

*Am J Hum Genet* 60:1411-1422, 1997.

**Summary:** A quantitative PCR assay for the determination of SMNT and SMNC gene-copy number is reported. Use of this method provides for accurate determination of SMNT copy number so as to improve risk estimates for the diagnosis and detection of SMA carriers.

Taton TA, Mirkin CA, Letsinger RL.

Scanometric DNA array detection with nanoparticle probes.

*Science* 289:1757-1760, 2000.

**Summary:** A method for analyzing DNA arrays using oligonucleotide-modified gold nanoparticle probes and a conventional flatbed scanner was described. Use of nanoparticle labels on oligonucleotide targets was found to sharpen the melting profiles of the targets, thereby imparting enhanced selectivity to the assay.

Walker NJ.

A technique whose time has come.

*Science* 296:557-559, 2002.

**Summary:** In this review and insight article, the technique of real-time PCR as a tool for a myriad of diagnostic applications in molecular biology, was discussed.

Watterson JH, Raha S, Kotoris CC, Wust CC, Gharabaghi F, Jantzi SC, Haynes NK, Gendron NH, Krull UJ, Mackenzie AE and Piuino PAE.

Rapid detection of single nucleotide polymorphisms associated with spinal muscular atrophy by use of a reusable fibre-optic biosensor.

*Nucleic Acids Res* 32:e18, 2004.

**Summary:** A reusable optical nucleic acid biosensor for rapid and quantitative genotyping for SNPs associated with spinal muscular atrophy was described. Control of surface chemistry was demonstrated to impart enhanced selectivity for SNP discrimination. Quantitative measurements were made in less than one minute by implementation of a kinetic response model.

## In vitro compartmentalization (IVC): A high-throughput screening technology using emulsions and FACS

Kalia Bernath<sup>1,2</sup>, Shlomo Magdassi<sup>1</sup>, and Dan S. Tawfik<sup>2,\*</sup>

1, Casali Institute of Applied Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel and 2, Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel.

\* Correspondence: tawfik@weizmann.ac.il

All screening approaches rely on ways of compartmentalizing assay reactions, and means of rapidly screening various molecules imbedded in these compartments. Miniaturization, which has become the hallmark of modern science and technology, has also been applied to screening, thus leading to a variety of high-throughput screening (HTS) technologies that aim at the smallest possible reaction volumes and the most sensitive and rapid means of detection. These demands are general and do not depend on the type of molecules (genes, proteins, small molecules, etc.) or activity (enzymatic, binding, inhibitory, etc.) that are being screened for, nor on the target of screening (functional genomics, directed evolution, drug discovery, etc.).

Conventional HTS approaches use either robotic 2D-arrays (e.g., microtitre plates), or living cells. In vitro compartmentalization (IVC) is a newly developed technology that uses the aqueous droplets of water-in-oil (w/o) emulsions as cell-like compartments. These droplets provide a facile means of compartmentalizing reactions – 1 milliliter of emulsion contains  $>10^{10}$  droplets, each of which serves as a discrete reaction vessel. The most distinct feature of IVC is the volume of the aqueous droplets, that is orders of magnitude smaller than in any other miniaturized assay format. Emulsions routinely contain droplets with mean diameters of 2-3  $\mu\text{m}$  and volumes around 10 fl ( $10 \times 10^{-15}$  liter). In such volumes, a single DNA or protein molecule is at a concentration of  $\sim 0.5 \text{ nM}$ . This concentration is high enough to drive transcription and translation from a single gene, and the ample detection of a single enzyme molecule.

