isolated by FACS, and the genes embedded in them (that encode the enzyme of interest) are isolated and amplified.

embedded in w/o emulsion droplets that do not contain a fluorescent marker.

## Experimental

### Preparation of w/o/w double emulsions

The primary water phase (80  $\mu\text{L}$  of 4.8% Tween-80 in phosphate-buffered saline (PBS: 50 mM sodium phosphate, 100 mM NaCl, pH 7.5)) was added to 0.8 mL of ice-cold oil mix (4.5% Span-80 in light mineral oil). The two phases were homogenized on ice in a 2-mL round-bottom cryotube (Corning) for 5 min at 9500 rpm (using IKA (Germany) T-25 homogenizer) to give the w/o emulsion. To this w/o emulsion, 0.8 mL of the second water phase was added (2% Tween-20 in PBS) and the mixture was homogenized for 2 min at 8000 rpm to give the double w/o/w emulsion.

Table 1  
Sorting of w/o/w emulsion droplets by FACS

Sample	Total events <sup>b</sup>		R1-gated events <sup>b</sup>	
	% Positives	Enrichment <sup>c</sup>	% Positives	Enrichment <sup>c</sup>
“Negative” emulsion <sup>a</sup>	0.06	—	0.06	—
“Positive” emulsion <sup>a</sup>	24.87	—	25.31	—
Presorted 1:5 mix	3.2	—	3.33	—
Sorted once	51.4	16	51.8	15.5
Sorted twice	79.7	24.9	80.0	24

<sup>a</sup> “Positive” w/o/w emulsions originated from w/o emulsions containing a fluorescent marker (FITC-BSA) in the aqueous droplets, and “negative” w/o/w emulsions from a w/o emulsion with no fluorescent marker.

<sup>b</sup> The statistics for total events relate to the overall droplet population with *no* gating by forward- and side-scattering, while the statistics for R1-gated events are restricted to a subpopulation that meets the forward- and side-scattering criteria as defined by the R1 gate (Fig. 2A).

<sup>c</sup> The enrichment (or “fold increase”) is the percentage of “positive” events (events gated through M1, Fig. 2B) after sorting, divided by the percentage “positive” events before sorting.

### Sorting of w/o/w emulsions by FACS

w/o/w emulsions were diluted in excess of PBS and run in a Vantage SE flow cytometer (Becton-Dickinson) using PBS as sheath fluid, at ~8000 events per second, with a 70- $\mu$ m nozzle, exciting with a 488-nm argon ion laser (coherent Innova 70) and measuring emissions passing a 530  $\pm$  20-nm bandpass filter. Single, unaggregated droplets were gated using forward- and side-scatter criteria. For analysis of the sorted droplets, several thousands droplets were analyzed in a FACScan cytometer (Becton-Dickinson) using the Becton-Dickinson Information Systems CellQuest Pro Software.

### Model enrichment of genes in w/o/w emulsions sorted by FACS

Cloning of the *M.HaeIII* and *FolA* genes (encoding, respectively, the DNA methyltransferase *HaeIII*, and *Escherichia coli* dihydrofolate reductase (DHFR)) is described elsewhere [14]. These genes were subcloned

into pIVEX2.2b vector [7]. The *M.HaeIII* and *FolA* genes were amplified from their respective pIVEX2.2b vectors using the forward primer LMB2-1-biotin labeled with biotin at its 5' end and the back primer pIVB-1 as described [7]. The “positive” w/o emulsion was prepared with a water phase composed of 0.3 nM *FolA* genes in PBS plus FITC-BSA (0.44 FITC/BSA mol/mol; at 2 mg/ml concentration). The water phase of the “negative” w/o emulsion contained 0.3 nM *M.HaeIII* gene diluted in 2 mg/mL BSA in PBS. The positive and negative w/o emulsions were then mixed 1:100 and this mix was converted into a w/o/w emulsion as described above. The w/o/w emulsions were sorted in the Vantage SE and 40,000–80,000 “positive” droplets (using the R1 + M1 gate; see Figs. 2 and 3 for examples) were collected.

