Nanobiotechnology and Biophysics

Single cell analysis

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Outline of the lecture

Part I:

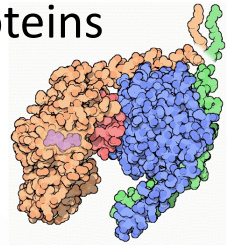
- Motivation: Why do we need single cell anaylsis and what are the requirements?
- Flow cytometry (non-invasive analysis)

Part II:

- Microfluidic devices for chemical analysis based on:
  - Nanowells
  - Valves
  - Droplets

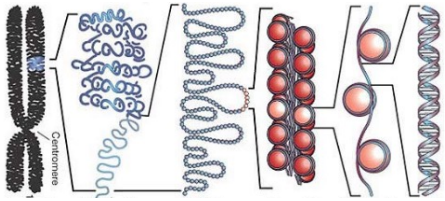
Analytes of interest in a single cell

Proteins

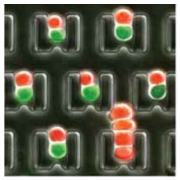


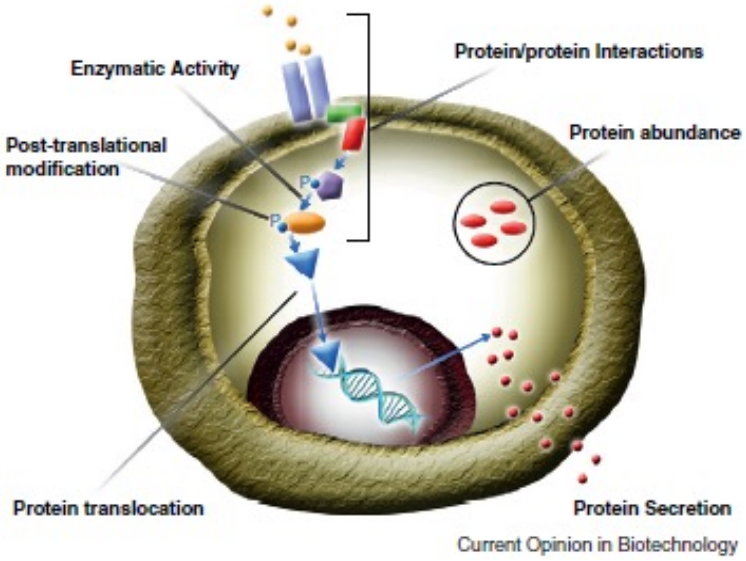
RNA

Genome and Epigenome

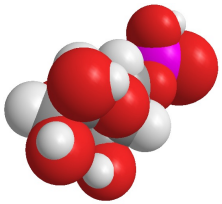


Manipulation

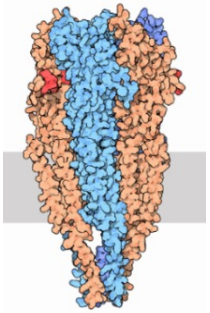




Metabolites



Signaling



Proteins:

quantification of protein levels, presence of rare / mutant proteins

RNA:

expression levels of proteins

Genome/Epigenome:

mutations, genetic and epigenetic defects, clonal origin of cells

Signaling:

presence of particular receptors, activity of distinct signaling pathways

Metabolites:

levels of metabolites, activity of the cell

all these single-cell analysis methods depend on **efficient manipulation methods**

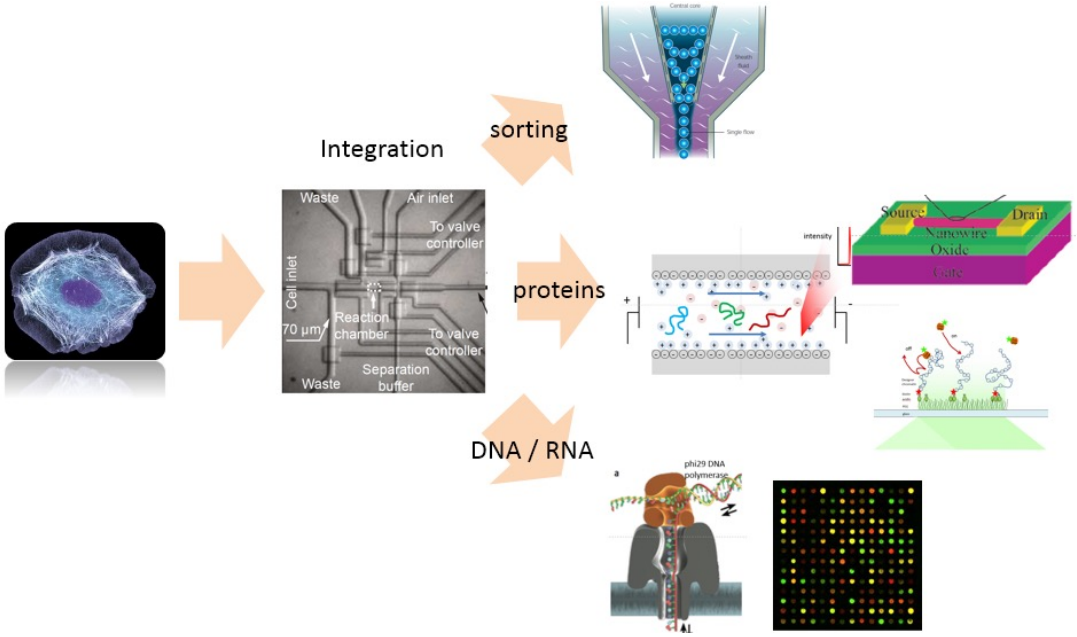
Motivations – Questions to be answered

- **Infection**: Why are some bacteria resistant to certain antibiotics? What is different in those cells?
- **Cancer**: Why are some cells resistant to chemotherapeutics? Is there heterogeneity in a tumor sample? Which cells are cancer stem cells?
- **Development:** Which cells are stem cells? Which daughter cells arose from a particular mother cell?

- **Microbial communities**: identification of species that cannot be cultured

Microfluidic Platforms for Single-Cell Analysis

- **Single cell biology:**
  - Flow cytometry
  - Surface analysis
  - Culturing & experimenting with single, living cells
- **Chemical analysis of single cells:**
  - Breakage of a cell and extraction/identification of its contents
  - Multicomponent analysis
  - Single cell version of biochemical assay



Cell manipulation: Flow cytometry

Aims:

- Counting / Quantifiying cells according to predefined markers
- Sorting of cells according to given characteristics

Procedure:

- Labeling
- Cells are suspended in a stream of fluid
- Production of a single-cell wide, focused stream of cells that can be analyzed
- Analysis using a detection apparatus

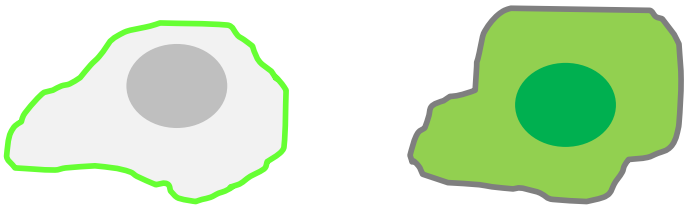
Methods: Flow Cytometry

Power of flow cytometry:

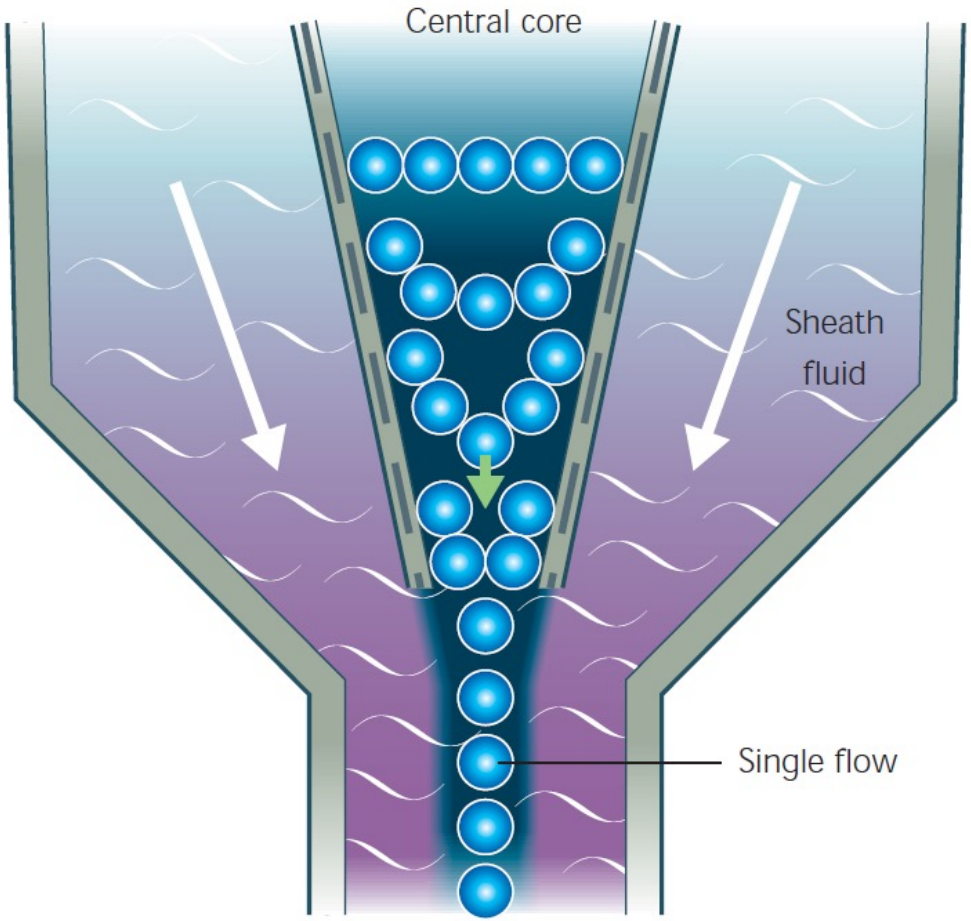
- Cells are kept intact → proteins are kept at high concentration
- Today: measurement up to 15 fluroescent species in a cell → multiparameter analysis
- Cell barcoding for HT drug screening: 3 dyes in different intensity levels (343 variations)

Labeling approaches:

- staining of surface proteins using antibodies coupled to fluorescent markers (fluorophores, quantum dots)
- chemical surface labeling (SNAP-tag, click-chemistry)
- expression of fluorescent proteins (GFPs, RFPs) fused to reporter genes



Flow cytometry, principles



M. Rahman et al., www.adbserotec.com

nozzle: 70 µm

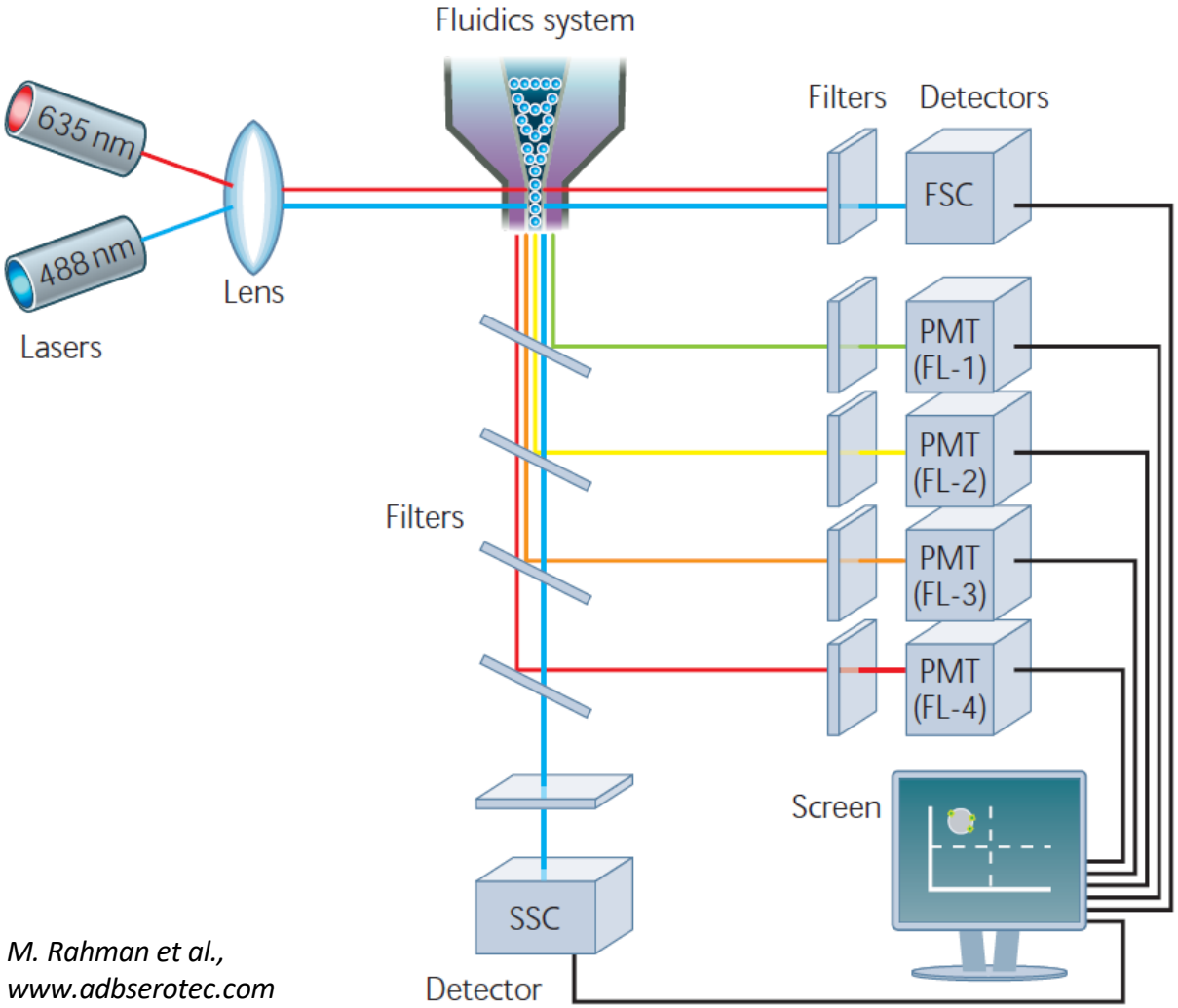
$$Re = \frac{\rho u L}{\mu}$$

ρ: density
u: velocity
μ: viscosity
L: characteristic length

At low Re (laminar flow):

- no mixing
- sheat fluid has greater velocity than particle solution
- hydrodynamic focusing of particle (cell) stream

