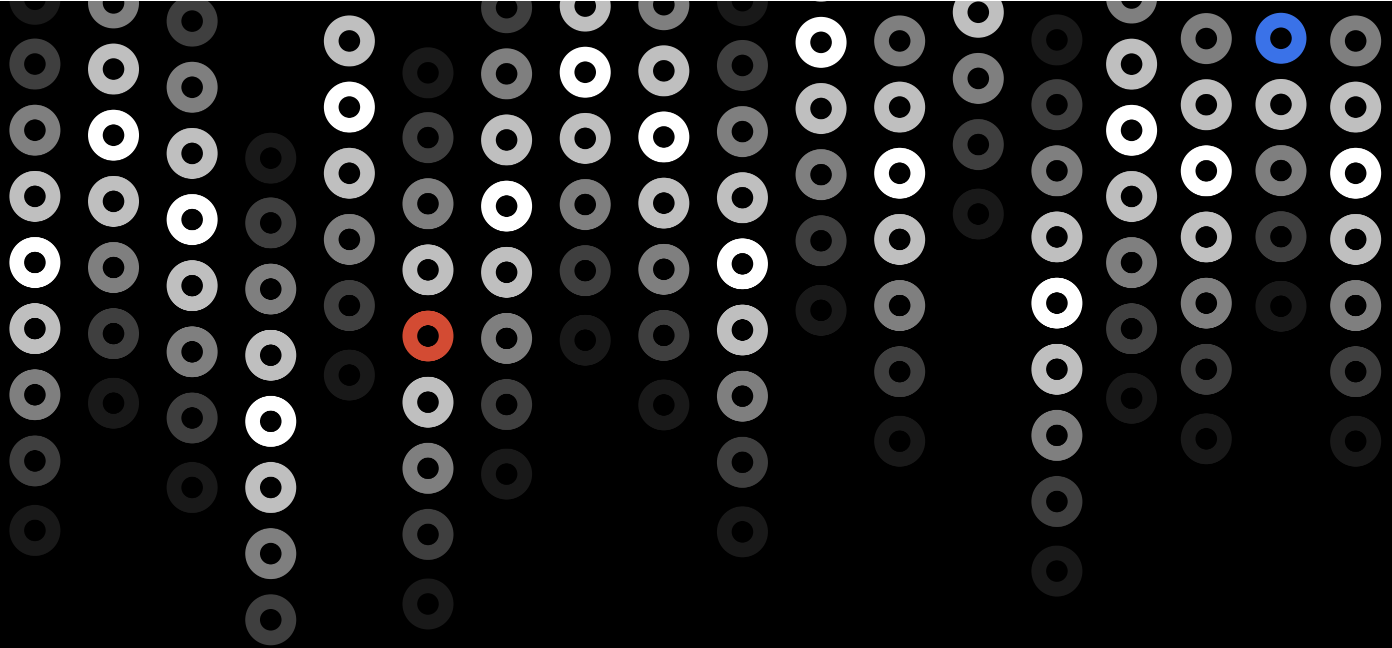


YOUR GROUND CONTROL IN HIGH-THROUGHPUT BIOLOGY

WHITE PAPER

DG-WP0001 - Rev A - White Paper

HIGH-THROUGHPUT FUNCTIONAL SCREENING FACILITATED BY DROPLET MICROFLUIDICS

Droplet Genomics

DROPLETGENOMICS.COM

High-throughput functional screening facilitated by droplet microfluidics

SUMMARY:

- In many instances, functional screening is a preferred selection strategy over the examination of complementary properties, such as binding affinity.
- Dielectrophoretic droplet sorting, unlike fluorescence-activated cell sorting, is compatible with water-in-oil emulsions.
- Functional screening employing fluorescence-activated droplet sorting advances antibody discovery, drug discovery, functional metagenomics, and protein engineering.

INTRODUCTION

Screening throughput is a common bottleneck of many research areas, including functional genomics, drug discovery, and *in vitro* evolution. Traditionally, high-throughput screening (HTS) is defined as the rapid analysis of unique samples, typically exceeding 10^3 samples per day, for the identification and selection of positive "hits"[1]. HTS techniques can be classified into two main categories: (i) affinity-based screening and (ii) functional screening. The first one relies on binding assays that provide information about the affinity of a test molecule for a target binding site. Binding assays are relatively easy to establish, however, they reveal no functional activity. In contrast, functional assays show an effect triggered by the interaction of a ligand at a target binding site. Functional assays might be based on a broad range of readouts, such as cell proliferation, reporter gene expression, downstream signaling, and other effects that are a consequence of ligand binding[2].