Water-in-oil emulsions afford several other distinct advantages:

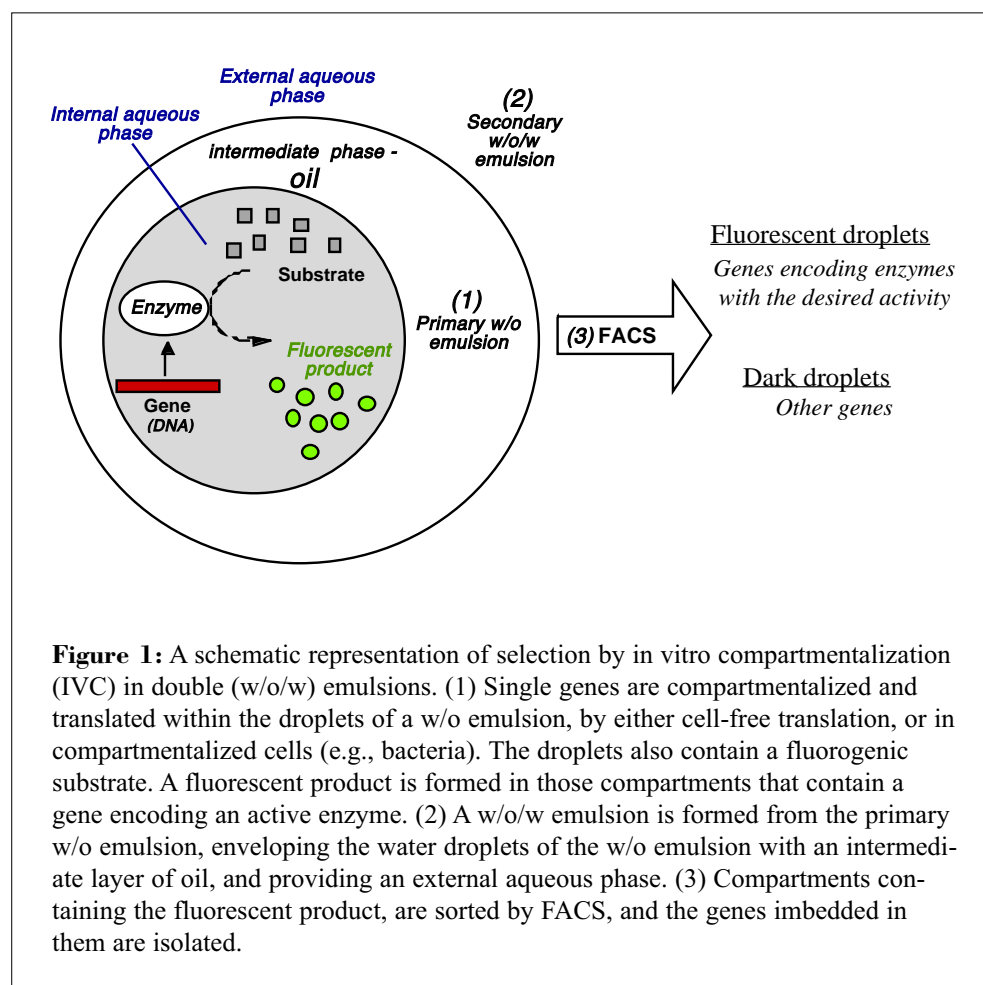
1. The external phase is biochemically inert. Thus, the entire volume of the aqueous reaction mixture is compartmentalized and there is no need to remove or inactivate the external phase.
2. The internal phase is biochemically active. Complex biochemical processes such as DNA replication, gene transcription and translation, as well as simpler enzymatic or binding processes, can be performed within the aqueous compartments with a comparable efficiency to a bulk solution.
3. Droplets of 1-50 $\mu\text{m}$  diameter can be generated. Making and breaking of emulsions takes 5-10 minutes and requires only basic laboratory equipment.
4. Emulsions are highly stable for many days, if not months, even at temperatures close to 100°C.

Coalescence of droplets, and the exchange of high-molecular weight components (e.g., genes and proteins) between droplets, are usually negligible.

IVC was initially developed as a mean of linking genotype to phenotype in a manner similar to living cells. Thus, in each of the aqueous droplets, 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. By applying an appropriate selection pressure, genes encoding proteins with the desired activity (binding or enzymatic) can be selected from large pools of genes. The capacity of IVC provides an opportunity to perform selections or screens of very large gene libraries ( $\geq 10^{10}$  using the smallest emulsion scale) whilst performing the entire selection in vitro with no need to clone and transform the gene library (Tawfik and Griffiths, 1998).

