Droplet-based microfluidics in high-throughput single-cell analysis

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Technical Journal Club, 10.11.2014

Tools for high-throughput single-cell studies:

- Fluorescence-based flow cytometry
- Fluorescence microscopy using automated image
- Microfluidic system

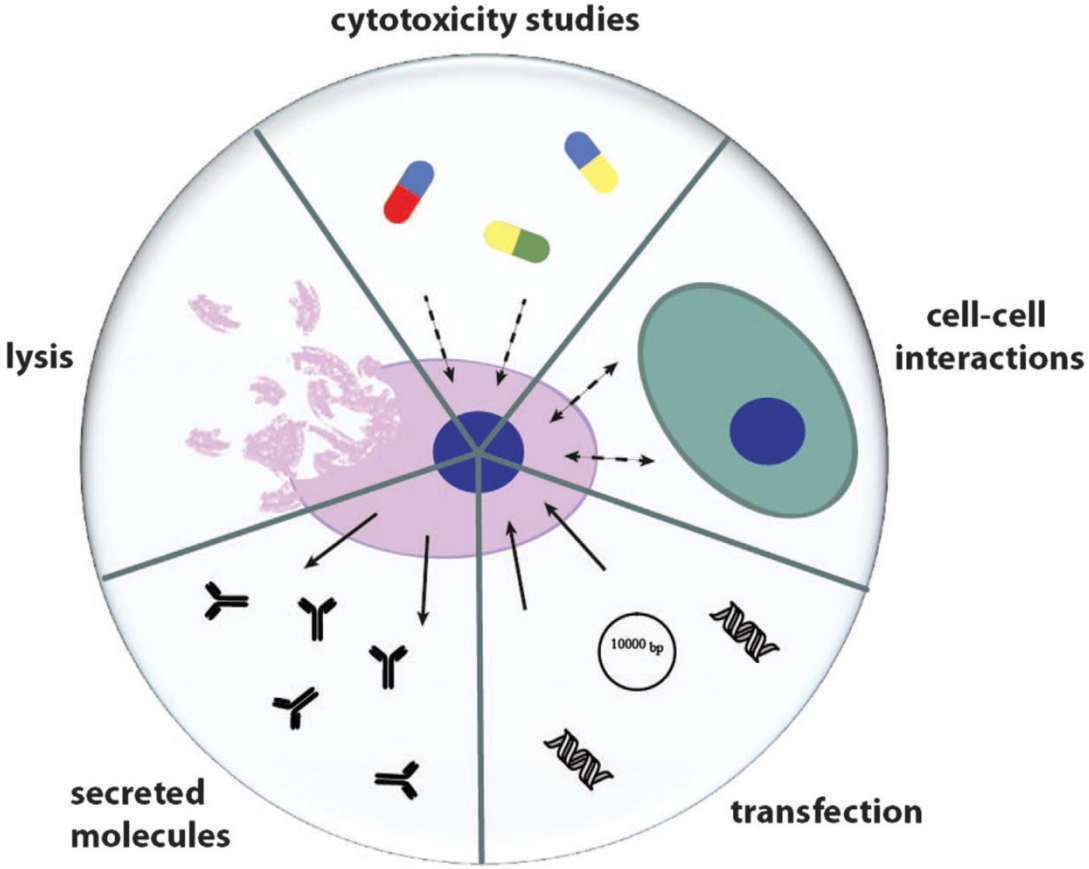
Advantages of microfluidics for single cell analysis.

Application	Challenges with traditional methods	Advantages of microfluidics
Single-cell RT-qPCR	Limited abundance of starting template	Concentration enhancement in small volumes
	Cost and throughput required for analysis of large numbers of cells and target genes	Parallelization, automation, and economy of scale
Single-cell genomics	Amplification bias and sensitivity	Improved reaction bias and sensitivity in nL volumes and reduced contaminant DNA
	Isolating individual cells	Integrated microfluidic cell sorting and processing
Single-cell measurements of intracellular proteins	Movement of living cells	Confinement of live cells in microfluidic structures
	Low amount of signal	Integrated single-cell handling allows lysate analysis
Single-cell measurements of secreted proteins	Small amounts of secreted products from single cells	Concentration enhancement in small volumes
	Difficult to co-localize multiple cells in defined chemical environments	Ease of confinement in droplets or microchambers
Signaling studies	Mostly limited to static conditions	Easy temporal stimulation
	Inability to rapidly exchange conditions on suspension cells	Laminar flow and proper design enables cell sequestration
Live cell imaging	Difficulties of tracking cells through multiple frames	Confinement of clones facilitates cell tracking

Curr Opin Chem Biol. 2012 Aug;16(3-4):381-90.

Recent advances in droplet microfluidics have greatly expanded their potential for single-cell studies:

- Droplets can be collected from a microfluidic device and incubated off-chip， after incubation droplets can be reinjected into another microfluidic device for further manipulation.
- Additional reagents can be added to a droplet at well-defined points in space and time by fusion or picoinjection.
- Splitting of droplets offers the opportunity to perform multiple assays on a single droplet and to dilute the content of droplets without increasing the reaction volume.
- Droplets can also be sorted passively because of the difference in their size or actively via fluorescence-activated droplet sorting, which allows for the separation of droplets of interest from the rest of the droplet stream.
- A wide spectrum of on-chip detection methods is available for the droplet format, including fluorescence measurements, ultraviolet-visible spectroscopy and electrochemical detection.



These ‘microtools’ provided by droplet microfluidics, alone or in conjunction with traditional single-cell technologies, are opening many new avenues of research into the function of single cells.

NPG Asia Materials (2014) 6, e133; doi:10.1038/am.2014.86

Encapusulate single cells into droplets

Stochastic encapsulation inside flow-focusing devices made from polydimethylsiloxane (PDMS) molds bonded to glass slides.

- Diluted cell suspensions and random cell encapsulation results in a Poisson distribution of encapsulated cells.
- If the average number of cells per droplets is low enough, the fraction of droplets containing more than one cell is negligible. But at the same time, the fraction of empty droplets is significant, lowering the throughput of the method and making analysis more difficult.

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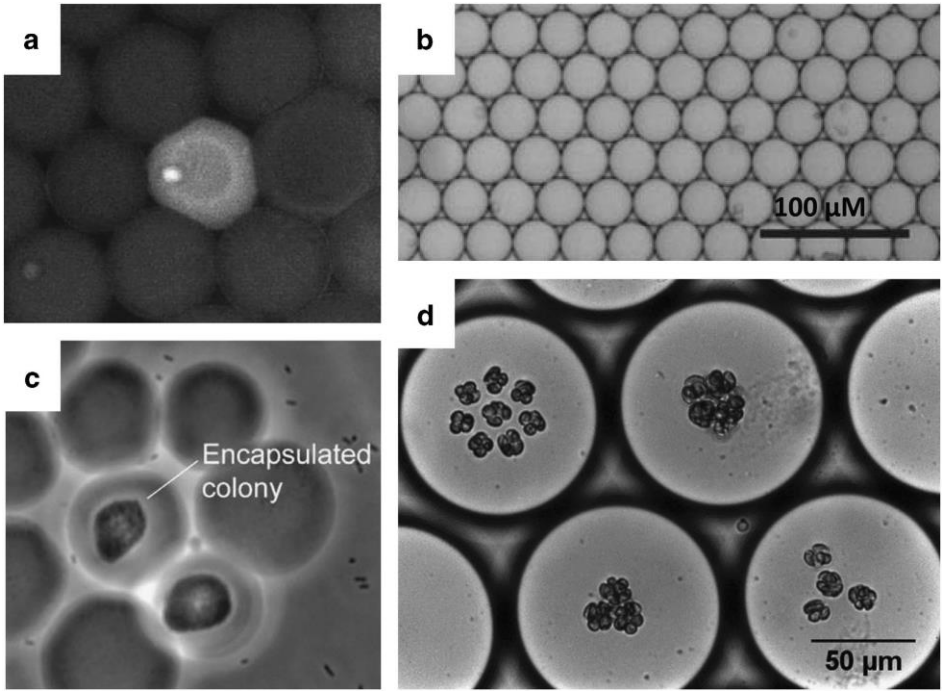
Manipulation of single cells inside droplets

- Cell culture inside droplets



a) multiple human cell lines, b) yeast, c) bacteria, d) algae

Several integrated modular designs for on-chip cell immobilization, culturing, transfection and transfer into droplets.