### PCR amplifications

The sorted w/o/w emulsion droplets were broken by adding an equal volume (~30  $\mu$ L) of B&Wx2 buffer (2 M

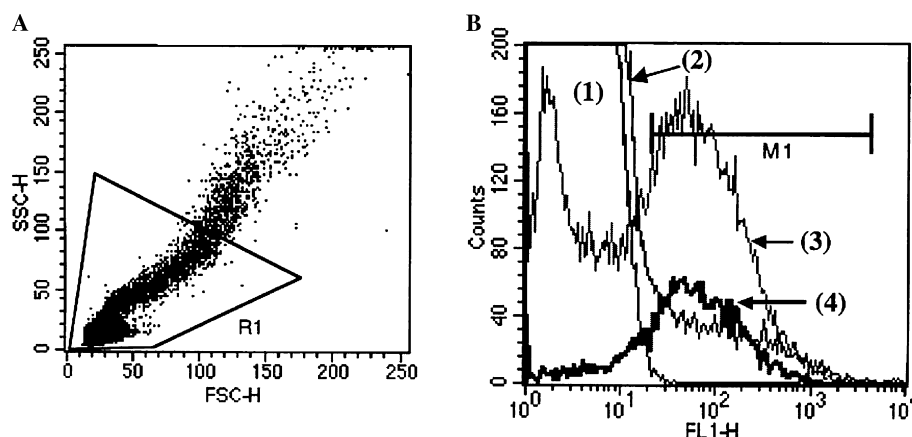


Fig. 2. Stability and enrichment of a double (w/o/w) emulsion sorted twice by FACS. A “positive” double emulsion containing FITC-BSA in its aqueous droplets was mixed 1:5 with a “blank” double emulsion containing buffer only. (A) Dot-plot FSC-H (forward-scatter) and SSC-H (side-scatter) analysis of the double emulsion of the first sort (for clarity, shown are 20% of events). Events gated in R1 (~90% of total events) were subjected to sorting and analysis. (B) Histogram analysis of different populations of the emulsion droplet fluorescence (for R1-gated events). Shown are population analyses. *Before* sorting: the “blank” w/o/w emulsion (1) and a 1:5 mix of “positive” and “blank” w/o/w emulsions (2); *after* sorting: the first (3) and second (4) sort. “Positive” events were gated and sorted through M1, and the statistics are given in Table 1.