Flow cytometry, detection



M. Rahman et al., www.adbserotec.com

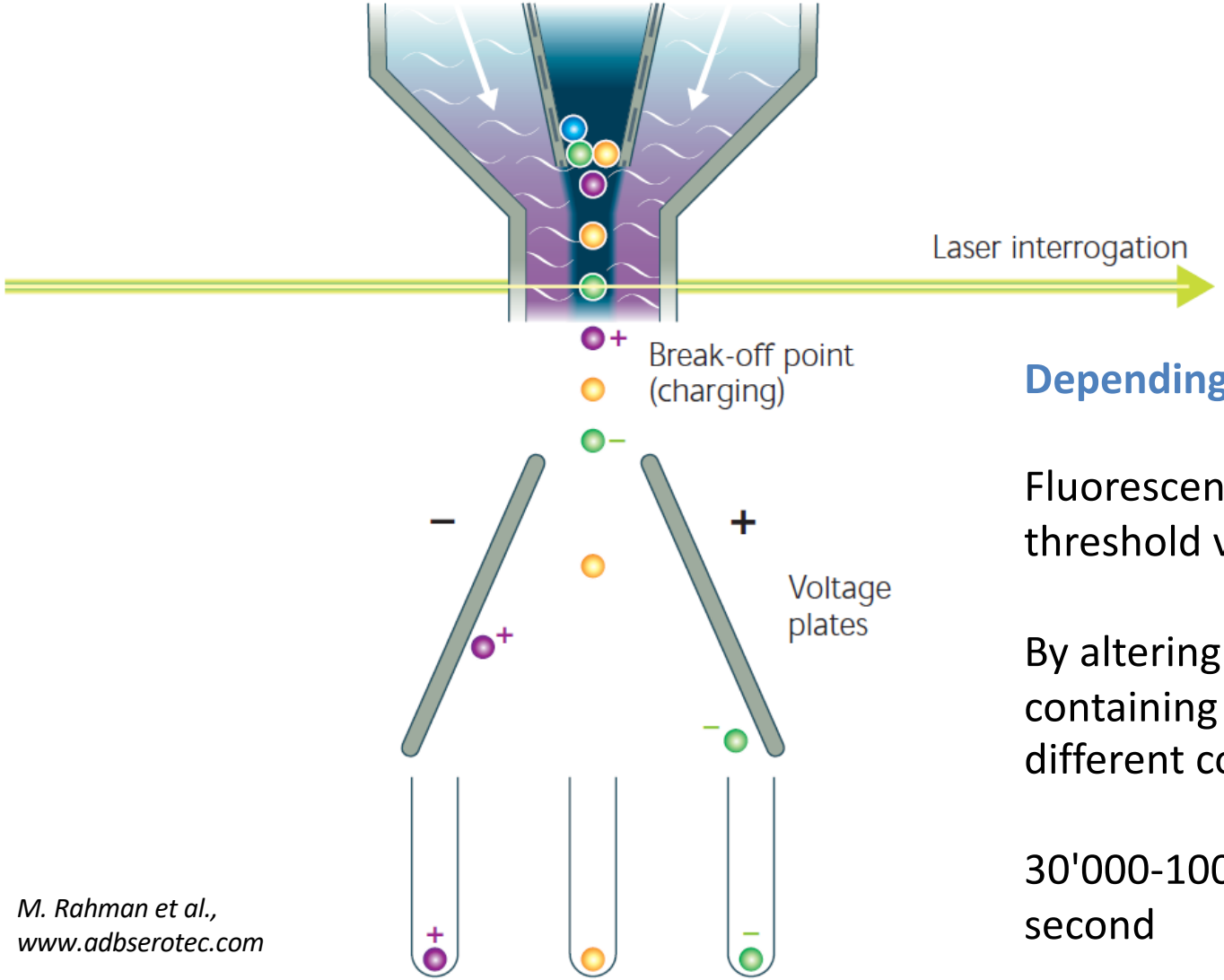
FSC: Forward scattering
→ size of particles

SSC: Side scattering
→ granularity of particles

Labeling of the cells using fluorescent antibodies

Multiparameter measurements using several dyes/wavelengths

Fluorescence-activated cell sorting FACS



M. Rahman et al.,
www.adbserotec.com

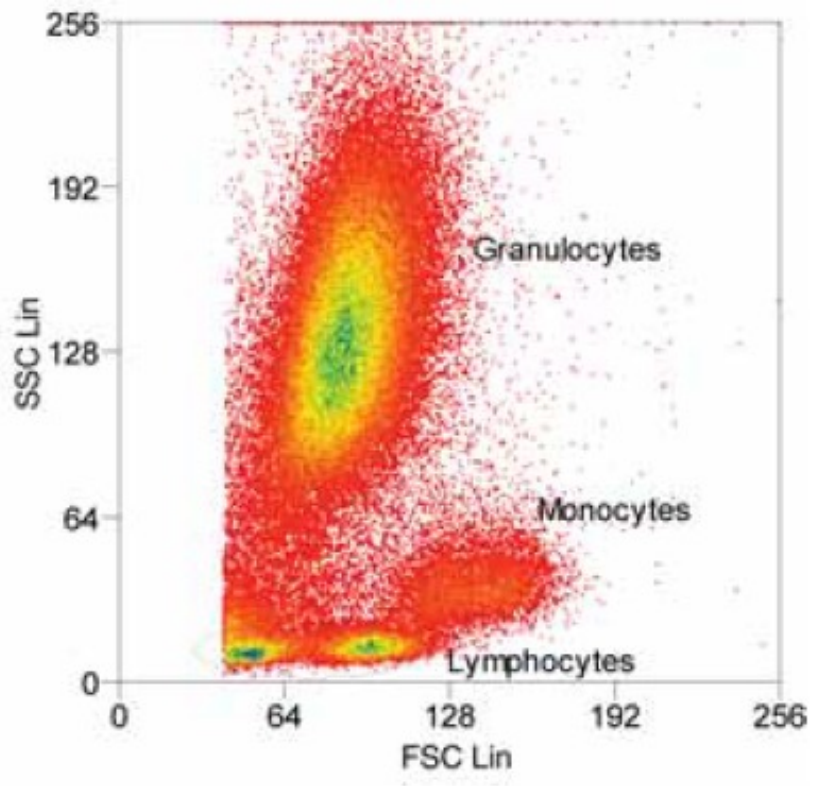
Depending on sorting criteria:

Fluorescence signal detects threshold values

By altering charge of nozzle, droplet containing cells is directed into different collection wells

30'000-100'000 droplets per second

Data analysis: Multiparameter measurements

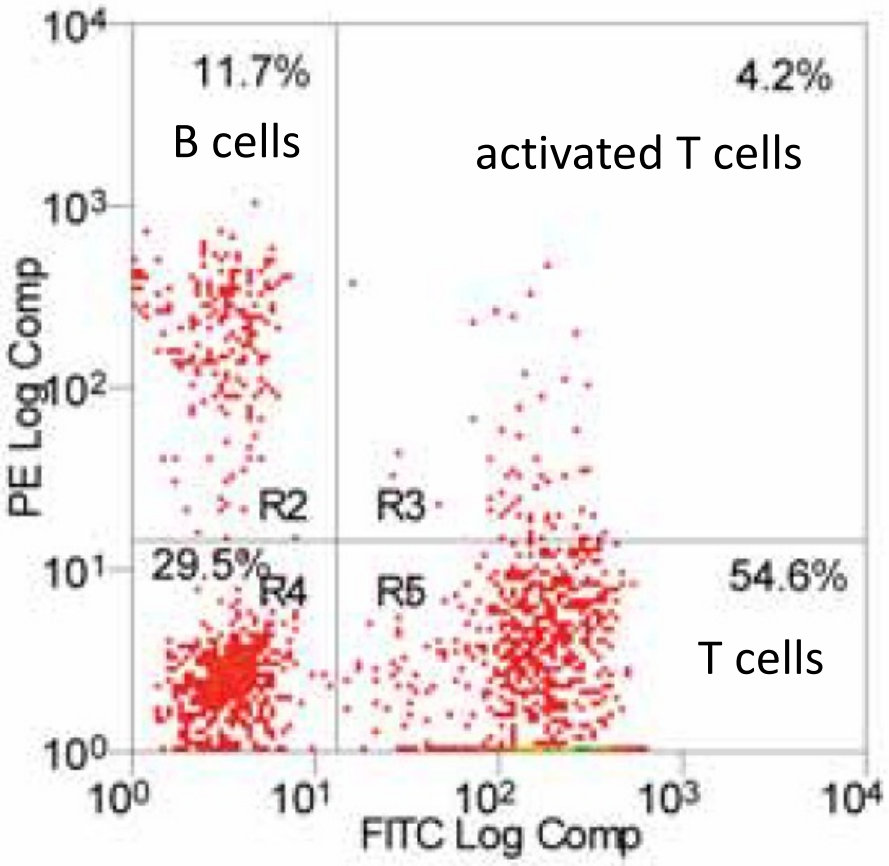


Whole blood analysis based on scattering information

Sub-cellular debris is sorted out (small forward scattering)