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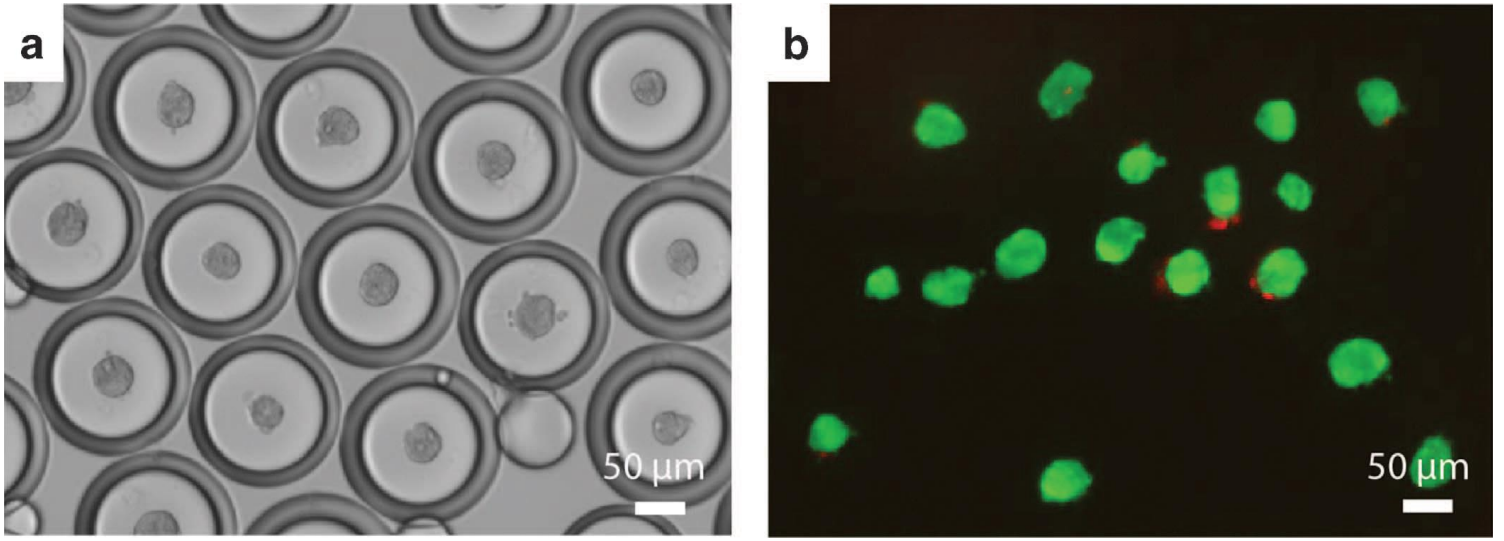
Manipulation of single cells inside droplets

- Cell culture inside droplets



Cells can be recovered from emulsions:

a) Phase image of human mesenchymal stem cells (hMSC) spheroids encapsulated in double emulsion droplets after 6 h.

b) Live/dead staining of spheroids after release from emulsion at 6 h using 1H,1H,2H,2Hperfluoro-1-octanol. Live cells were labeled with calcein AM (green), and dead cells were labeled with propidium iodide (red).

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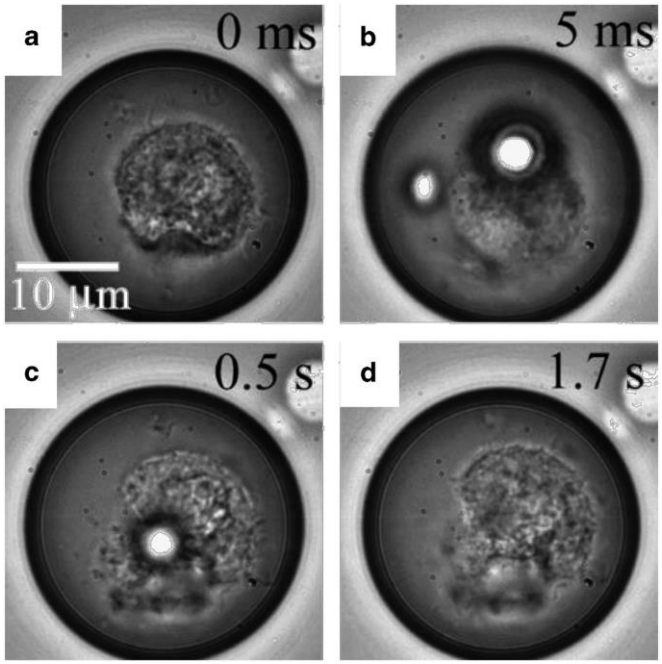
Manipulation of single cells inside droplets

- Cell lysis inside droplets



Physical (optical) lysis: laser-induced plasma formation and the subsequent generation of a shock wave, followed by a cavitation bubble, results in cellular disruption.

Detergent-based or enzymatic lysis agents have been applied to study the content released from single cells.

e.g., to release genomic DNA or mRNA, **proteinase K** digestion and **sodium dodecyl** or **Tween** lysis have been demonstrated to be useful.

Physical lysis methods appear to be challenging because it is relatively easy to perturb monodisperse water-in-oil emulsions.

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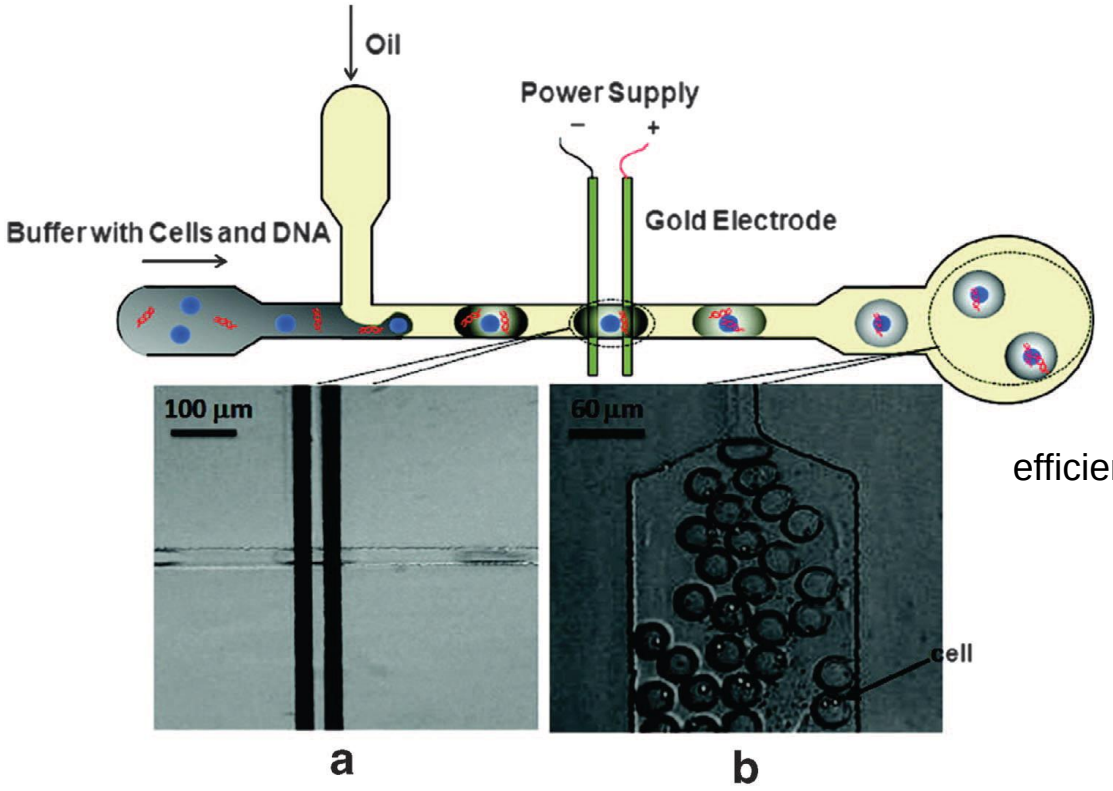
Manipulation of single cells inside droplets

- Transfection of single cells



efficiency was about 11-25%

a) Electroporation occurs when the cell containing droplets in oil flow through a pair of microelectrodes with a constant voltage established in between.

b) The droplets with encapsulated cells after electroporation at the exit of the device.

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Functional studies on single cells

- Drug studies on single cells in droplets
- Single-cell genomics
- Single-cell immunology
- Evolution of microorganisms in droplets

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Table 1 Single-cell genetic analysis in microfluidic droplets

Reactor	*Type of analysis*	*Nucleic acid of interest*	*Target*	*Innovative aspect*	*Through-put*
Nanoliter aqueous droplets	Sanger sequencing, long read pyrosequencing	DNA	Human, *E. coli*	Production of amplicons suitable for Sanger sequencing and long read pyrosequencing from single cell	High
Nanoliter aqueous droplets	Multiplex PCR	DNA	*E. coli*	Analysis of more than one gene per droplet	High
Nanoliter agarose droplets	Multiplex PCR, next-generation sequencing	DNA	Human	Sequencing of multiple target genes within single cell, production of amplicons suitable for next-generation sequencing	High
Nanoliter aqueous droplets	RT-qPCR	RNA	Mammalian cells (*Canis familiaris*)	RT-qPCR from single cell	Low
Picoliter agarose droplets	RT-PCR	RNA	Human	Physical trapping of RT-PCR products in agarose by grafting primers to polymer matrix	High
Nanoliter aqueous droplets in chambers	qPCR, sequencing, whole genome amplification	DNA	Different microbial species	Versatility	Low
Aqueous droplets	RT-PCR	RNA	Human	Elimination of bias caused by cell lysis prior encapsulation	Ultrahigh
Nanoliter agarose droplets	STR typing	DNA	Human	STR typing of individual cells	High