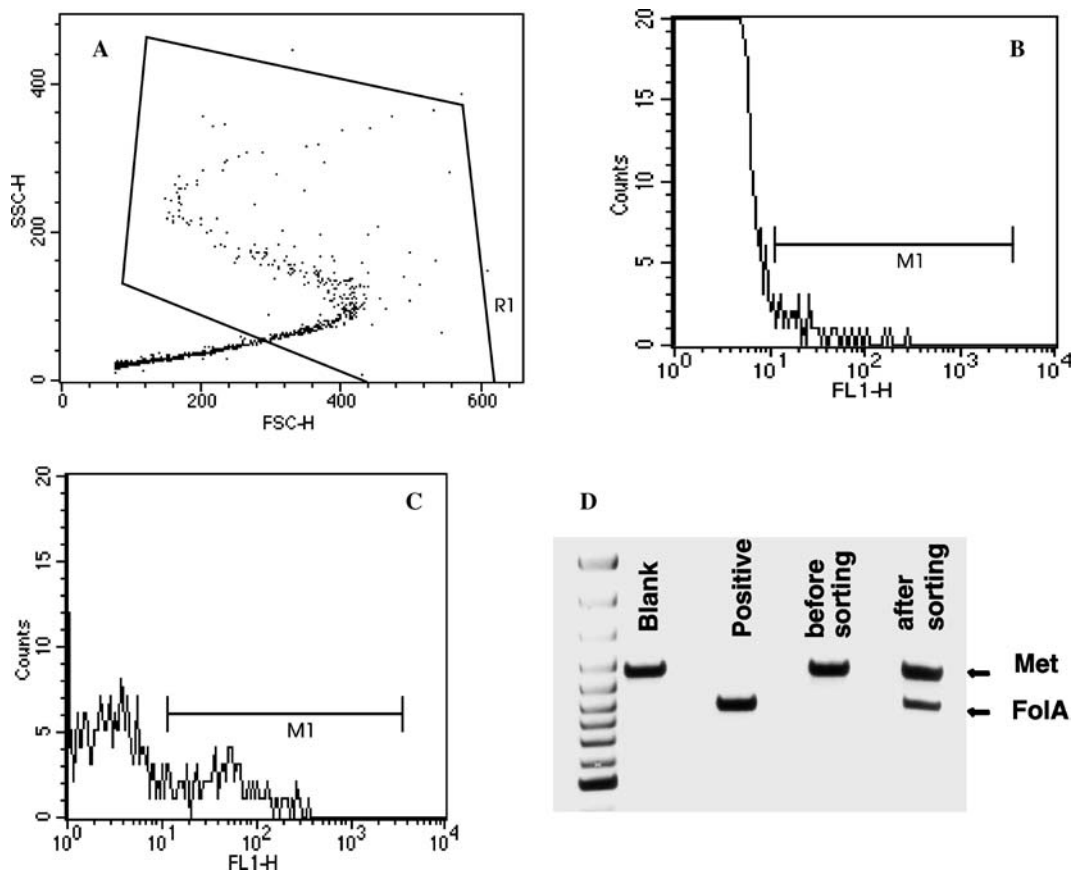


Fig. 3. Model selection of genes in a double w/o/w emulsion system. A “positive” w/o emulsion containing *FoIA* genes and a fluorescent marker was mixed at a 1:100 ratio with a “negative” w/o emulsion containing buffer and *M.HaeIII* genes. The mixed w/o emulsion was converted into a w/o/w emulsion that was then sorted by FACS. (A) FSC-H (forward-scatter) and SSC-H (side-scatter) of the mixed w/o/w emulsion (for clarity, only 5% of all events are shown). The subpopulation gated through R1 was subjected to sorting and analysis. (B) Histogram analysis of the presorted w/o/w emulsion. The M1 marker indicates the range of high fluorescence chosen for sorting of “positive” droplets. (C) Histogram analysis of the sorted w/o/w emulsion. The statistical analysis of the presorted and sorted population is provided in Table 2. (D) Analysis by gel electrophoresis of the PCR amplification of genes isolated from the different w/o/w emulsions: a separate “negative” emulsion containing the *M.HaeIII* genes only (yielding an amplification product of 1477 bp annotated as *Met*); a separate “positive” emulsion containing the *FoIA* genes only (yielding an amplification product of 1214 bp); the 1:100 mixture of “positive” and “negative” emulsions before sorting and after sorting. The ratio between the *FoIA* and *Met* genes in the mixed emulsion is 1:100 before sorting (at this ratio, the amplification product of the *FoIA* gene is not visible) and  $\sim$ 1:3 after sorting (estimated by eye in comparison to DNA mixtures of known ratios) indicating an enrichment of  $\sim$ 30-fold. M, marker DNA (100-bp DNA ladder; Fermentas).

Table 2  
Model selections of w/o/w emulsions by FACS

Sample	Total events <sup>a</sup>		R1-gated events <sup>a</sup>	
	% Positives	Enrichment <sup>b</sup>	% Positives	Enrichment <sup>b</sup>
“Negative”	0.01		0.01	
“Positive”	6.97		15.8	
1:100 mix <i>before</i> sorting	0.21		0.59	
1:100 mix <i>after</i> sorting	7.05	33.6	22.7	38.5

<sup>a</sup> The statistics for total events relate to the overall droplet population with *no* gating by forward- and side-scattering, while the statistics for R1-gated events are restricted to a sub population that meets the forward- and side-scattering criteria as defined by the R1 gate (Fig. 3A).

<sup>b</sup> The enrichment (or “fold increase”) is the percentage of “positive” events after sorting (Fig. 3C, events gated through M1) divided by the percentage “positive” events before sorting (Fig. 3B, events gated through M1).