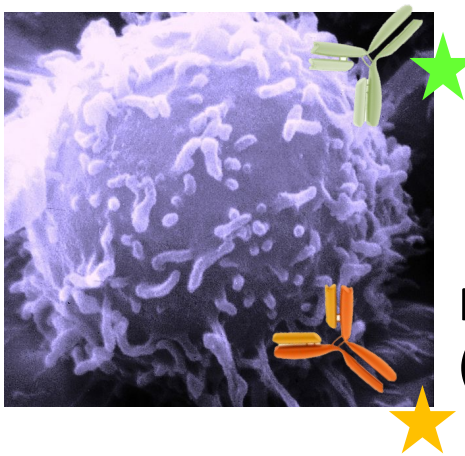
M. Rahman et al.,
www.adbserotec.com

Fluorescence analysis



M. Rahman et al., www.adbserotec.com

Leukocyte sorting



FITC-α-CD3 *marker for T cells*

PE-α-HLA-DR (MHC II) *marker for B cells*

Live cell sorting based on surface receptors

Multiparameter measurements: up to 15 parameters can be detected simultaneously (this is done **using tandem dyes**)

Intracellular proteins: cell fixation required for staining

Summary: FACS

FACS technology enables the isolation of specific cell populations from heterogeneous samples

- By utilizing fluorescently labelled antibodies or dyes to target specific cell surface markers.
- Through the use of high-speed fluidics and laser systems to identify and sort cells based on their fluorescence properties.
- By employing electrostatic deflection to physically separate cells based on their charge and size.

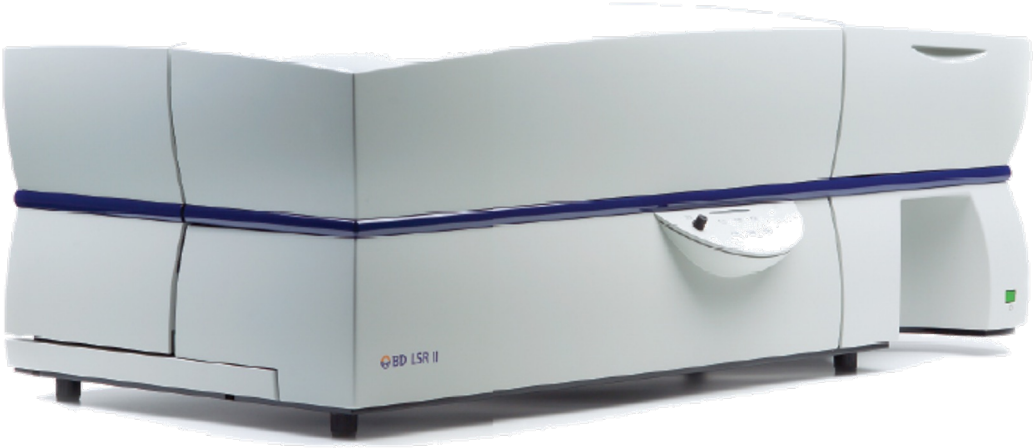
FACS has revolutionized research in fields such as immunology, stem cell biology, and cancer research

- Through the isolation of rare cell populations, facilitating in-depth studies of cell heterogeneity and functional characterization.
- Advancing personalized medicine by enabling the sorting of patient-derived cells for diagnostics, treatment monitoring, and therapeutic applications such as cell-based therapies.

Microfluidic Flow Cytometry

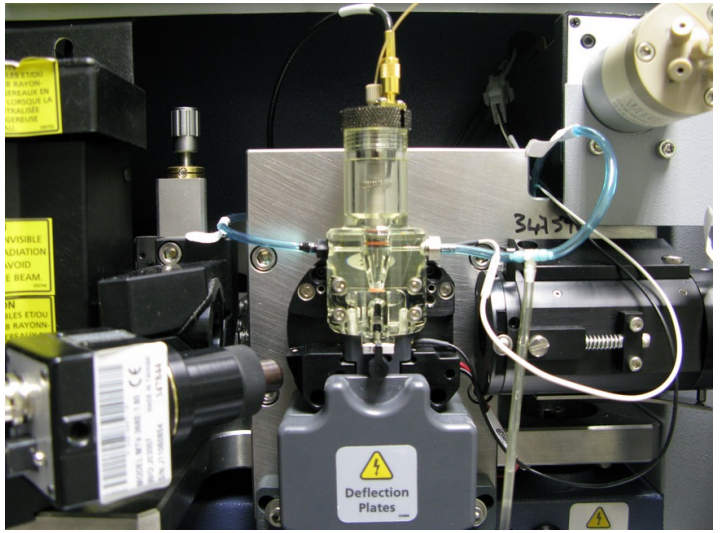
Conventional flow cytometry:

- complex machinery, expensive
- Requires experimental preparation of cell samples
- Large number of cells required



Microfluidics

- Cell sized channels
- Small cell numbers can be handled (e.g. from biopsy, 100-1000 cells)
- Sample preparation can be integrated