Whilst 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. Selections by IVC usually rely on the formation of a physical linkage between a given product and the gene encoding the desired protein that leads to the formation of this product. This format of IVC has already been demonstrated in the directed evolution of new enzymes (Cohen et al., 2004; Griffiths and Tawfik, 2003).

Other applications for IVC have been demonstrated. Most notably, the emulsion droplets can be used to perform single molecule PCRs, thereby allowing the parallel amplification of many millions of DNA molecules, each imbedded in a discrete compartment (Nakano et al., 2003). This capability led to a powerful application in the transformation of single DNA molecules into magnetic beads,



each carrying >10,000 identical copies of the original DNA molecule. This approach, dubbed BEAMing, allows rare variations in a population of DNA molecules to be identified and quantified by flow cytometry of the magnetic beads. Rare genetic events can be isolated by sorting and analyzed further. The utility of BEAMing in the identification and quantification of rare mutations in large gene pools, has been recently demonstrated. Future applications of this technology include the analysis of gene and transcript variations (e.g., occurrence and distribution of polymorphisms) in different populations and tissues (Dressman et al., 2003). Emulsions can also be used to create repertoires of beads, each of which displays a different gene together with multiple copies of the peptide or protein that this gene encodes. Using FACS (fluorescence activated cell sorting), these bead-display libraries could be selected for either binding or enzymatic activity (Griffiths and Tawfik, 2003; Sepp et al., 2002).

Conjugates of genes to their encoded peptides have also been obtained in emulsions, by using biotinylated DNA and streptavidin-fused peptides (Yonezawa et al., 2003). Recently, we have described a format of IVC that significantly increases the scope of this technology and provides a facile means for direct sorting of emulsion droplets by FACS. The ability of modern FACS instruments to analyze and sort up to 100,000 events per second, has given this technology a wide potential in the area of high throughput screening, functional genomics and directed evolution. After all, fluorescence is one of the most sensitive and versatile ways of detecting biological activities. Both binding interactions (of small ligands and proteins labeled with a fluorescent tag) and enzymatic activities (using fluorogenic substrates, i.e., 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., GFP) that are expressed in line with the binding pair) as well as enzymatic activities.

We have therefore developed an IVC system based on water-in-oil-in-water (w/o/w) emulsions that afford an external aqueous phase. W/o/w emulsions (or double emulsions) allow the creation of an external aqueous phase without the alteration of the aqueous droplets imbedded in the primary w/o emulsion. Thus, a variety of biochemical reactions can be performed in the primary w/o emulsions, including the translation of single genes to give active enzymes that generate a fluorogenic product.

Subsequent conversion of the primary w/o emulsions into a double emulsion makes it amenable to sorting by flow cytometry without compromising the integrity of the inner aqueous droplets within the oil phase. Thus, genes imbedded 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 imbedded in w/o emulsion droplets that do not contain a fluorescent marker (Figure 1) (Bernath et al., 2004).