NaCl, 10 mM Tris, pH 7.5, 10 mM EDTA) followed by 100  $\mu$ l of B&W buffer (1 M NaCl, 5 mM Tris, pH 7.5, 5 mM EDTA). Streptavidin-coated magnetic beads

(Dynal M280, 5  $\mu$ L) were added and incubated for 3 h at room temperature while sonicating in a bath sonicator (every 30 min for 20 s each time). The beads were then

rinsed three times with 200  $\mu$ L of B&Wx2 and twice with 200  $\mu$ L of PCR buffer (16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 67 mM Tris–HCl, pH 8.8, 0.1% Tween-20). The rinsed beads were resuspended in 10  $\mu$ L PCR buffer. For the controls, pure (unmixed) “positive” and “negative” w/o/w emulsions and the w/o/w emulsions prepared from the 1:100 mix (before sorting) were all diluted 1000-fold to give approximately the same number of droplets as isolated by the sorter. The diluted w/o/w emulsions were broken and the genes captured as described above.

PCRs were set up at 50  $\mu$ L total volume, with PCR buffer supplemented with template DNA,  $\text{MgCl}_2$  (1.5 mM), primers (500  $\mu$ M), dNTPs (200  $\mu$ M), and polymerase (2 U, BioTaq (BioLine)). Bead suspensions (5  $\mu$ L from each sample) were used as templates for PCR amplifications with primers LMB2-9 (GTAAAACGACGGCCAGT) and pIVB10 (TTTTTTGCTGAAAGGAG) [8]. Reactions were cycled 20 times (95  $^\circ\text{C}$ , 0.5 min; 60  $^\circ\text{C}$ , 0.5 min; 72  $^\circ\text{C}$ , 2 min) with a final step at 68  $^\circ\text{C}$  for 7 min. This PCR was diluted 100 times in water, and 1  $\mu$ L was used for a nested PCR, using primers that anneal to the T7 promoter and the T7 terminator (5'-TAATACGACTCACTATAGG and 5'-CCCGTTTAAAGGCCCCAAGGGG; respectively). The nested reactions were cycled 25 times (95  $^\circ\text{C}$ , 0.5 min; 60  $^\circ\text{C}$ , 0.5 min; 72  $^\circ\text{C}$ , 1.5 min) with a final step at 68  $^\circ\text{C}$  for 7 min. The reactions were loaded on a 1.2% TAE agarose gel using ethidium bromide for DNA visualization.

## Results

### *w/o/w emulsion droplets can be sorted by FACS*

Passage through sorters involves high pressures and shear forces: a sample sorted by FACS is injected into a direct fluid stream (sheath fluid) at high speed and pressure and then passes through a narrow vibrating nozzle to create a stream of separate droplets. After illumination by a laser beam, a fluorescent droplet is electrically charged and deflected by an electric field to be collected [1]. The w/o/w droplets must stay intact during FACS sorting so that their contents (and the enzyme-encoding gene, in particular) remain compartmentalized. We therefore examined the preparation and stability of w/o/w emulsion droplets and their amenability to sorting. A w/o/w emulsion was prepared from a w/o emulsion containing FITC-BSA as a fluorescent marker, and was then mixed (at 1:5 ratio) with a w/o/w emulsion prepared from a w/o emulsion containing no fluorescent marker. Light microscopy indicated an average of  $\sim 5$  w/o droplets per w/o/w droplet (results not shown). The w/o/w emulsions were sorted by FACS by defining a region of 90% of the population by criteria of shape and size as dictated by the forward- and side-scattering parameters (R1 gate, Fig. 2A) and a marker

for the “positive” peak of fluorescence (M1 gate, Fig. 2B). The sorter was allowed to collect about 100,000 droplets that met the criteria defined by *both* the R1 and M1 gates. The droplets isolated by the first sort were analyzed, resorted, and analyzed again. The results of this experiment are summarized in Fig. 2 and Table 1.