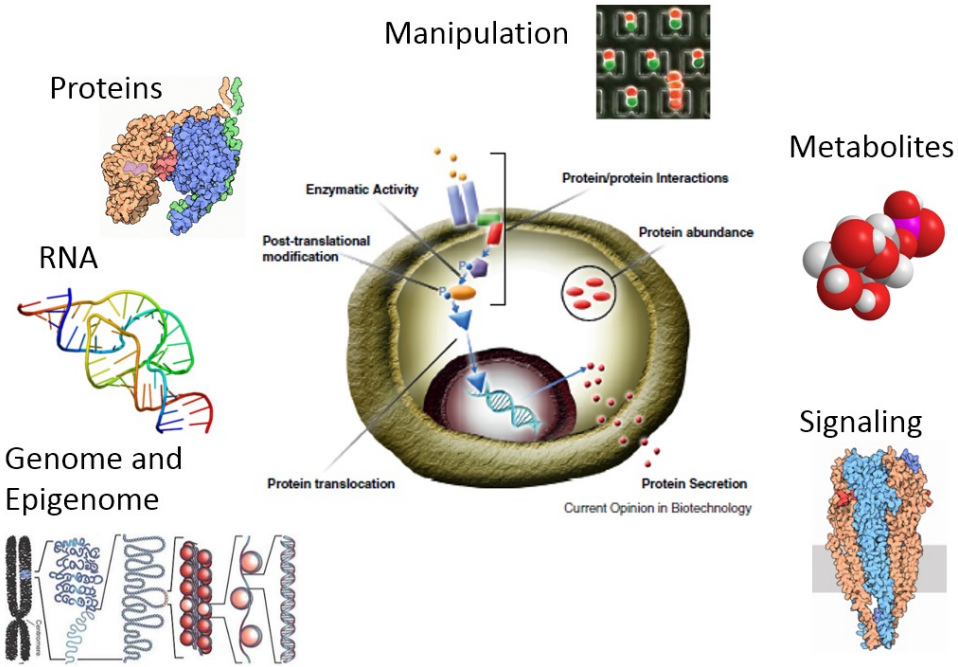


Chemical cytometry of single cells

Chemical analysis of contents of the cell

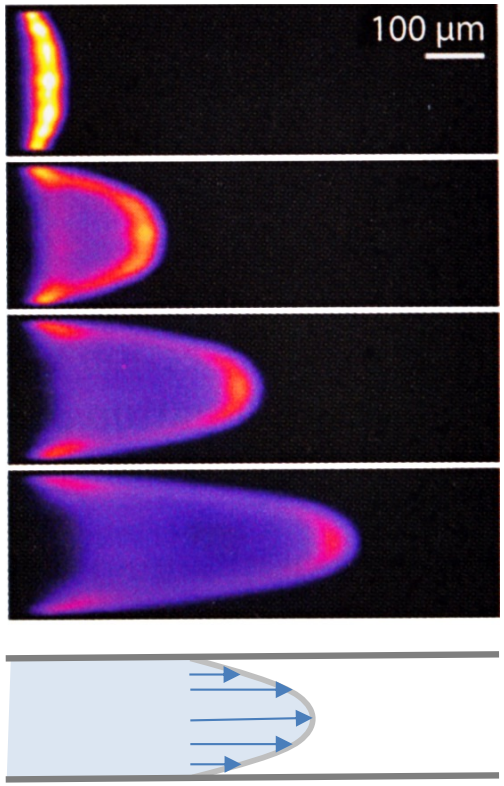
instead of solely analyzing surface features, gaining insight into the complete chemical composition of a single cell (genome, RNA, proteins, metabolites)

- **Microfluidics:**
  - Processing of single cells
  - Very small sample volumes
  - Little dilution, thus low detection limits
  - Accurate liquid handling, reproducibility
- **Requirements:**
  - Accurate microvalves, picopipettes
  - Integrated separation methodologies
  - Integrated analysis



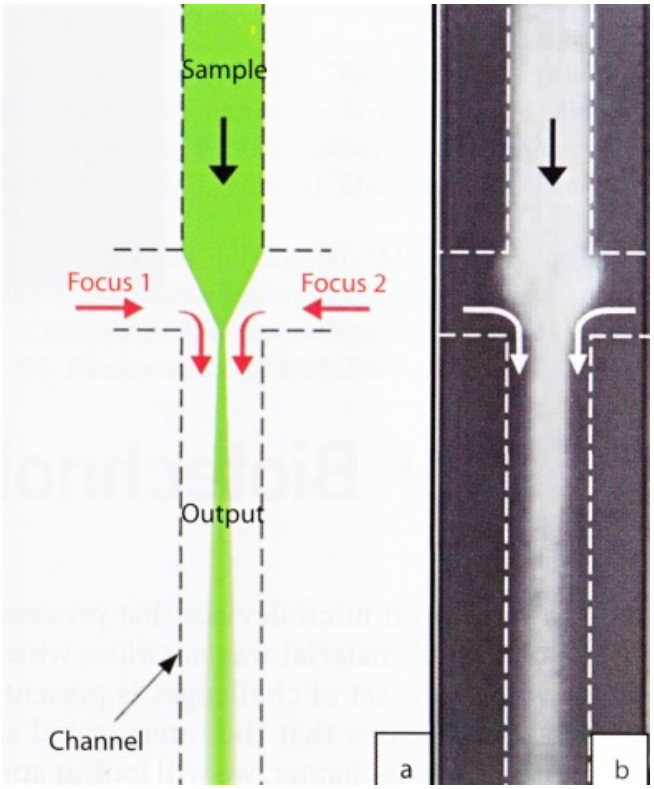
Single cell stream in microchannels

Laminar flow:



Velocity distribution

Focusing by sheath flow

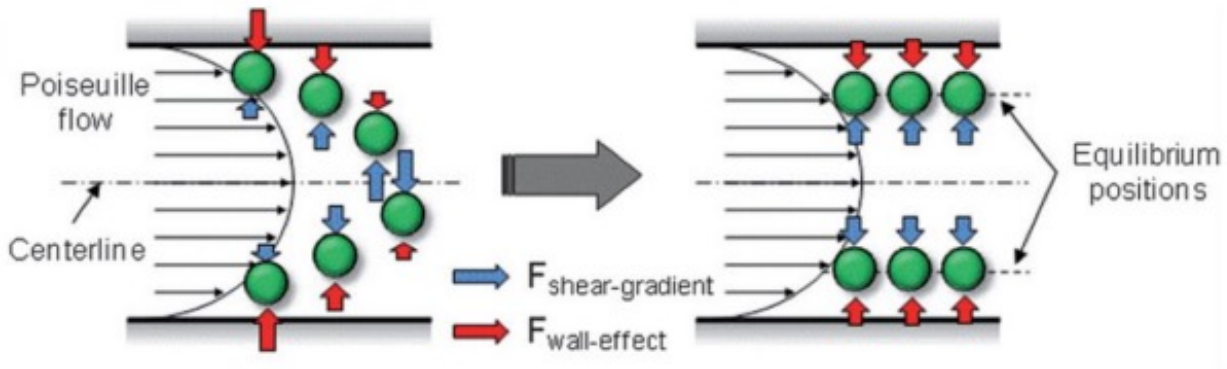


Folch, Introduction to bioMEMS, CRC press

Integrating **the nozzle** of a macroscopic flow cytometer within a microfluidic system

Requirement:
Form a focused stream of cells, delimited by either channel walls or sheet flow

Massively parallel cytometer

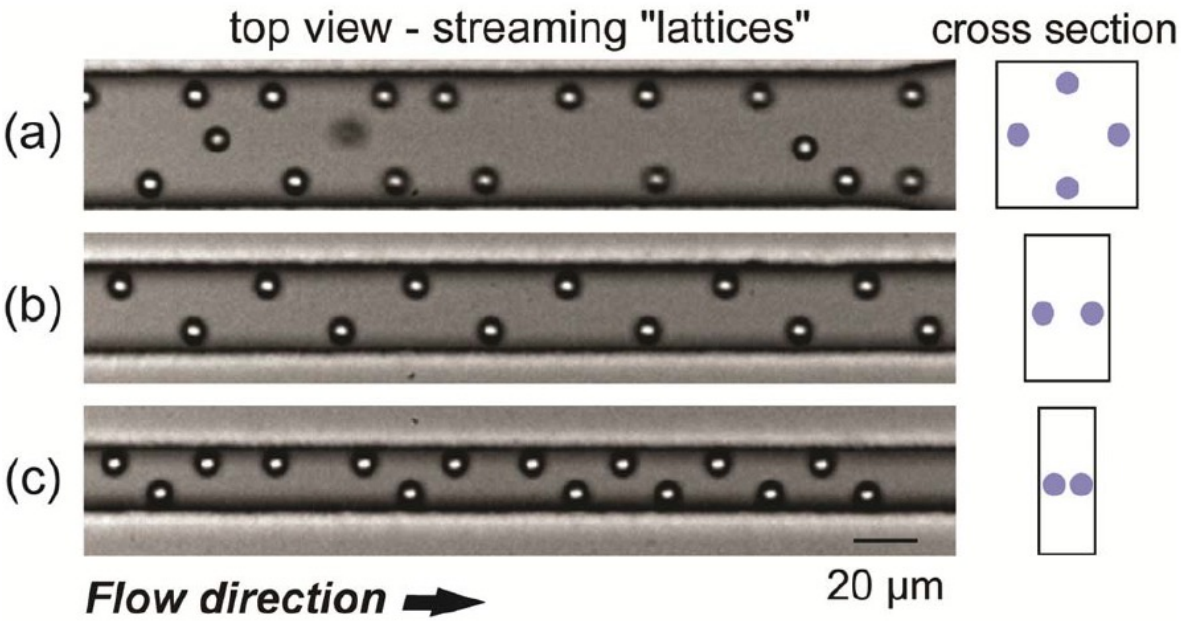


$$F_{SG} = \pi \rho_f R_H^{\,3}\, \vec{\omega} \times (\vec{U}_p - \vec{U}_f),$$

$$\omega = \partial u / \partial y \approx U_f / d,$$

$$F_{WE} = 9.22 \left(36 \frac{U^2}{d^2} \right) \rho_f R_H^{\,4}$$

ρ: density of fluid
R_H: radius of the particle
U: average velocities of fluid and particle
d: channel depth