Droplet microfluidic technology for single-cell high-throughput screening

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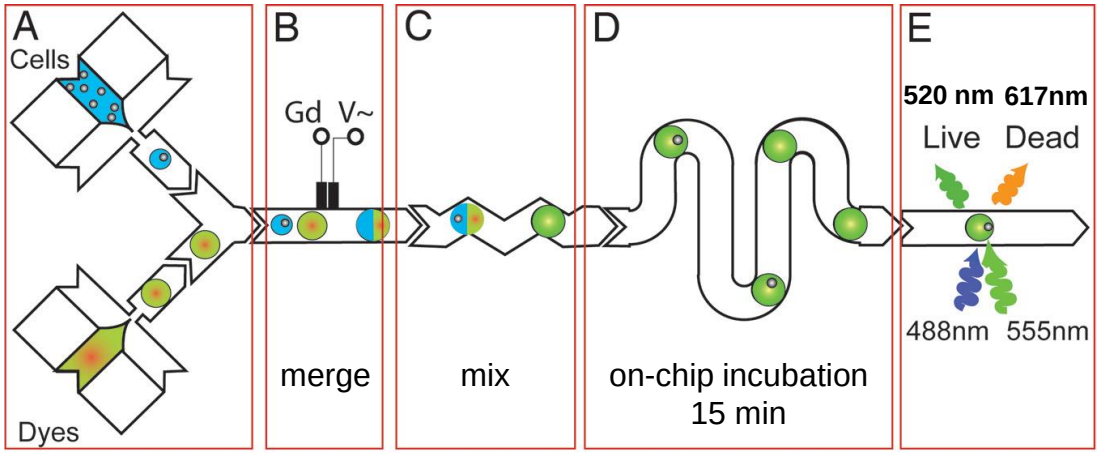
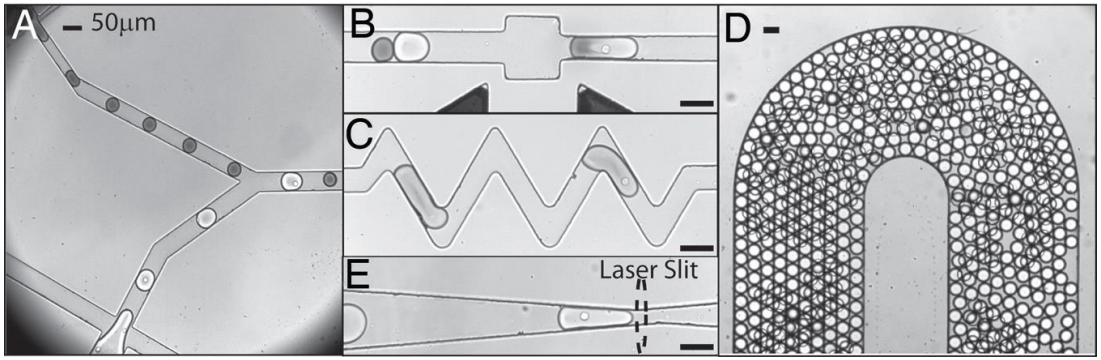
Edited by Noel A. Clark, University of Colorado, Boulder, CO, and approved June 2, 2009 (received for review March 31, 2009)





The viability assay chip integrated a series of 5 modules:

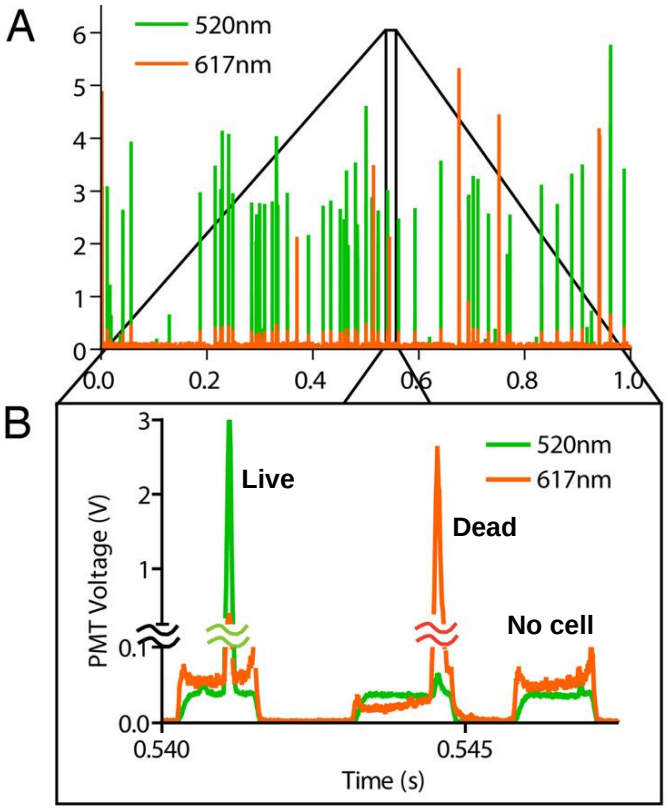
a) encapsulated cells and live/dead fluorescent dyes respectively. The fork enabled the interdigitation of the streams resulting in cell-containing droplets alternating with dye-containing droplets.

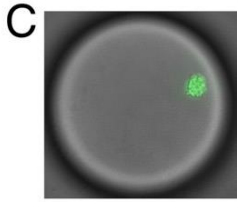
b) delivered a field permitted electrically-controlled merging of pairs of dye-containing droplets and cell-containing droplets.

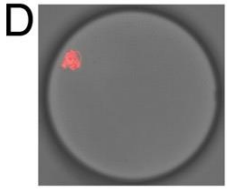
c) facilitated rapid and thorough mixing of cells with dyes.

d) optimized cell staining by enabling on-chip incubation of the droplet for 15 min in a delay line.

e) A detection module confined droplet laterally and vertically to collect the fluorescent signals excited with a laser slit . Live and dead cells were scored with Calcein-AM and Sytox Orange, respectively.







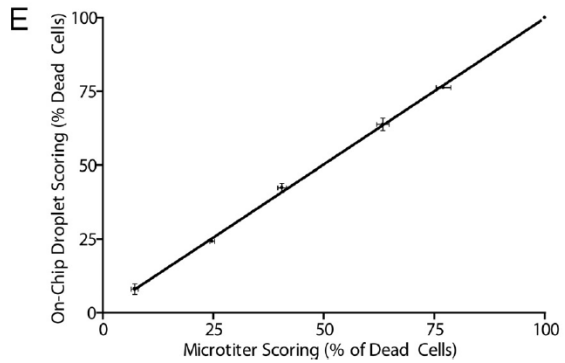
On-chip viability assay:

a) Typical 1 second raw signal trace collected by the photomultiplier tubes (PMT).

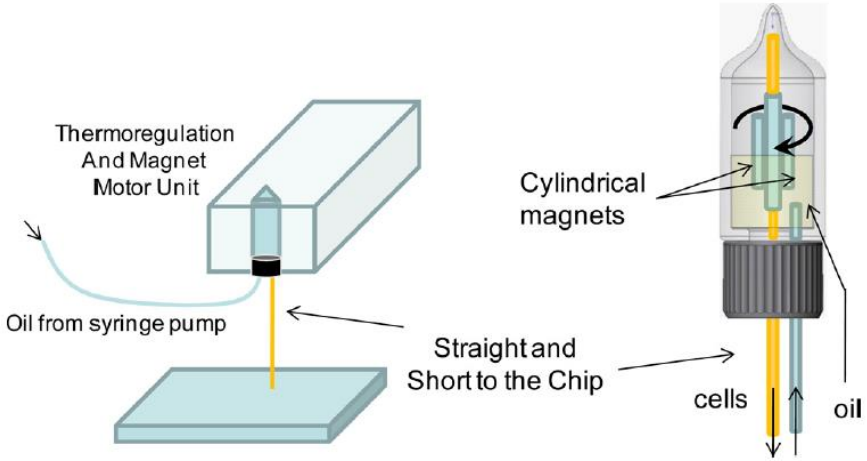
b) Individual droplet signals decompose into a plateau that corresponds to the homogeneous droplet signal overlaid by a narrow peak that corresponds to a cell signal. In this trace, we could see 3 droplets, containing a live cell, a dead cell, and no cell, respectively.

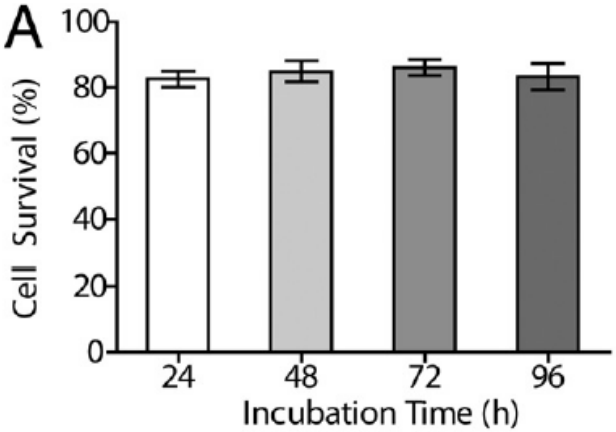
c) Images of a live cell and of a dead cell inside droplets.

e) Different mixtures of live and dead cells were scored, and the results were compared with control reactions performed in a microplate assay.