Prior to sorting, the percentage of positive events in the 1:5 mix was 3.33 (out of the R1-gated events). The first sort resulted in 51.8% of the droplets appearing at the high-fluorescence (“positive”) gate M1 (a 15.5-fold enrichment). The second round of sorting gave an additional 50% enrichment to a total of 80% positives. These results show that the FACS sorts the correct droplets and can reach a high level of enrichment of w/o/w emulsion droplets containing a fluorescent marker. The droplets remained intact after sorting given that there was no change in forward- and side-scatter (the same R1 gate was applied in all sorts and analyses) nor in the fluorescence intensity (FL1-H parameter; Fig. 2B). The droplets appear to be stable during sorting and while stored in the sheath fluid, and could be taken through another round of sorting.

### *Model enrichment of genes in w/o/w emulsions sorted by FACS*

w/o/w emulsions have the potential to be applied for the selection or screening of a particular molecular phenotype as suggested above (Fig. 1). To do so, the content of the droplets containing the “positive” genes that encode active enzyme molecules (and thereby contain the fluorescent product) must not mix with droplets carrying “negative” genes that encode inactive proteins and contain no fluorescent product. Otherwise, the genotype–phenotype linkage that is vital for all evolutionary processes (and for HTS processes related to functional genomics, for example) would be lost. To demonstrate the capability of this new IVC system to maintain this linkage, we performed a model selection that aims at enriching genes embedded in aqueous droplets together with a fluorescently labeled protein (FITC-BSA) from a large excess of other genes embedded in aqueous droplets with no marker. Enrichment was tested through mixing of two w/o emulsions (each containing a different gene) and re-emulsification to give a w/o/w emulsion that is amenable to FACS.

We first prepared two separate w/o emulsions: the “positive” emulsion contained *FoIA* genes and FITC-BSA; the “negative” w/o emulsion contained genes of a different length (*M.HaeIII* genes) and no fluorescent marker. Both genes were amplified from the same cloning vector and were tagged with biotin at their 5' end. Next, the two w/o emulsions were mixed at a ratio of 1:100 (“positives” to “negatives,” respectively) and re-emulsified to give a w/o/w emulsion. The w/o/w emulsion was sorted by FACS under forward- and

side-scattering parameters that defined a subpopulation of 42% of the total events (Fig. 3A, R1 gate). Sorting the subpopulation of medium-size droplets (40–50% of the total population) while avoiding the very large and small droplets yielded the highest enrichment. The very large oil droplets contain a large number of water droplets and therefore compromise the enrichment. The small oil droplets appear to contain no water droplets within them and their sorting seems pointless (see below). Droplets sorted through the M1 high-fluorescence gate (Fig. 3B) were collected. These emulsion droplets were then broken, and the genes contained within them captured onto streptavidin-coated magnetic beads. The beads were rinsed and the captured genes were amplified by PCR using primers that anneal to the identical sequence regions flanking both the *FolA* and *M.HaeIII* genes. The genes isolated from the sorted droplets and amplified by PCR appear at  $\sim 1:3$  *FolA*:*M.HaeIII* ratio, indicating an enrichment of  $\sim 30$  fold from a starting ratio of 1:100 (Fig. 3D). Analysis of the sorted droplets (prior to breaking) by FACS indicated that the percentage of positives increased by 38.5-fold relative to the presorted w/o/w emulsion (R1-gated events, Table 2). These results indicate that little or no mixing occurs, of either DNA or FITC-BSA, between w/o droplets on formation and processing of the w/o/w emulsion, and that the genotype–phenotype linkage could be kept in this system.