The preparation of double emulsions is simple and rapid: A second water phase containing a hydrophilic surfactant is added to the primary w/o emulsion, and the mixture is homogenized for a few minutes to give the w/o/w emulsion. The droplets of these double emulsions are stable and amenable to sorting. This could be easily demonstrated with w/o/w emulsions prepared from a mixture of w/o emulsions: one containing a fluorescent marker (FITC-BSA) and the other only buffer. The droplets containing the fluorescent marker could be sorted and enriched for by FACS. Fluorescent droplets isolated from the first sort were stable enough to be re-sorted and enriched to almost 100%. To demonstrate the capability of this new IVC system to maintain the linkage between genes (e.g., enzyme-encoding genes) and fluorescent products, we performed a model selection aimed at enriching genes imbedded in aqueous droplets together with a fluorescent marker, from a large excess of other genes imbedded in aqueous droplets with no marker. Enrichment was tested through mixing of two primary w/o emulsions and re-emulsification to give a w/o/w emulsion that is amenable to FACS. Droplets sorted through the high fluorescence gate were collected and subsequently analyzed by flow cytometry. The sorted w/o/w emulsion droplets were then broken, and the genes contained within them amplified by PCR. Analysis of the sorted w/o/w droplets, by both flow cytometry and PCR, indicated an enrichment of ~30 fold in favor of the gene contained in the w/o emulsion droplets that carried the fluorescent marker (from a starting ratio of 1:100 before sorting, to ~1:3 after sorting). These results indicated that little or no mixing occurs, of either DNA or the fluorescent marker, between w/o droplets upon formation and processing of the w/o/w emulsion, and that the linkage between genes and markers is kept in this system.

Compartmentalization in double emulsions for the direct sorting by fluorescent signals is a unique technology. It allows various activities to be detected and selected with a wide range of commercially available fluorogenic sub-

strates that require no further modification. Selection in this system may be completely in vitro, namely, the protein molecules can be expressed from gene libraries generated by PCR, using cell-free extract translation; such a process involves no cloning or transformation. Emulsions, and double emulsions, could also be used to compartmentalize cells. Of particular interest are various display libraries, i.e., libraries of proteins that are physically linked to their coding gene, for example, protein libraries displayed on yeast or bacteria (Wittrup, 2001). Other types of display libraries could be compartmentalized as well (microbead-, phage-, plasmid-, or ribosome-display, or mRNA-peptide fusion libraries), together with soluble fluorogenic substrates.

Advances in combinatorial chemistry and molecular biology created numerous opportunities for screening large repertoires of different kinds of molecules (e.g., synthetic ligands, drug candidates, genes, proteins) for a variety of different properties (Ansede and Thakker, 2004). The ability to create miniature aqueous compartments of a few microns diameter, and then sort these compartments by FACS, widens the scope and capacity of in vitro compartmentalization and opens new opportunities in the area of HTS. This technology provides yet another powerful tool for the in vitro evolution of new proteins, but the scope of this system is in fact much wider. Double emulsions could be applied for many types of screens that involve fluorogenic marker detection, and provide a high-throughput, facile and cheap screen formats. Examples of this potential include the fields of drug discovery and analysis, for instance, in screens for enzyme inhibitors. Other applications may be in screens of genomic libraries, using fluorescently-labeled oligos or DNA beacons, or in the detection of viral and bacterial antigens.

## References

- Ansede J, Thakker D.  
High-throughput screening for stability and inhibitory activity of compounds towards cytochrome P450-mediated metabolism.  
*J Pharmac Sci* 93:239-255, 2004.  
**Summary:** A recent review on HTS technologies related to drug metabolism. The article also provides a broad introduction and references regarding the role of HTS in drug discovery and analysis.
- Bernath K, Hai MT, Mastrobattista E, Griffiths AD, Magdassi S, Tawfik DS.  
In vitro compartmentalization by double emulsions: sorting and gene enrichment by fluorescence activated cell sorting.  
*Anal Biochem* 325:151-157, 2004.  
**Summary:** The article describes the development of a double emulsion system for sorting by FACS, as described in the text and Figure 1.
- Cohen M, Tawfik D, Griffiths A.  
Altering the sequence specificity of HaeIII methyltransferase by directed evolution using in vitro compartmentalization.  
*Protein Eng Des & Select* 17:3-11, 2004.  
**Summary:** IVC was applied for the selection of gene libraries derived from the DNA-methyltransferase M.HaeIII for the ability to methylate the non-palindromic target sequence AGCC. The selection mode was based on the substrate being an integral part of the gene as described below (Tawfik & Griffiths, 1998). A variant was isolated exhibiting an 800-fold improvement in catalytic efficiency for AGCC and a preference for AGCC over GGCC (the original target sequence of M.HaeIII).
- Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B.  
Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations.  
*PNAS* 100:8817-8822, 2003.  
**Summary:** The article describes the application of in vitro compartmentalization for the transformation of single DNA molecules into magnetic particles that can be sorted by FACS. This approach, dubbed BEAMing, allows rare variations in a population of DNA molecules to be identified and quantified.
- Griffiths AD, Tawfik DS.  
Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization.  
*EMBO J* 22:24-35, 2003.  
**Summary:** The article describes the directed evolution by IVC of a phosphotriesterase that degrades the organophosphate paraoxon, with a very fast turnover number. Selection was based on coupling the product, and any unreacted substrate, to beads imbedded within the emulsion compartments. Product-coated beads, displaying active enzymes and the genes that encode them, were labeled with anti-product antibodies and selected by flow cytometry. This process was shown to select for all enzymatic features simultaneously – substrate recognition,