- ✓ High degree of specificity: less than 0.1% of the dead cells and 1% of the live cells showing double-staining with both live and dead cell stains.
- ✓ Sensitivity: more than 99% of dead cells and 98% of live cells injected into the chip were detected.

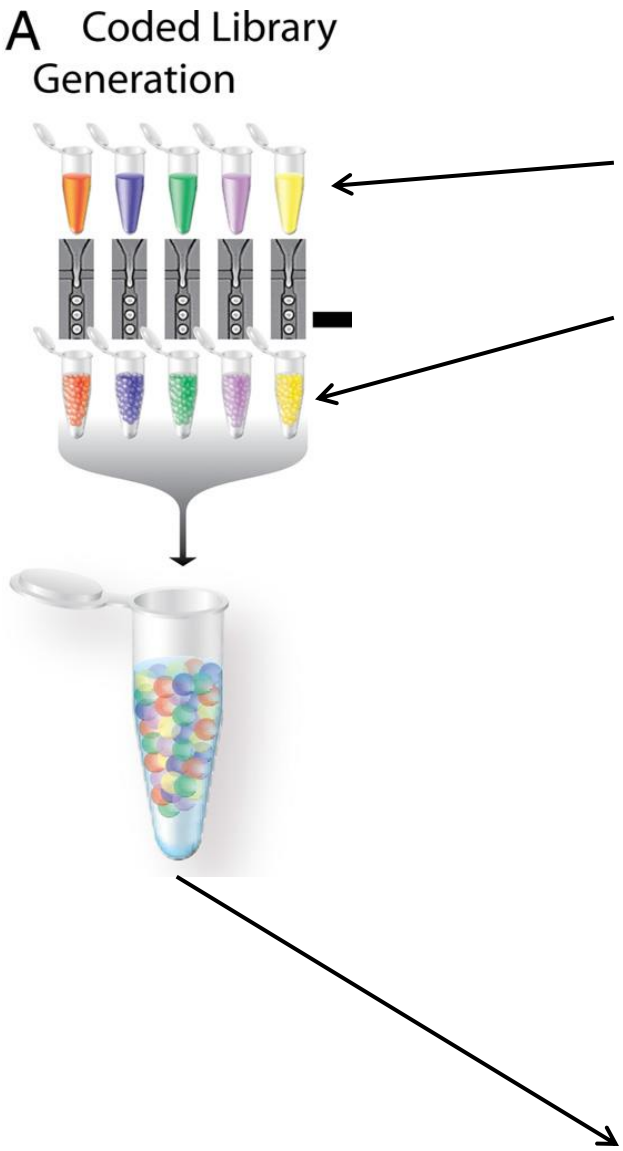




A magnetically stirred injection system that maintained cells in suspension before on-chip injection. Cells have been injected for more than 4 h using this system.

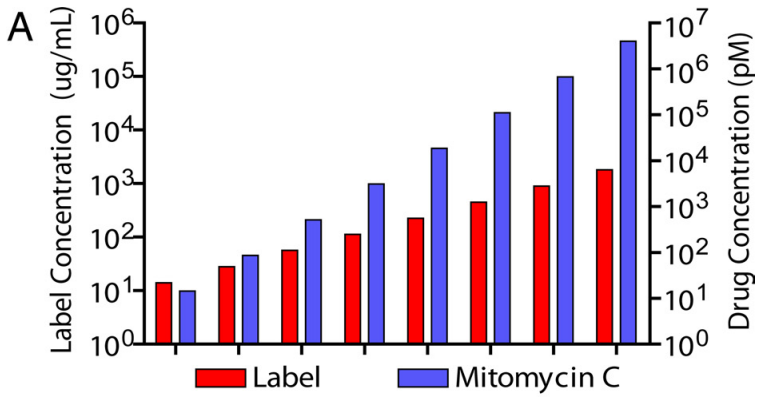
Off-chip incubation test (for drug screen):

- The cells were first encapsulated and collected into a syringe that was subsequently transferred to a conventional CO_2 incubator.
- The emulsion was then reinjected into the quantitative viability assay for scoring. Encapsulated cells maintained 80% survival up to 4 days post-encapsulation.



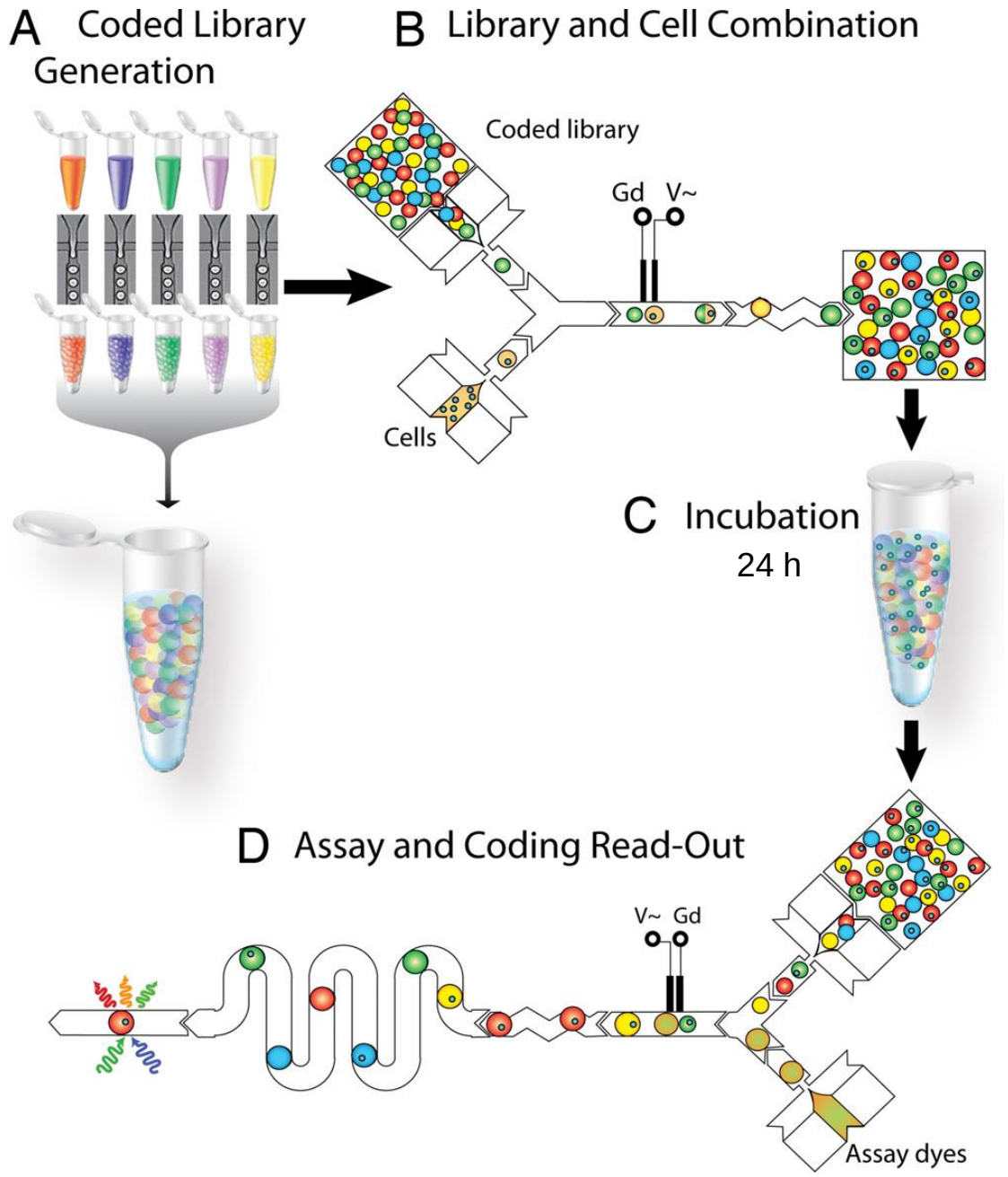
Mitomycin C Cytotoxicity Screen:

- Each mitomycin C drug concentration was labeled with a specific concentration of a soluble red dye fluorescent at 710nm (Alexa Fluor 680 R-phycoerythrin).
- Each drug-label combination was encapsulated into 200 pL droplets using the library generation chip.



The resulting 8-member library contained the mitomycin C drug diluted 6-fold stepwise and the coding dye diluted 2-fold stepwise.

- Tested this encoded drug library for its cytotoxic effects on human monocytic U937 cells.