The observed level of enrichment is consistent with no exchange of genes or fluorescent markers between droplets, as well as with the droplet-size distribution (5 w/o droplets per w/o/w droplet, on average). Thus, if the primary w/o droplets are evenly distributed in the secondary w/o/w emulsion, one should expect that mixing a “positive” and a “blank” w/o emulsion at 1:100 ratio would yield w/o/w droplets containing, on average, one positive aqueous droplet together with four blank droplets. Assuming that the “positive” droplets have been enriched by sorting to 100%, we should therefore expect a maximal ratio of 1:4 *FolA* to *M.HaeIII* genes, namely, a 25-fold enrichment relative to the 1:100 presorted mix. The observed gene enrichment is indeed in the anticipated range ( $\sim 30$ -fold, Fig. 3D) and this enrichment of genes is mirrored in the enrichment for highly fluorescent droplets (M1-gated w/o/w droplets) observed after sorting by FACS (38.5-fold, Table 2). However, the percentage of highly fluorescent droplets after sorting is only 22.7% (Table 2). And indeed, the histogram shows a clear peak of a nonfluorescent population (Fig. 3C). We assume that this nonfluorescent population consists mostly of oil droplets with no aqueous droplets within them. This is supported by the fact that the percentage of positives in a w/o/w emulsion prepared from a w/o emulsion containing a fluorescent marker only (Fig. 3C, “positive” emulsion) is not 100% but 15.8%. Thus, a large fraction of the w/o/w droplets

comprises oil only and exhibits no fluorescence. As these droplets carry no genes, their presence reduces the FACS enrichment, but does not compromise the gene enrichment.

## Discussion

We have previously shown that large gene libraries can be compartmentalized in w/o emulsions to give, on average one gene per aqueous droplet. Single, compartmentalized genes can then be transcribed and translated to yield many copies (10–100) of the encoded protein. Here we show that such w/o droplets could be emulsified again to yield a w/o/w double emulsion with a continuous aqueous phase. The droplets of these w/o/w emulsions can be sorted by FACS while the content of the original w/o aqueous droplets seems to remain undisturbed. For this to happen, the following two requirements must be fulfilled. First, the w/o/w emulsion droplets must be stable enough to withstand the pressure and shear force of FACS. Second, there should be no mixing of contents between droplets of the primary w/o emulsion. Our results indicate that both these requirements are met. Sorting of w/o/w emulsions allows highly fluorescent droplets to be isolated and enriched by many fold, while the droplet size and shape distribution remain intact. Further, w/o/w droplets isolated by FACS can be resorted to show yet additional enrichment while the physical characteristics of the droplets remain unchanged. The histograms (e.g., Fig. 2B) also show that the low-fluorescence population is much decreased in the first sort and becomes negligible after the second sort, whereas the mean fluorescence of the “positive” population remains unchanged. This suggests that there is no significant “leakage” of fluorescent marker during and inbetween the sorts. It is also apparent from the model selection presented in Fig. 3 that under the experimental conditions described here, shuttling, diffusion, or mixing of contents between droplets of this system is slow enough to enable selection. It has to be noted, though, that only high-molecular-weight components were used here (both DNA and the fluorescent marker). Low-molecular-weight molecules (e.g., fluorescent dyes that are not linked to a carrier protein as with FITC-BSA) are more likely to diffuse between droplets and we are currently investigating the use of such markers.

To conclude, our results constitute the first described usage of compartmentalization in double emulsions for the direct sorting by fluorescent signals. This technology is unique in allowing enzymatic activities to be detected and selected with a wide range of available soluble fluorogenic substrates that require no immobilization or attachment. Selection in this system may be completely *in vitro*—namely, the enzyme molecules can be expressed

from gene libraries generated by PCR, using a cell-free extract embedded in the aqueous droplets of the primary w/o emulsion; such processes involve no cloning or transformation. It may also be possible to compartmentalize in w/o emulsions various display libraries (libraries of proteins that are physically linked to their coding gene, e.g., cell, bacterial, microbead, phage, plasmid, or ribosome display, or mRNA-peptide fusion libraries) [2,3,7,15–19] together with soluble fluorogenic substrates. Display libraries cannot be selected directly for enzymatic activity [11] except in those cases where the fluorescent product associates with the cell surface [6]. Emulsification in w/o/w emulsions may enable the subsequent isolation of genes encoding the desired enzyme, while circumventing the need to have the product physically linked to the displayed protein. All screening and selection procedures make use of compartmentalization, be it in tubes, wells of microtiter plates or other two-dimensional arrays, or nanodroplets [20]. The ability to create miniature aqueous compartments a few microns in diameter, and then sort these compartments by FACS, may therefore widen the scope and capacity of HTS and provide yet another powerful tool for the in vitro evolution of enzymes.

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