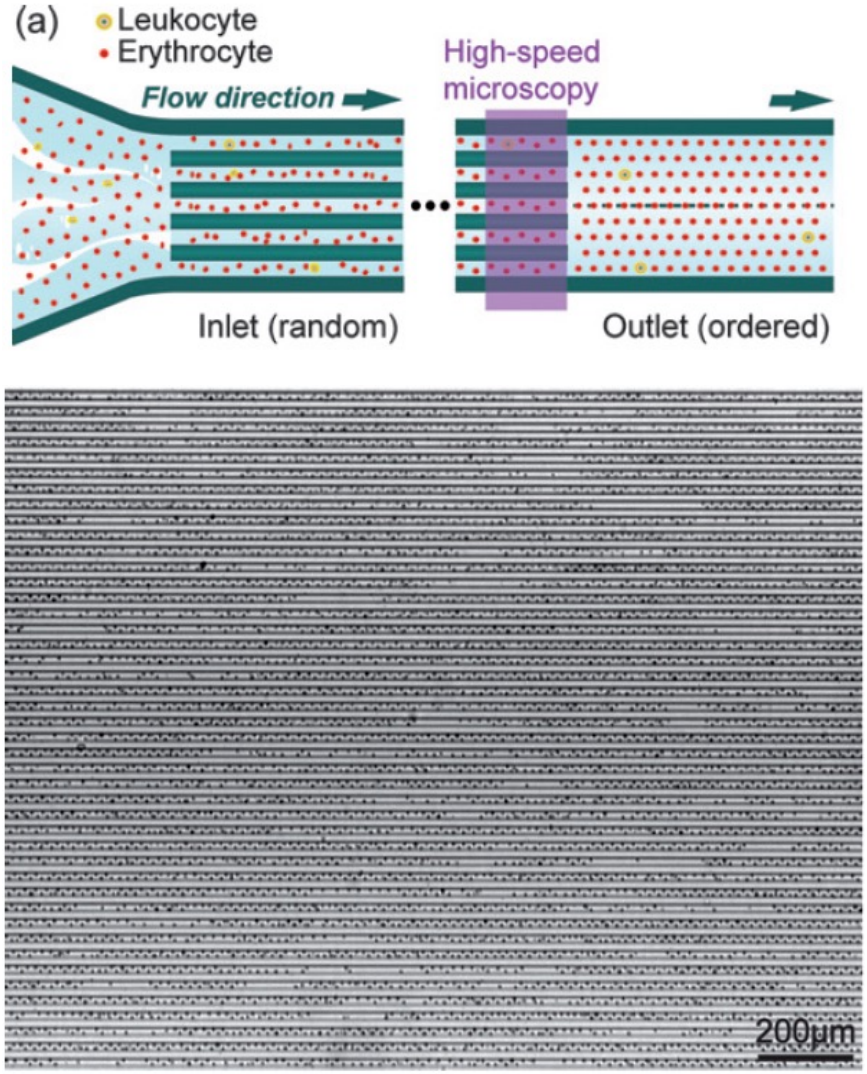
Finite number of stable positions of cells in channels

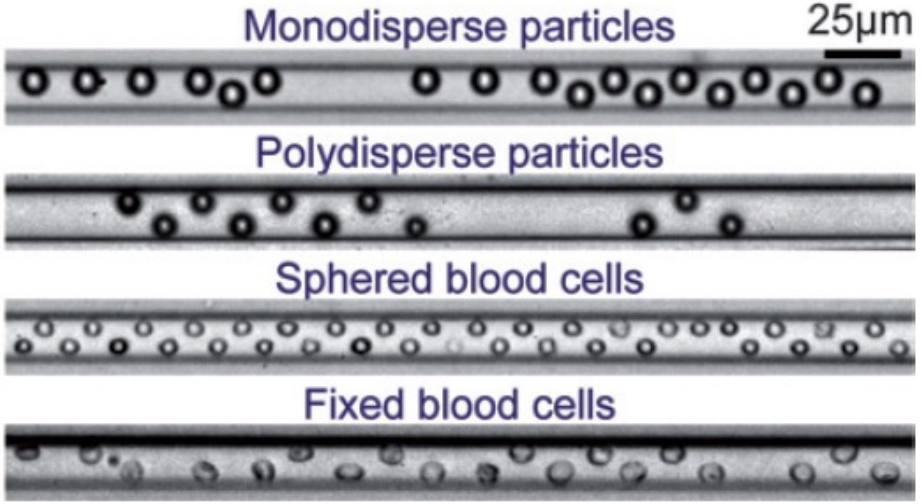
Depends on flow rate and aspect ratio of channels