Droplet screening workflow:

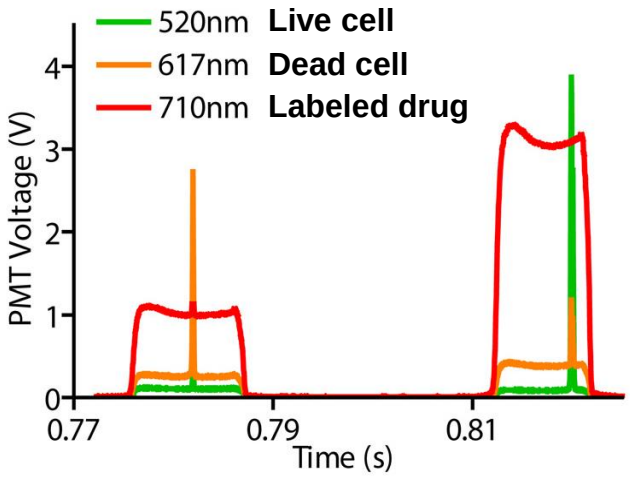
a) A reformatting step to emulsify compound-code pairs and pool them into a droplet library.

b) Merging each library member with one of the cell containing droplets that are continuously generated.

c) Off-chip Incubation.

d) Droplets are reinjected into the integrated viability assay chip for quantitative fluorescent based cytotoxicity and drug coding read-out.

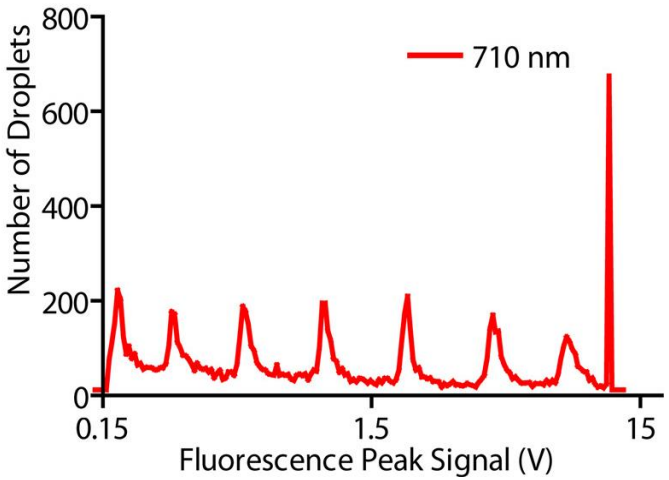
B

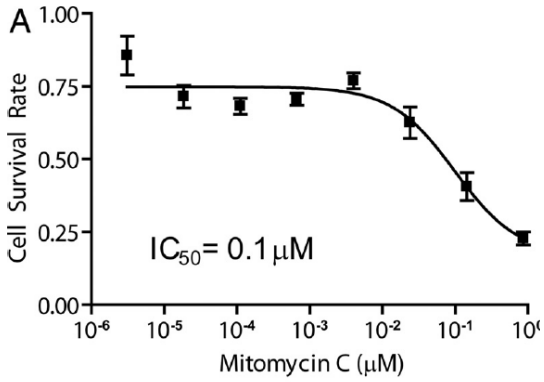


b) The coding signal is collected in 710nm detection channel, in addition to the signal traces of the viability stained cells in 520nm and 617nm detection channels.

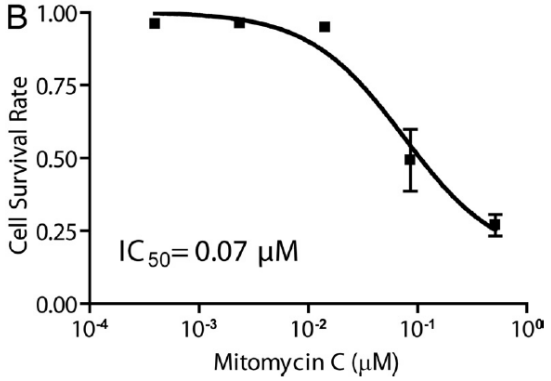
c) The histogram clearly shows that the 8 members of the library are optically resolved during the read-out after the 2 merges during the experiment. Each condition tested roughly equal numbers of cells (900 cells corresponding to 900 droplets per condition). (The signal of the highest concentration member was clipped because of PMT saturation.)

C





A dose-response curve by binning droplets based on their optical code.



Performed in a microliter plate.

Summary-1

1) developed a droplet viability assay that permits the quantitative scoring of cell viability and growth within intact droplets.
2) demonstrated the high viability of encapsulated human monocytic U937 cells over a period of 4 days off-chip incubation.
3) developed an optically-coded droplet library enabling the identification of the droplets composition during the assay read-out.
4) screened a drug library for its cytotoxic effect against U937 cells.

analytical chemistry

Article

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Ultrahigh-Throughput Mammalian Single-Cell Reverse-Transcriptase Polymerase Chain Reaction in Microfluidic Drops

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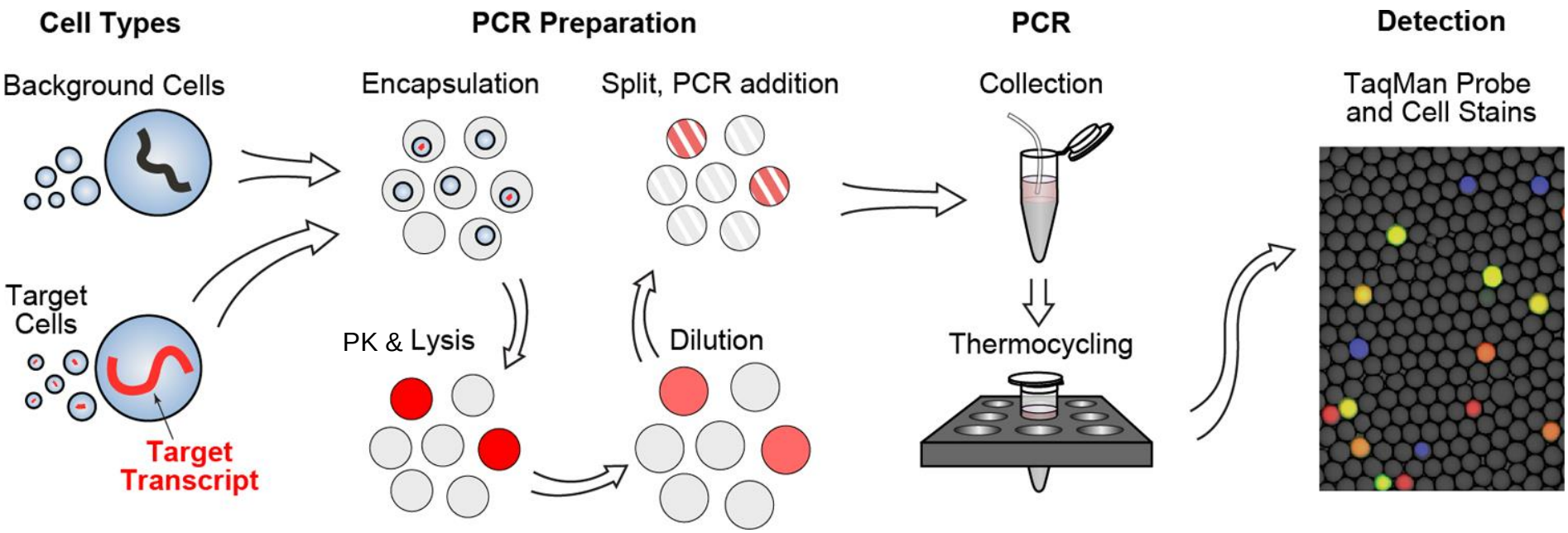
Scientific questions:

- Cellular heterogeneity and its impact on biological function and disease is becoming increasingly important for questions in human immunology, stem cell biology, and cancer research.
- RT-PCR is a widely used method that enables transcriptional profiling and sequencing analysis on bulk populations of cells.
- The ultrahigh-throughput capability of droplet-based microfluidics is ideal for single-cell analysis applications. But a barrier to realizing the potential of this approach is that, at concentrations of a single mammalian cell in a microdroplet, cell lysate is a potent inhibitor of RT-PCR.
- Methods using large droplets (2 nL) have only been able to analyze ~100 cells in total.