Affinity-based screening is suitable for the interrogation of many macromolecules, such as DNA and proteins, that have been demonstrated as pharmaceutical targets. This screening approach does not require separating every component from a complex mixture. The assay focuses specifically on bound ligands-targets, while unbound molecules are washed away.

Affinity-based screening assays can investigate multiple interacting pairs involved in biological systems, including antigen-antibody, receptor-ligand, enzyme-inhibitor or activator, protein-protein. Methods typically include affinity chromatography, ligand fishing, affinity electrophoresis, and others. Affinity-guided screening undeniably accelerates the discovery process[3]. Nevertheless, non-specific interaction of a ligand with the target is possible. It is indistinguishable whether binding occurs at the active or allosteric site of a target, or at an agonistic or antagonistic receptor binding site until characterization by secondary assays is performed[4]. This, in turn, precludes functional interpretation of affinity-based screens.

Functional HTS and selection require spatial co-localization of the target molecule and information carrier that encodes its structure. Compartmentalization can be achieved either *in vivo* or *in vitro*. *In vivo* compartmentalization uses biological compartments, such as cells and phage particles, while *in vitro* compartmentalization uses artificial compartments, in most cases water-in-oil emulsion droplets or water-in-oil-in-water double emulsion (DE) droplets[5].

Fluorescence-activated cell sorting (FACS) is the gold standard for phenotypic HTS and isolation of different cell types from a heterogeneous cell population based on fluorescence, size, or granularity. Despite the wide adoption for both basic and clinical research, FACS is limited to cell-based functional assays that interrogate processes inside the cell or on the cell surface. The repertoire of feasible functional HTS assays has been substantially broadened by lab-on-a-chip techniques.

Functional HTS based on on-chip droplet cytometry and sorting of water-in-oil droplets according to fluorescence readout has become possible with the emergence of the fluorescence-activated droplet sorting (FADS) technique. In FADS, droplets are introduced into a microfluidic chip with an asymmetric Y-shaped junction. By default, droplets flow into a wider arm of the sorting junction due to lower hydraulic resistance. If a fluorescent signal triggers a sorting event, a pulse of high voltage alternating current is applied across the electrodes adjacent to the sorting junction. The resulting electric field deflects the droplet into a narrow arm of the sorting junction by dielectrophoresis[6]. Sorting mostly depends on the dielectric contrast between water and oil, independently of additives in these phases, which makes it compatible with virtually any functional assay. Moreover, FADS allows the screening and sorting of a broad range of droplet volumes (typically, fL to nL) at a rate comparable to FACS devices[7].

Screening of large cell or gene libraries based on direct activity rather than complementary properties (e.g. binding affinity) is now a preferred strategy in many areas of research as functional assays more closely resemble context where entities of interest are anticipated to act. Next, several areas of research that have successfully adopted FADS will be briefly reviewed.

ANTIBODY DISCOVERY

Monoclonal antibody (mAb) screening is an exciting application of droplet microfluidics in therapeutics development. mAbs are promising agents against infectious, autoimmune, and cancerous diseases, however, the discovery of mAbs with desirable functionalities remains challenging.

The binding of mAbs to their targets is a prerequisite for their functional activity. mAbs can be screened for binding affinity using in vitro display techniques, yet therapeutic mAbs must also modulate the activity of their targets thus selection on the basis of binding alone is not sufficient.

Best binders must be evaluated for their functional properties, which conventionally involves individual screening using microtiter plates and liquid handling systems, severely limiting functional screening throughput, success rate, and turnaround time. Currently, there are no general rules for guiding functional screens – mAbs that are able to bind the same or similar epitopes can result in different biological outcomes, with some acting as agonists while others – as antagonists. This intrinsic complexity requires screening as many mAbs as possible[8].

Microfluidics-assisted screening platforms allow performing phenotypic analysis of individual mAbs-secreting cells in the presence of co-encapsulated reporters with exceptionally high throughput. Functional mAbs activate reporters to produce fluorescence signal that serves as a trigger to sort "positive" droplets from a heterogeneous population.