All cells stay in focal plane for microscopy

Claire Hur et al., Lab Chip. 2010

Massively parallel cytometer



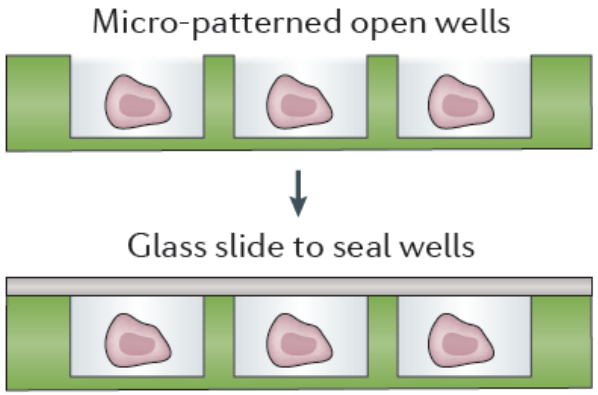


256 Channels
Counting rate: 1 Mio cells/s
Detection: microscope, fast camera

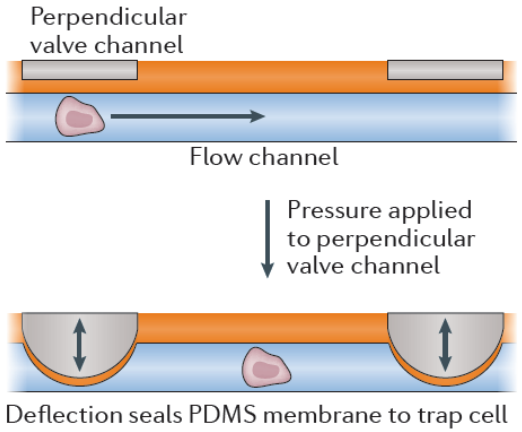
Claire Hur et al., Lab Chip. 2010

Single-cell analysis

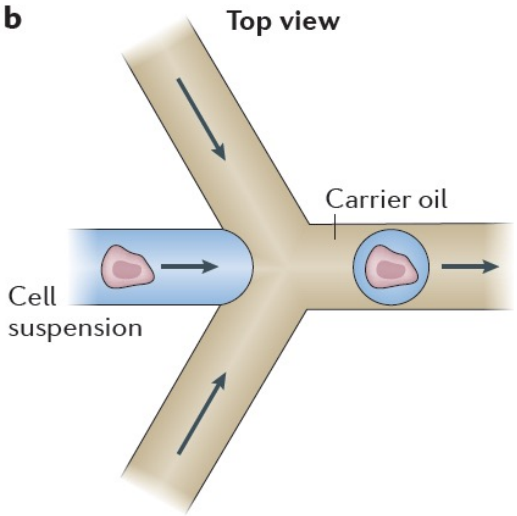
Based on nanowells



Based on valves



Based on droplets



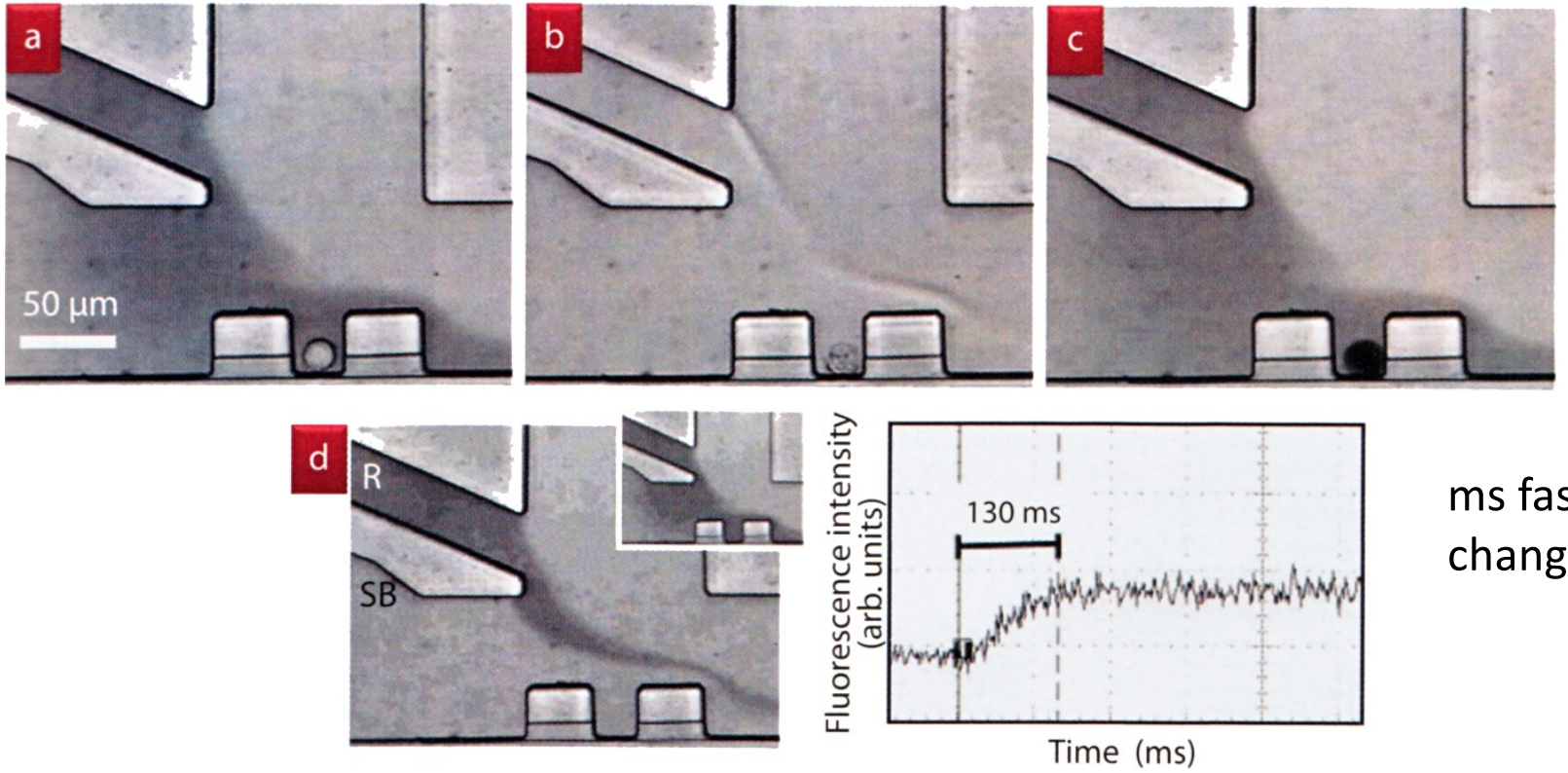
Prakadan, Nat Rev Genet 2017

Hydrodynamic trapping of single cells

trapping

Assay (MeOH cell death)

Analysis (staining)

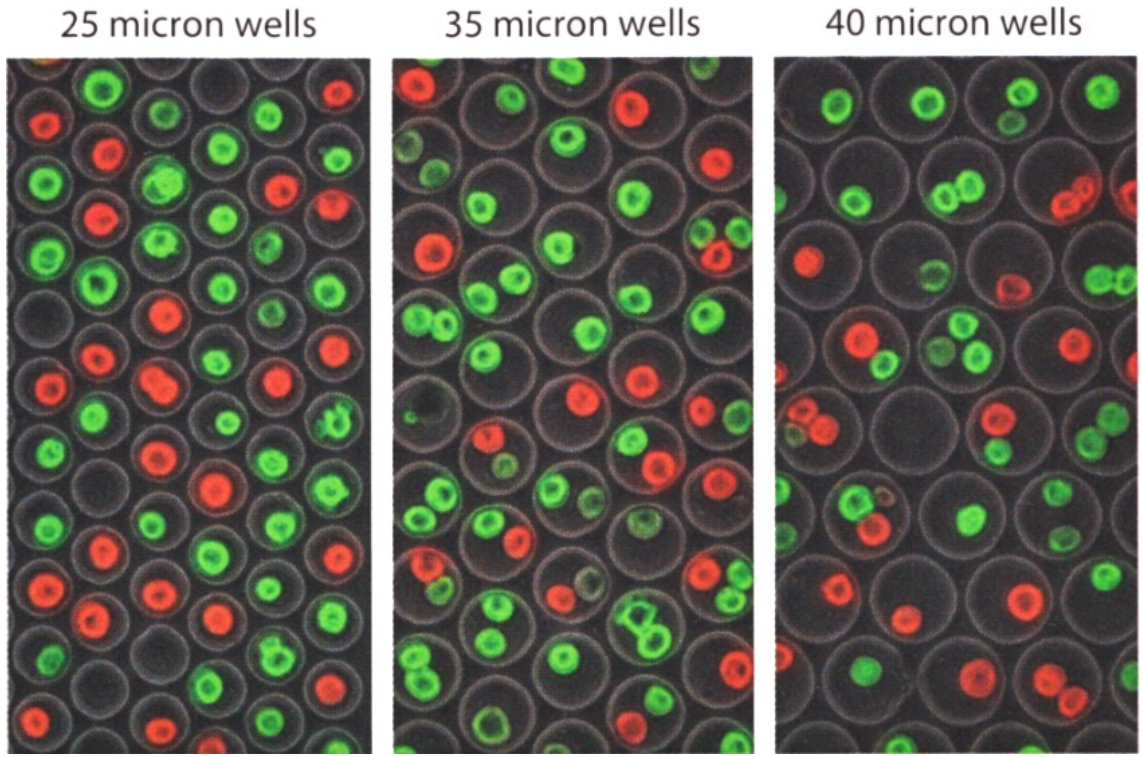


ms fast solution changes

Zare lab:
Wheeler et al., Anal. Chem 2003

- Streamlines change in full trap, cells are not washed out
- Fast assays possible, fast solution exchange