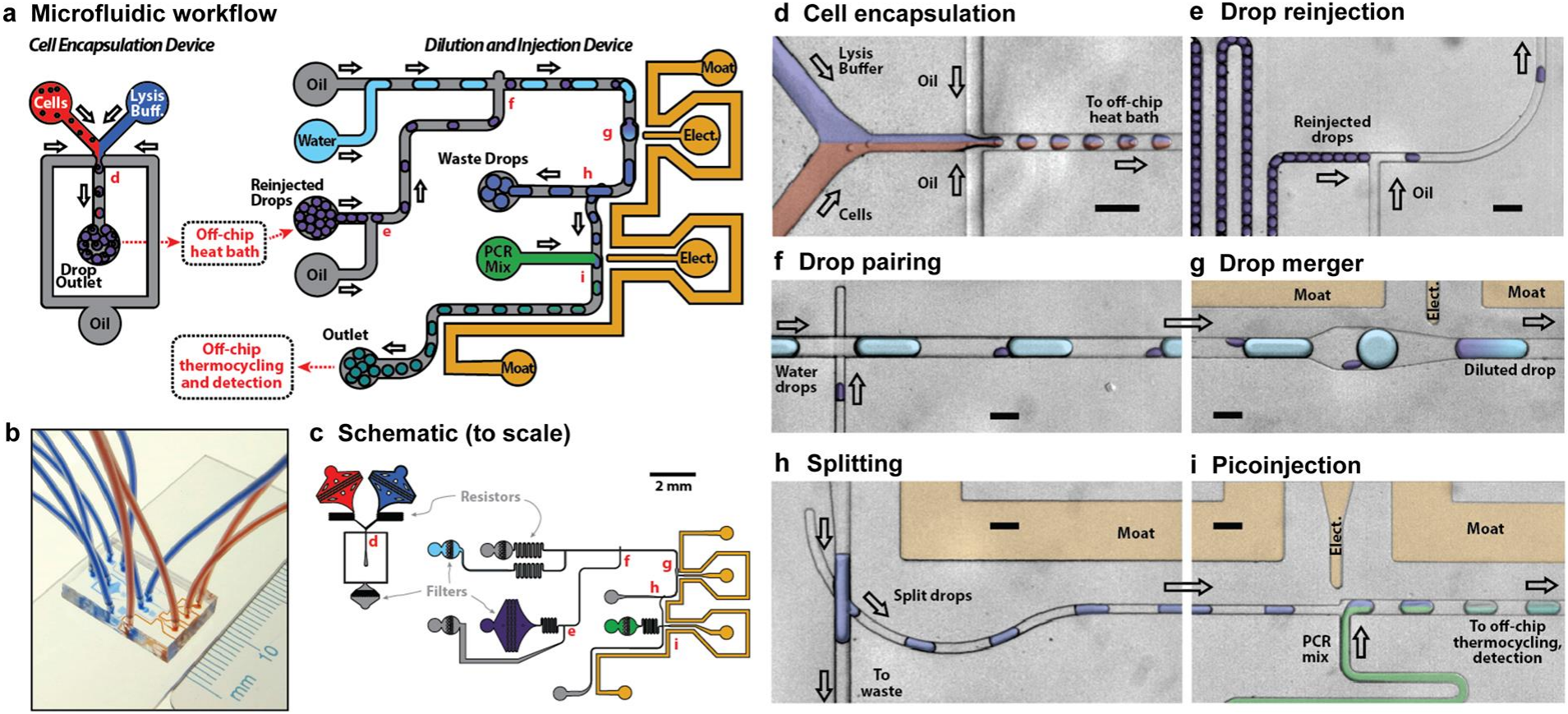
➢ To overcome cell lysate-mediated inhibition of RT-PCR in microdroplets, a new microfluidic workflow was developed.

Strategy:



A mixed population of human cells labelled with dyes:

- Raji cells: specific express PTPRC (protein tyrosine phosphatase, receptor type, C).
- PC3 prostate cancer cells: do not express PTPRC.



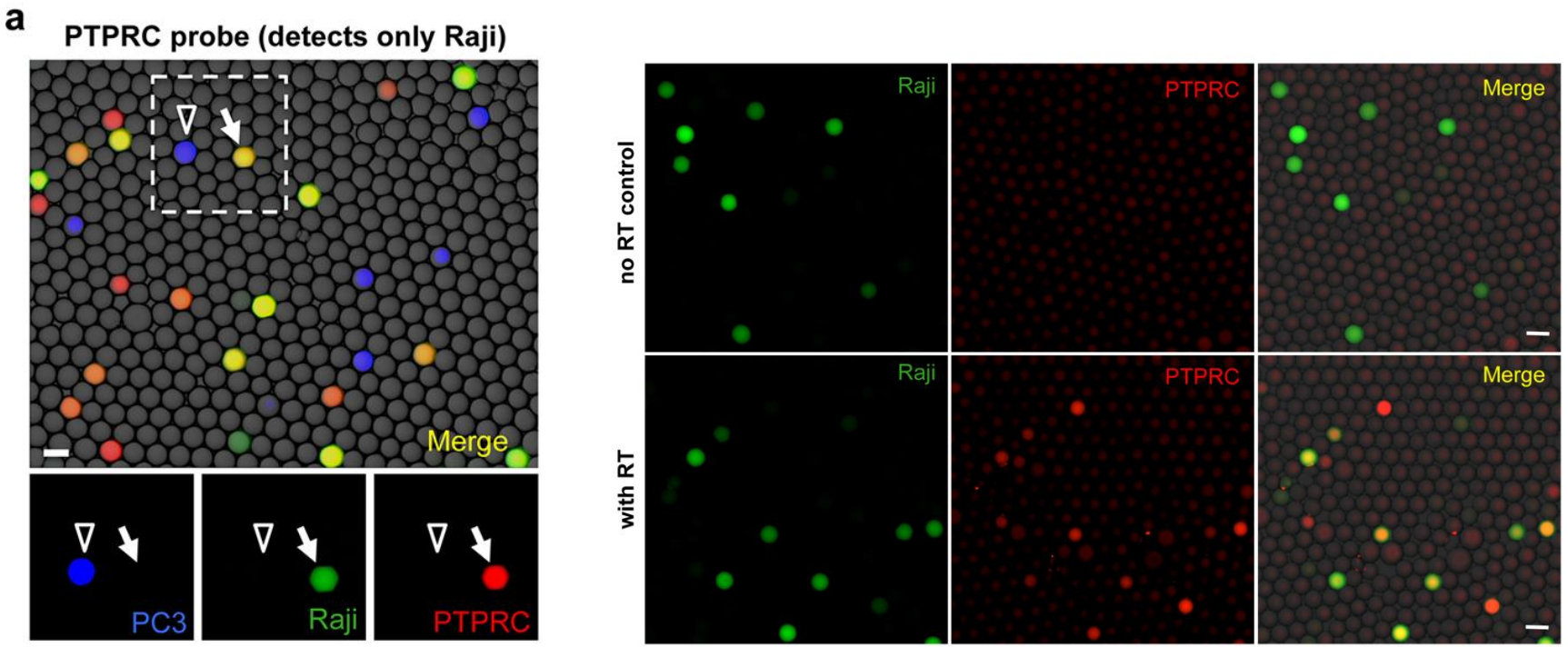
d) Cell suspension is coflowed into a microfluidic drop maker with lysis buffer containing proteinase K. The cells are encapsulated at limiting dilution such that most drops are empty but ~1−5% contain single cells, in a process governed by Poisson statistics. Following lysis and proteinase K digestion off-chip, PK inactivation in a heat bath.

e) The lysate-containing drops are introduced into a second device, f) paired with a large water drop,

g) then merged with an electric field for an ~20-fold dilution.

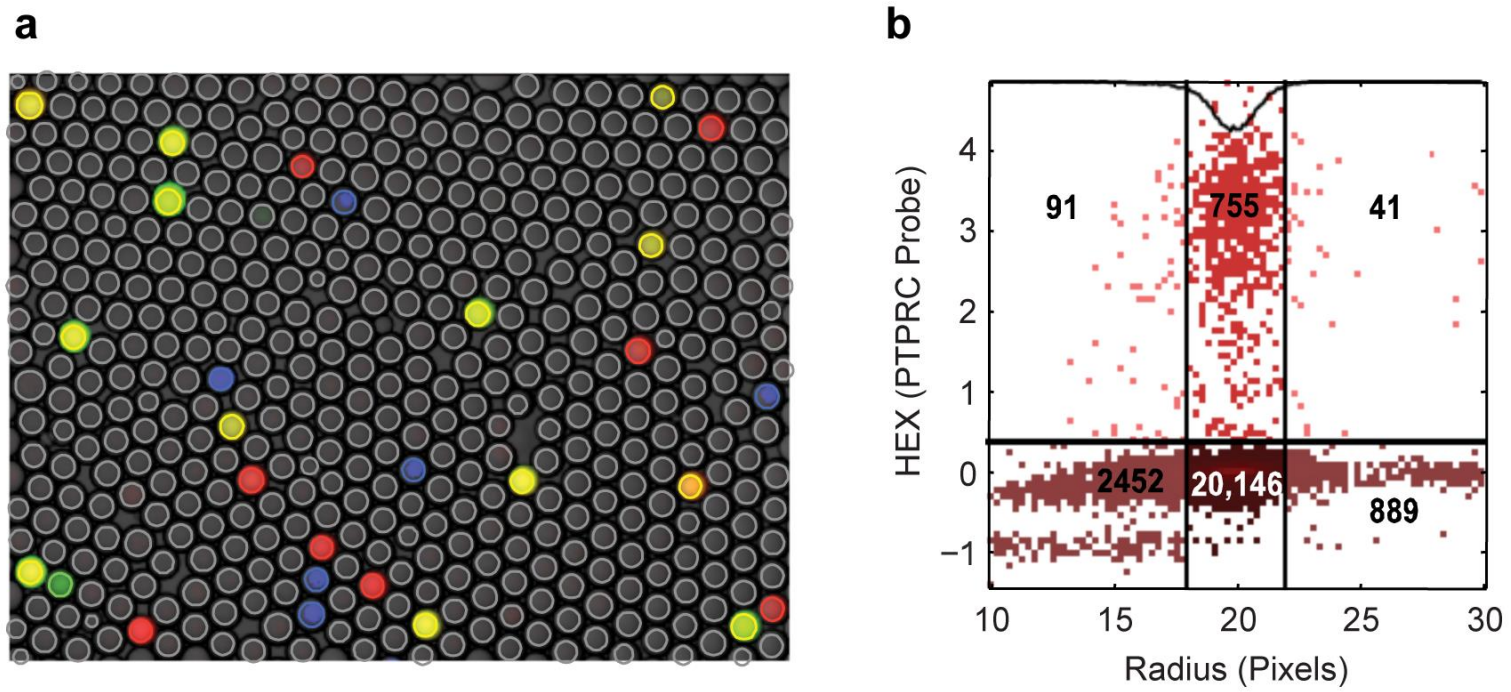
h) After in-droplet mixing, a portion of the diluted lysate is split off,

i) RT-PCR reagents are added with picoinjection via a second electric field.