Notable advantages of employing droplet microfluidics for functional mAbs screens over conventional techniques involve:

- **Analysis speed.** The microfluidics approach allows functional screening of hundreds of thousands to millions of single cells in a day[9,10].
- **Combinatorial screens of bi- or multi-specific mAbs.** Efficient identification of active bispecific T cell engager mAbs was demonstrated employing a droplet-based microfluidic screening system with a functional readout[8]. Moreover, combinatorial screens might help to decipher the design principles for bispecific mAbs[11].
- **Adaptability and scalability.** Direct interrogation of functional properties eliminates the need for prior knowledge of binding characteristics which gives more flexibility to the discovery of bi- or multi-specific mAbs[11].
- **Coupling of phenotypic screening with VH-VL sequencing.** Re-compartmentalization of sorted cells with barcoded beads and reverse transcription reagents enables to couple phenotypic screen with transcriptomic profiling[12].

DRUG DISCOVERY

DNA-encoded library (DEL) screening is a widespread method for early-stage drug discovery. DELs comprise millions to billions of unique small molecules covalently attached to DNA barcodes. Small molecule-DNA conjugates are produced via split-pool combinatorial chemistry which includes both synthesis and DNA ligation steps before pooling. DNA tags encode the structural information of each associated small-molecule moiety, and catenated barcode describes the synthesis history of each compound.

DELs can be created by either the solution-phase or solid-phase synthesis. Traditionally more popular solution-phase synthesis yields an inseparable mixture of compounds at very small concentrations. Screening of such DELs is limited to affinity-based identification of "hits" similar to *in vitro* display: immobilized target protein is incubated with the library; following washing steps DNA barcodes that are conjugated with the compounds that remain associated with the immobilized protein are amplified and sequenced[13]. This approach is suitable only for soluble target proteins thereby excluding many pharmaceutically relevant targets, e.g. membrane-bound proteins, protein complexes, signal transduction pathways[14].

To enable functional screening of DELs, many copies of individual library members should be allocated to discrete volumes. One-bead-one-compound (OBOC) solid-phase combinatorial synthesis is an effective strategy to parse library members into discrete entities using

beads, where each bead displays 10^{-15}-10^{-9} moles of a library member[15]. In biochemical or cellular functional screens of OBOC DELs, small molecules may be released from the beads and thus freely interact with their targets without potential interference from a DNA barcode while compartmentalization within microdroplets retains chemotype-genotype connection. The presence of the desired functional activity can be translated through fluorescence intensity, and droplets containing positive “hits” can be sorted depending on their level of fluorescence.

An illustrative example of using droplet microfluidics for functional OBOC DEL screening is a study by Cochrane et al. who identified novel inhibitors of autotaxin from a 67,100–member DEL[15]. Microfluidics-assisted functional screens open up the possibility to tackle more complex assays than simple binding and **democratize small molecule discovery**.

FUNCTIONAL METAGENOMICS

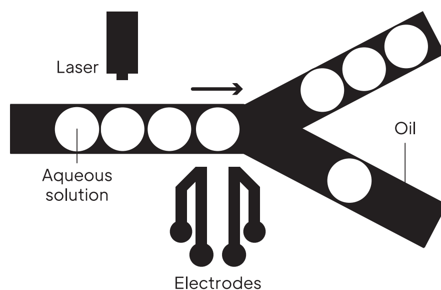
The community of environmental microbes contains billions of unique life forms. Standard laboratory culture techniques support the growth of less than 1% of the environmental bacteria. Metagenomics has long served as a tool to screen for enzymes with potential for industrial use from both cultured and uncultured microorganisms.

Metagenome libraries might be screened based on either the sequence or function. Sequence-based screens involve direct sequencing of metagenomic DNA, e.g. extracted from soil. This approach is limited to the detection of gene variants with conserved motifs and does not allow the identification of novel enzyme encoding genes. As a consequence, the fraction of genes with unknown function in newly sequenced microbial genomes has remained constantly high[16]. Function-based screening overcomes this by assaying enzyme activity, traditionally on agar

Binding assay

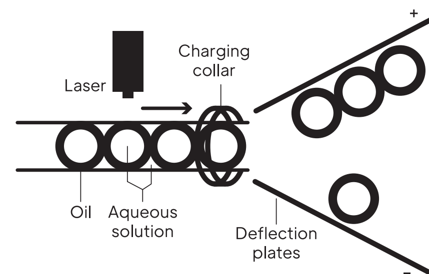
- 💡 DEL screening; antibody discovery
- ✓ Very high throughput
- ✗ No functional information

