

# Overview: methods and applications for droplet compartmentalization of biology

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Three protocols in this issue highlight applications of emulsification procedures, which deliver high-throughput potential to the molecular biology laboratory, without the need for automation. These procedures have already generated interesting results and spurred the development of exciting new technologies, while requiring only readily available laboratory equipment.

The spontaneous compartmentalization of materials in picoliter-sized vesicles is thought to be one of the key features of the early primordial soup that enabled the emergence of life<sup>1</sup>. In this process vesicles were important for several reasons: they provided a mechanism to link phenotype and genotype, a means to subdivide space into a virtually infinite array of subchambers for essentially limitless numbers of simultaneous parallel reactions, and through division, a mechanism for heredity and evolution over generations. In the laboratory, biochemists have sought to reproduce these encapsulation properties in robust, highly controlled systems through the use of emulsions. The emulsions of interest are essentially a mayonnaise, in which instead of oil droplets dispersed in water, scientists use droplets of bioreagents dispersed in oil. With the addition of specialized surfactants, these droplets can be made extremely resistant to coalescence during heating, cooling and processing steps, and through the right selection of materials, they can form ideal microreactors supporting a wide variety of biochemistry. In this issue, three protocols are described for using water-in-oil emulsions as a massively parallel array of bioreactors suitable for directed evolution<sup>2</sup>, emulsion PCR<sup>3</sup> and BEAMing<sup>4</sup>.

Emulsification provides one universal benefit to each of the three applications described in this issue, and that is the staggering throughput generated by the billions of independent microreactors contained within a single emulsion. Although emulsification also affords other application-specific advantages, the high-density, parallel reactions produced by compartmentalization allow single-molecule translation, amplification and interrogation without resorting to microfabrication<sup>5–7</sup>.

Emulsification has been instrumental to directed evolution—the use of successive rounds of selective pressure under controlled laboratory conditions to obtain protein functionalities unlikely or impossible in living systems. Emulsions are important for several reasons. First, emulsification directly aids the biochemical process by creating small reaction volumes, simultaneously increasing both the effective concentration of the reagents in the aqueous phase<sup>8</sup> and the reaction rates within the droplets as diffusional distances decrease<sup>9</sup>. Emulsion-based systems also exhibit extraordinarily high potential throughput, as approximately 10<sup>10</sup> separate microreactors are formed within 1 ml of emulsion<sup>10</sup>. This is particularly relevant to directed evolution studies, in which individual variants derived from single molecules of a heterogeneous population of DNA are selected for a particular functionality. The number of potential reactions generated within an emulsion permits template dilution to concentrations that favor droplets containing only a single tem-

plate copy and thus a single phenotype, and similarly increase the probability that even statistically rare or unlikely events (mutations, recombinations) may occur within any given reaction.

Equally important is that although reactions found in living organisms can be successfully replicated within an emulsified microreactor, the reactors themselves are nonliving synthetic constructs of completely known composition. This allows enzyme evolution and selection under conditions that are incompatible with life or in which cells are difficult to culture (such as extremes in temperature, pressure or pH), with the potential to design enzyme functions impossible in the natural world. The process has been successfully used to control the evolution of a variety of proteins<sup>10–19</sup>; one obvious area of current interest is the evolution of enzymes that can efficiently convert biomass into accessible fuel supplies<sup>20</sup>.

Emulsified microreactors can be used for reactions other than directed evolution; the development of thermostable emulsions permitted emulsion PCR, in which reactions are conducted within water-in-oil emulsions. Initially described as a methodology for directing the evolution of DNA polymerases<sup>11</sup>, emulsion PCR was first used specifically for amplification of single molecules by Nakano *et al.*<sup>21</sup>. Commercial development of emulsion PCR commenced with the launch of 454 Life Sciences with the aim of generating clonally amplified templates from complex populations to facilitate sequencing. The immobilized PCR product

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served as a template for sequencing organisms ranging from the bacteria *Mycoplasma genitalium*<sup>22</sup> and *Myxococcus xanthus*<sup>23</sup> to woolly mammoth<sup>24</sup> and neanderthal<sup>25</sup>. Emulsion PCR has also been successfully used for a variety of applications including reverse-transcriptase PCR-based detection of 10 copies of viral RNA<sup>26</sup>, haplotyping human paraoxonase-1 (ref. 27), ligation-based bacterial resequencing<sup>28</sup> and enrichment and analysis of specific transcription factors from complex populations<sup>29</sup>.