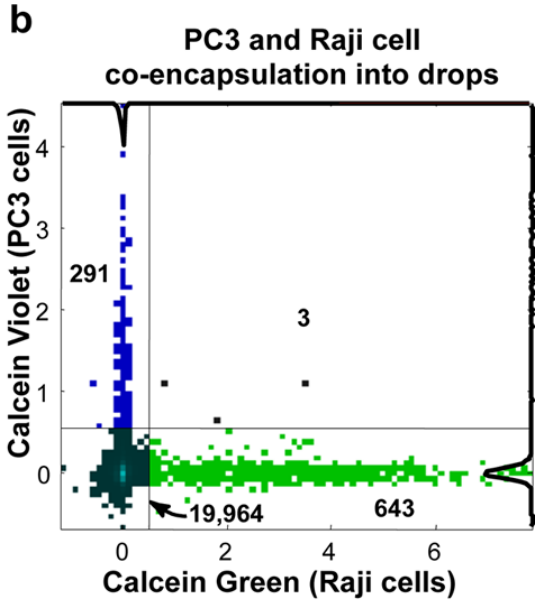
Merged bright-field and three-fluorescence microscopy images: 10 µL of thermocycled droplets was pipetted into Countess chambered coverglass slides. The slides were imaged on a Nikon Eclipse Ti.

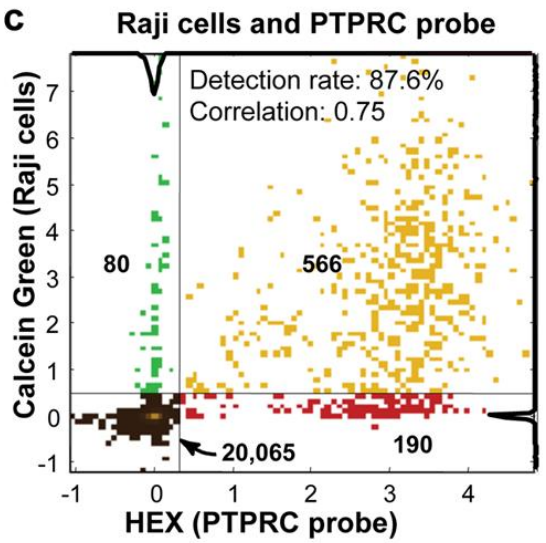
- Before encapsulation in drops, Raji cells were labeled with calcein green viable cell dye and PC3 prostate cancer cells were labelled with calcein violet dye.
- Raji cell lysate frequently displayed the presence of PTPRC probe HEX fluorescence.

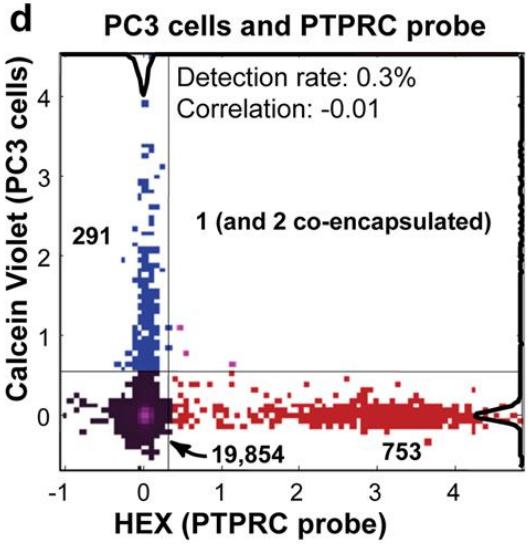


a) Computer-determined drop locations and binary fluorescence values (colored circle outlines) overlayed on top of **merged bright-field and three-fluorescence microscopy images** (solid background). The outline colors identify drops as having no cell lysate or probe fluorescence (gray), or containing PC3 lysate (blue), Raji lysate (green), PTPRC probe (red), or Raji lysate and PTPRC probe (yellow).

b) A plot of the PTPRC probe fluorescence signal as a function of drop-size. They excluded drops from analysis that were outside the central size range marked by the two vertical lines.



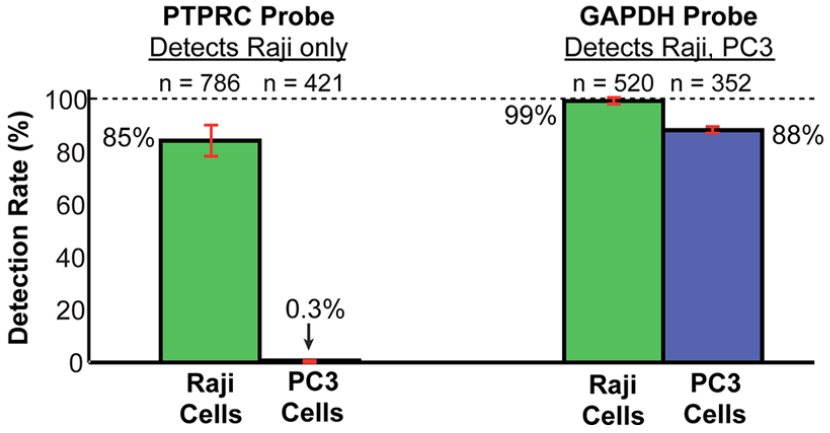




566/(566+80) = 87.6%

1/(1+291) = 0.3%

e

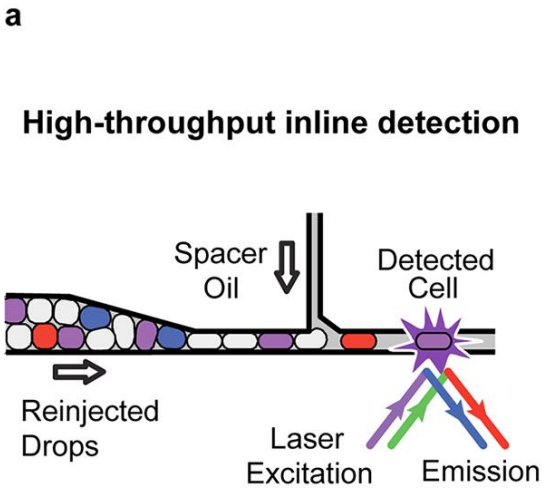


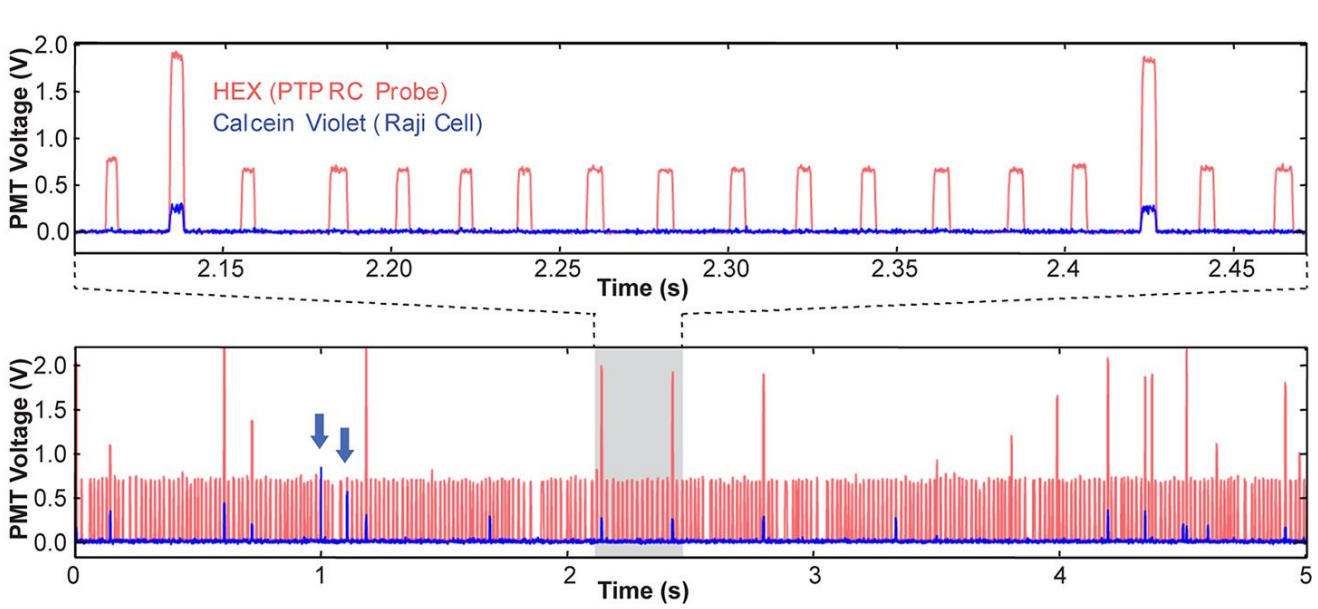
b) Raji and PC3 coencapsulation is rare.

c) PTPRC probe is seen in 87.6% of drops with Raji cell lysate,

d) PTPRC probe is seen in only 0.3% of drops with PC3 cell lysate, excluding the two drops that show Raji and PC3 coencapsulation.

e) Detection rates of Raji or PC3 cells are shown for PTPRC and GAPDH TaqMan probes.