FADS



- 💡 Any functional screening
- ✓ Easy to manipulate droplet contents
- ✗ Cannot dispense

FACS



- 💡 Phenotypic cell screening
- ✓ Multiparametric analysis
- ✗ Liquid-phase screening is complicated

plates or microtiter plates supplemented with an appropriate substrate. To perform function-based screening, environmental DNA must be cloned into a surrogate host if source microbes are not culturable. Due to labor intensiveness and low throughput, traditional functional analyses are impractical.

Functional metagenomic screens using droplet microfluidics has proved to be instrumental for efficient identification of rare genes and activities that could have not been previously predicted by sequence homology:

- **Miniaturization** of cell lysate assays allows obtaining single-cell level insights.
- **Beating the odds:** efficient high-throughput screening of large metagenomic libraries enables to identify less widespread catalytic activities. For example, Colin et al. used droplet-based screening to find rare enzymes with sulfatase and phosphotriesterase activities[17].

PROTEIN ENGINEERING

Protein engineering has emerged as a powerful tool to improve the catalytic properties of enzymes and study protein structure-function relationships. Engineering via directed evolution requires iterative rounds of *in vitro* selection and amplification. The outcome of *in vitro* evolution campaign largely depends on the number of variants that can be screened and the quality of enrichment technique that separates functional variants from the background. The benefits of miniaturization and compartmentalization in the context of directed evolution were realized quite early, with the compartmentalized self-replication (CSR) approach reported back in 1998[18]. Nevertheless, bulk emulsification resulting in polydisperse emulsions does not permit further manipulation with droplets due to massive differences in droplet size and enzyme-substrate concentrations.

Microfluidic devices generate monodisperse droplets that are amenable to further manipulations, such as merging, picoinjection, or sorting. This in turn paves the way to design complex functional screening assays that do not necessarily include gene self-replication. Any fluorogenic substrate responsive to target protein activity will allow sorting positive “hits” from the population[19].

Successful functional assay designs created for directed evolution of enzymes in droplet microfluidic systems include primer extension, strand displacement, ligation, restriction digestion[20], and *in vitro* transcription[21] in the presence of fluorescently-labeled probe that gets separated from a quencher during the course or after the enzymatic reaction.

FLOW CYTOMETRY AND FACS

FACS technologies can routinely analyze and sort >10^7 events per hour and thus are widely adopted for HTS of single cells based on their fluorescence or light-scattering properties. Here, cells suspended in a fluid flow pass through a laser beam one by one and are detected based on the scatter or emission of light energy from fluorescent reporters. FACS allows isolating cells with desired properties from heterogeneous population into separate containers. FACS has already facilitated significant advances in biomedical sciences, however, flow cytometry-based analyses are limited to readouts that are confined within the cell or on the cell surface. Screening of liquid-

phase reactions where there is no physical linkage between the genotype, phenotype, and assay readout impedes the use of FACS. In addition, typical FACS instruments require more than 10^5 cells in a starting population, are expensive, and may produce hazardous aerosols.

The pairing of flow cytometry with droplet microfluidics can solve the shortcomings related to the lack of physical compartmentalization given that generated microdroplets may be suspended in aqueous solution – compatible designs are water-in-oil-in-water DE and hydrogels[22]. DE is produced when the primary water-in-oil emulsion is injected into a hydrophilic microfluidic chip containing an aqueous continuous phase. Physical separation is also preserved when cells or other contents of the compartments are entrapped in a hydrogel. The presence of polymers does not impair the droplet generation process, and, importantly, solidified hydrogels retain cell viability. Nevertheless, the formation and manipulation of DEs and hydrogels are lengthy and cumbersome, and the capacity to modify the contents of these compartments is limited, thus technologies compatible with direct manipulation with water-in-oil droplets are preferred for liquid-phase screening assays.

CONCLUSIONS

- Fluorescence-activated cell sorting is useful for phenotypic cell screening based on the markers expressed either inside the cell or on the cell surface.
- Fluorescence-activated droplet sorting employs dielectrophoresis to select water-in-oil droplets based on their fluorescence intensity.
- The major benefits of using FADS in various functional screening contexts include miniaturization, improved analysis speed, cost-efficiency, adaptability, scalability, opportunity to design complex multistep assays thanks to the technical ability to modulate droplet contents.