Emulsification provides specific benefits for PCR beyond substantial increases in throughput. Although amplification bias in bulk PCRs is common, single-template microreactors permit unbiased PCR amplification of complex, heterogeneous template populations by preventing competition between multiple amplicons. As each amplicon is isolated within its own microreactor, preferential amplification of one template over another based on amplification efficiency<sup>30,31</sup>, differences in amplicon G+C content<sup>32</sup> or amplicon reannealing<sup>33</sup> are avoided, and all templates are uniformly amplified<sup>3</sup>. This lack of bias has been demonstrated by the 99.5% coverage of non-repeat regions obtained from sequencing the emulsion PCR-amplified *M. genitalium* genome<sup>22</sup>. The lack of bias is essential to the successful amplification of complex libraries, or indeed, whole-genome amplification, as described in the protocol by Williams *et al.*<sup>3</sup> in this issue. Through compartmentalized amplification, emulsification provides a solution to the commonplace problem of quick, efficient and democratic amplification of PCR amplicons, cDNA or entire genomic libraries for laboratories of all resource levels.

Intact emulsions provide a vehicle through which the phenotype and genotype are linked, yet for many PCR-based applications, the emulsion must be broken after amplification to recover the product for subsequent manipulation, creating a single heterogeneous pool of amplicons. Although this is acceptable for applications such as whole-genome amplification, this is not desirable when the clonal nature of the amplified material must be preserved. In these instances, retention of product amplified within each individual microreactor is achieved by capturing the product on solid supports, or DNA capture beads, providing a substrate for subsequent sequencing without reliance upon the bacterial cloning process<sup>22,28</sup> and avoiding the potential bias that cloning introduces<sup>34</sup>.

Immobilization of the amplified material also provides a means to differentiate between microreactors containing templates and those without templates. Owing to the high throughput afforded by emulsion-based reactions, DNA concentrations in emulsion PCRs can be lowered to generate predominantly single template-containing droplets, permitting the amplification and detection of rare alleles without interference from more prevalent wild-type sequences<sup>35</sup>. But because distribution of template molecules in droplets follows the Poisson distribution, this level of dilution yields a large percentage of negative (empty) microreactors in which no template is present, and the beads contained within them possess no amplified template or sequence information. As such, subsequent processing of the entire DNA capture bead population would be extremely inefficient or expensive for some applications, such as sequencing.

Recovery and subsequent use of the DNA capture beads can be accomplished through any of several methods. BEAMing, as used by Dressman *et al.*<sup>36</sup> and described in this issue<sup>4</sup>, makes use of paramagnetic DNA capture beads permitting bulk enrichment of the total bead population, both PCR-positive (with amplified product immobilized on the surface) and PCR-negative (without PCR product as a result of a lack of template or poor amplification, among other reasons). Probes complementary to regions of known allelic variations are then added to the reactions, and allelic discrimination is determined upon hybridization by the probes' allele-specific fluorescence, assayed by cytometry or fluorescence-activated cell sorting. Alternatively, emulsion PCR, as described by Margulies *et al.*<sup>22</sup>, uses nonmagnetic DNA capture beads, but biotinylated probes complementary to the PCR primers are used in conjunction with paramagnetic beads to selectively enrich the PCR-positive DNA capture beads from the PCR-negative beads. As PCR primers are used as the hybridization target, any amplifiable product can be enriched without *a priori* sequence knowledge, permitting true *de novo* sequencing or analysis of complex genomic mixtures (for example, pools of hundreds of amplicons). Shendure *et al.*<sup>28</sup> modified this process by using the density increase resulting from large nonmagnetic beads binding to PCR-positive beads via biotinylated probes complementary to the PCR primers to permit enrichment via centrifugation. Regardless of the methodology, enrichment of immobilized emulsion PCR

products allows efficient segregation and use of unbiased, clonally amplified templates in a variety of applications ranging from fluorescent enumeration<sup>36</sup> and resequencing<sup>28</sup> to viral-strain identification<sup>35</sup>, whole-genome comparisons<sup>37</sup> and whole-genome *de novo* sequencing<sup>22</sup>.

Emulsion droplets as massively parallel bioreactors have proven to be a powerful research tool for biologists and biochemists, comprising the heart of new technologies that are dramatically increasing our ability to design new enzymes, to manipulate complex mixtures of reagents and to study whole genomes. Although bulk-emulsion techniques have proven successful in many applications, many more applications would benefit from improved droplet-processing technology. Processing bulk emulsions limits the uniformity in droplet size and composition that can be achieved, and precludes multistep chemistries where reagents are added incrementally, reducing their overall utility. To overcome these restrictions, researchers at Harvard University have developed techniques to incorporate electric fields and electric-field gradients into microfluidic channels to achieve high-speed active control over droplets<sup>38,39</sup>. The control afforded by the active use of electric fields has allowed RainDance Technologies to initiate commercial development of an instrument platform with the ability to combine and sort individual droplets containing different reagents based on the results of biochemical reactions (see <http://www.raindancetechnologies.com>) at processing speeds exceeding thousands of droplets per second. With the advent of precise microfluidic control over bioreagents encapsulated in droplets, we can expect that great new things will be achieved from mayonnaise, the modern equivalent of the primordial soup.

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