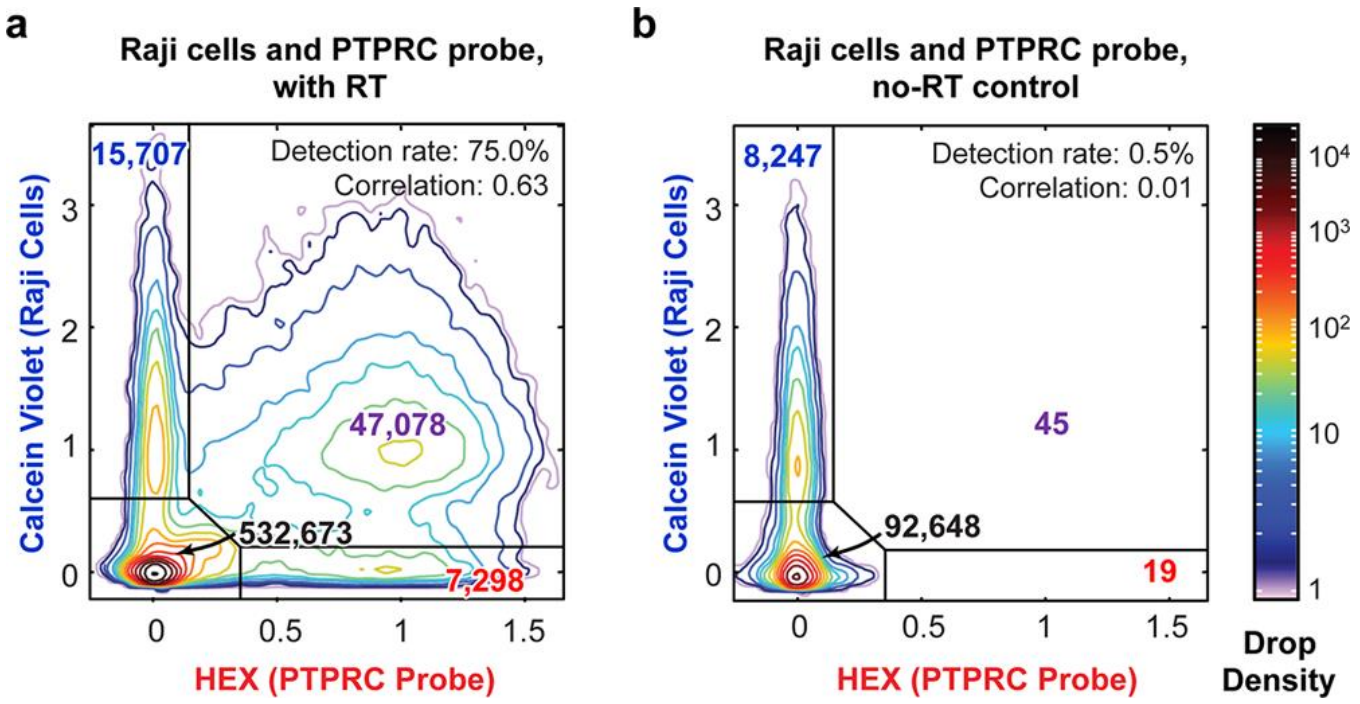




Due to the number of drops generated by the microfluidic system (~300'000 drops/h), it is not possible to image all drops with fluorescence microscopy. So, they built a high-throughput fluorescence-based droplet inline detector that excites fluorescent dyes in the drops with lasers and records the resultant emitted light with photomultiplier tubes (MPT).

b) Fluorescence drop signals are recorded as peaks in PMT voltage versus time. Raji positive drop fluorescence (blue peaks) correlates with PTPRC probe fluorescence (red peaks).

c) A longer time series, containing (b) in the gray region, indicating the data quality. Blue arrows show examples of false negatives demonstrating the relative rarity of these events.



47708/(47708+15707) = 75%

45/(45+8247) = 0.5%

Fluorescence data acquired with the ultrahigh-throughput detector was plotted using a heat map.

a) 47’078 Raji cell lysate drops, also have a PTPRC probe signal following single cell RT-PCR in the presence of reverse transcriptase.

b) No-RT control drops show minimal PTPRC probe fluorescence.

Summary-2

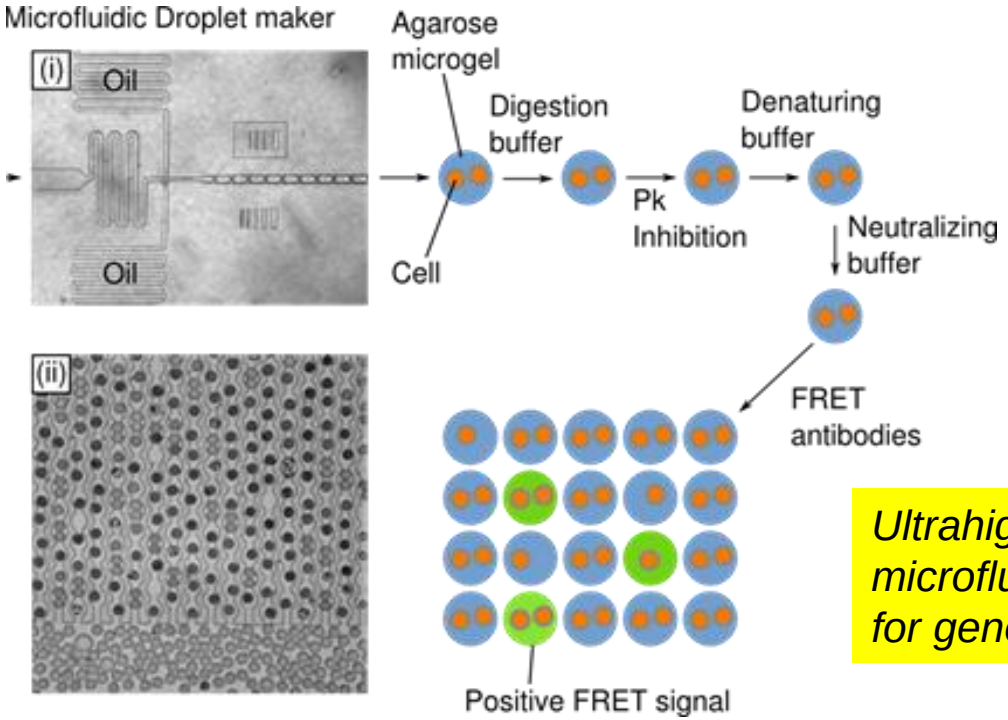
1) described a novel droplet-based microfluidic system for performing ~50’000 single-cell RT-PCR reactions in a single experiment.
2) demonstrated the identification of specific cells from a mixed human cell population, using cell type-specific staining and TaqMan RT-PCR probes.
3) this method was well-suited for studying large, heterogeneous populations of cells at the transcriptional level.

Conclusions

- Droplet-based microfluidics is one of the most powerful approaches to studying the functional behavior of single cells, especially to associate secreted molecules.
- The possibility of manipulating cell-containing droplets by sorting, splitting and fusion will also enable scientists to study how cellular heterogeneity affects cell–cell communication and cellular decision making.
- Conventional approaches to studying evolution can be obscured by populations of cells having mutations that rapidly overgrow less rapidly dividing cell populations, a problem easily solved by single-cell encapsulation.
- Droplet microfluidics leads to a huge reduction in the time and labor requirements.

Outlook

- One of the major obstacles: the inability to perform washing steps inside droplets, which is crucial for many biological protocols. To improve it:
  - use low-melting-temperature agarose and hydrogels.
  - develop new and smart biosensors such as aptamers or Förster Resonance Energy Transfer (FRET)-based sensors that will circumvent the need to wash droplets and will allow for the dynamic monitoring of responses over time.



Ultrahigh-throughput cell-based microfluidic prion protein assay for genome wide screenings ?

Thank you !