REFERENCES

1. Payne EM, Holland-Moritz DA, Sun S, Kennedy RT. High-throughput screening by droplet microfluidics: perspective into key challenges and future prospects. *Lab Chip*. 2020;20(13):2247–2262.
2. Gabriel J, Höfner G, Wanner KT. A Library Screening Strategy Combining the Concepts of MS Binding Assays and Affinity Selection Mass Spectrometry. *Front Chem*. 2019;7:665.
3. Hou X et al. Recent advances in screening active components from natural products based on bioaffinity techniques. *Acta Pharm Sin B*. 2020;10(10):1800–1813.
4. Mayr LM, Zehender H. Affinity-based screening. 2006. European Pharmaceutical Review. https://www.europeanpharmaceuticalreview.com/article/2657/affinity-based-screening/ (accessed 25 Jan 2022).
5. Xiao H, Bao Z, Zhao H. High Throughput Screening and Selection Methods for Directed Enzyme Evolution. *Ind Eng Chem Res*. 2015;54(16):4011–4020.
6. Weng L, Spoonamore JE. Droplet Microfluidics-Enabled High-Throughput Screening for Protein Engineering. *Micromachines (Basel)*. 2019;10(11):734.
7. Caen O et al. High-throughput multiplexed fluorescence-activated droplet sorting. *Microsyst Nanoeng*. 2018;4:33.
8. Wang Y et al. High-throughput functional screening for next-generation cancer immunotherapy using droplet-based microfluidics. *Sci Adv*. 2021;7(24):eabe3839.
9. El Debs B, Utharala R, Balyasnikova IV, Griffiths AD, Merten CA. Functional single-cell hybridoma screening using droplet-based microfluidics. *Proc Natl Acad Sci U S A*. 2012;109(29):11570–5.

10. Mazutis L et al. Single-cell analysis and sorting using droplet-based microfluidics. *Nat Protoc.* 2013;8(5):870–91.
11. Segaliny AI et al. A high throughput bispecific antibody discovery pipeline. BioRxiv [Preprint]. 2021. DOI: 10.1101/2021.09.07.459213
12. Gérard A et al. High-throughput single-cell activity-based screening and sequencing of antibodies using droplet microfluidics. *Nat Biotechnol.* 2020;38(6):715–721.
13. Kodadek T, Paciaroni NG, Balzarini M, Dickson P. Beyond protein binding: recent advances in screening DNA-encoded libraries. *Chem Commun (Camb).* 2019;55(89):13330–13341.
14. Goodnow RA Jr, Dumelin CE, Keefe AD. DNA-encoded chemistry: enabling the deeper sampling of chemical space. *Nat Rev Drug Discov.* 2017;16(2):131–147.
15. Cochrane WG et al. Activity-Based DNA-Encoded Library Screening. *ACS Comb Sci.* 2019;21(5):425–435.
16. Galperin MY, Koonin EV. From complete genome sequence to 'complete' understanding?. *Trends Biotechnol.* 2010;28(8):398–406.
17. Colin PY et al. Ultrahigh-throughput discovery of promiscuous enzymes by picodroplet functional metagenomics. *Nat Commun.* 2015;6:10008.
18. Tawfik DS, Griffiths AD. Man-made cell-like compartments for molecular evolution. *Nat Biotechnol.* 1998;16(7):652–6.
19. Larsen AC et al. A general strategy for expanding polymerase function by droplet microfluidics. *Nat Commun.* 2016;7:11235.
20. Vallejo D, Nikoomanzar A, Paegel BM, Chaput JC. Fluorescence-Activated Droplet Sorting for Single-Cell Directed Evolution. *ACS Synth Biol.* 2019;8(6):1430–1440.
21. Kapustina Ž, Jasponė A, Dubovskaja V, Mackevičius G, Lubys A. Enzymatic Synthesis of Chimeric DNA Oligonucleotides by in Vitro Transcription with dTTP, dCTP, dATP, and 2'-Fluoro Modified dGTP. *ACS Synth Biol.* 2021;10(7):1625–1632.
22. Li M, Liu H, Zhuang S, Goda K. Droplet flow cytometry for single-cell analysis. *RSC Adv.* 2021;11:20944–20960.

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