product formation, rate acceleration and turnover. Furthermore, the sensitivity of the process and the miniscule volume of the droplets enable the detection and isolation of a single enzyme molecule. Also described is a method for creating microbead-display libraries by IVC. These are libraries of  $>10^8$  different microbeads, where each microbead displays a single gene and multiple copies of the protein it encodes (see also, Sepp et al., 2002).

Nakano M, Komatsu J, Matsuura S, Takashima K, Katsura S, Mizuno A.

Single-molecule PCR using water-in-oil emulsion. *J Biotechnol* 102:117-124, 2003.

**Summary:** The article describes the use of w/o emulsion droplets as microreactors, in each of which, a single DNA molecule can be amplified.

Sepp A, Tawfik DS, Griffiths AD.

Microbead display by in vitro compartmentalization: selection for binding using flow cytometry. *FEBS Lett* 532:455-458, 2002.

**Summary:** The article describes a method of creating, within w/o emulsion droplets, microbeads, each of which displays a single gene and  $>200$  copies of the peptide encoded by this gene (see also, Griffiths & Tawfik, 2003). Flow cytometry can then be used to sort single microbeads for binding of an anti-peptide antibody.

Tawfik DS, Griffiths AD.

Man-made cell-like compartments for molecular evolution.

*Nat Biotechnol* 16:652-656, 1998.

**Summary:** The article describes the first application of water-in-oil emulsions for compartmentalizing genes and proteins, and linking genotype to phenotype. It was shown that a single gene could be transcribed and translated within the emulsion droplets to give many copies of the encoded enzyme, and that, these enzyme molecules are active within the droplets. A selection was demonstrated, of genes encoding a DNA-methyltransferase, from an excess of 107 genes encoding an enzyme that does not methylate DNA. By virtue of compartmentalization, only genes encoding a methylase were methylated and thereby protected from digestion by a cognate restriction enzyme; genes that do not encode a methylase, remained unmethylated and were subsequently digested.

Wittrup KD.

Protein engineering by cell-surface display.

*Curt Opin Biotechnol* 12:395-399, 2001.

**Summary:** A review describing various approaches by which, protein libraries displayed on cell surfaces are labeled with fluorescent ligands and then screened by flow cytometry. Applications of cell-surface display in the directed evolution of protein-ligand binding, stability, expression and enzymatic activity, are described.

Yonezawa M, Doi N, Kawahashi Y, Higashinakagawa T, Yanagawa H.

DNA display for in vitro selection of diverse peptide libraries.

*Nucleic Acids Res* 31:e118, 2003.

**Summary:** A display system is described, based on translating within w/o emulsion droplets, biotinylated genes encoding a peptide library fused to streptavidin. By virtue of compartmentalization, DNA-peptide conjugates are formed (i.e., a library of genes, each conjugated to the peptide it encodes) which can then be selected by panning onto a target protein. This paper also reports the usage of a cell-free translation system from wheat germ in emulsions.

#### Acknowledgments

Financial support by the Israel Ministry of Science is gratefully acknowledged.