Arrays PDMS microwells for cell trapping

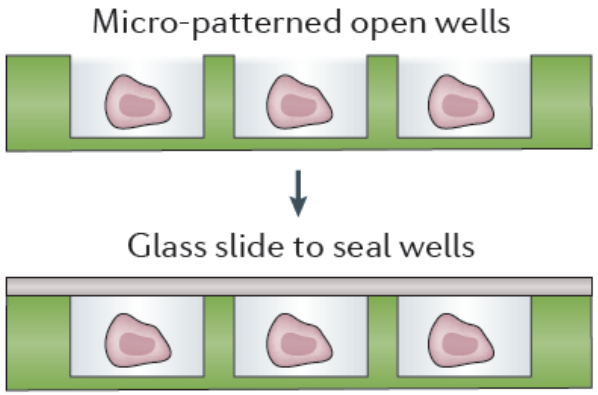


Rettig and Folch, Anal Chem 2005

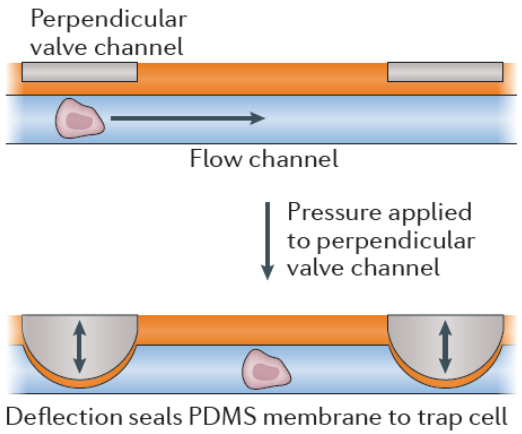
- PDMS molded array of microwells
- Optically translucent
- Once cell falls in well it cannot be easily flushed out (laminar flow)
- High seeding density possible
- Dependent on size: multiple cells present
- With CCD camera possible to observe up to 100'000 cells simultaneously

Single-cell analysis

Based on nanowells



Based on valves



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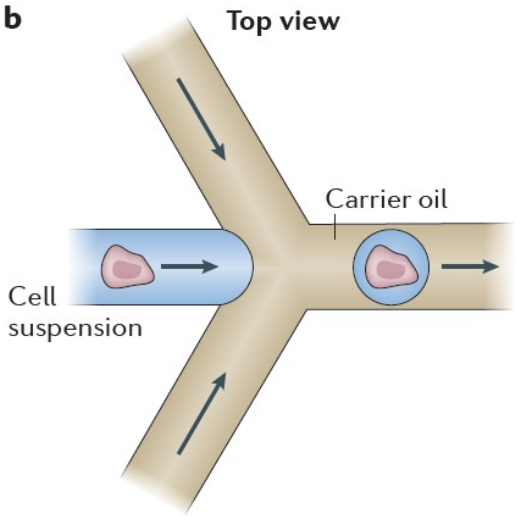
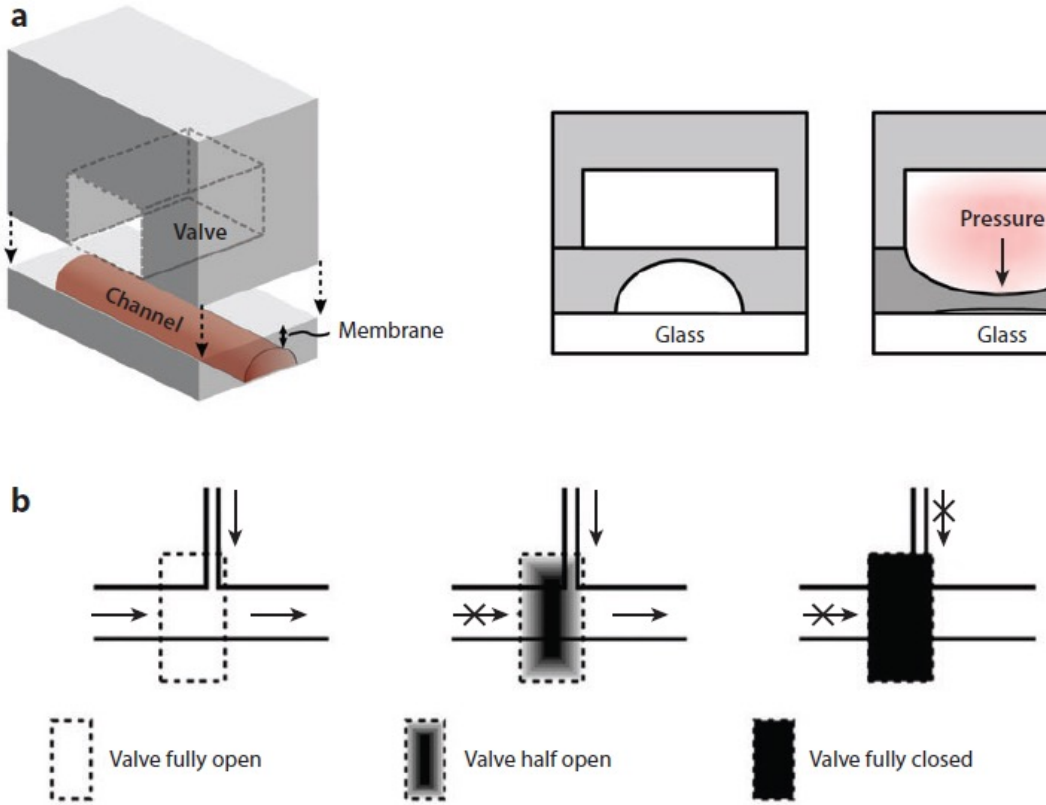


Prakadan, Nat Rev Genet 2017

Cell trapping and chemical lysis: Microfluidic 3-state valve



Wu, Wheeler & Zare, PNAS 2004

3-state valve in PDMS

Half open state: cells do not pass

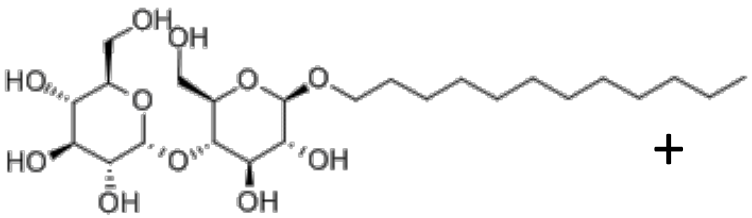
Allows facile buffer exchange

Lysis: can be used to flow in a lysis buffer after cell trapping and washes

Biomolecule purification on chip

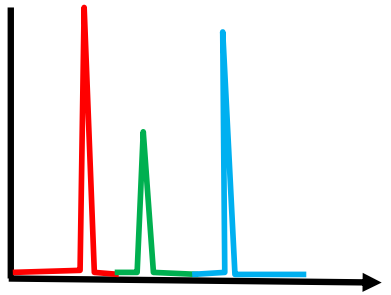
- **DNA**:
  - solid state extraction using silica beads
  - amplification using PCR
- **RNA**:
  - capture using poly-dT solid-state features (beads)
  - solid state extraction followed by selective DNA degradation (enzymatic)
  - amplification
- **Proteins**:
  - purification using CE

Protein purification: CE using passivated channels

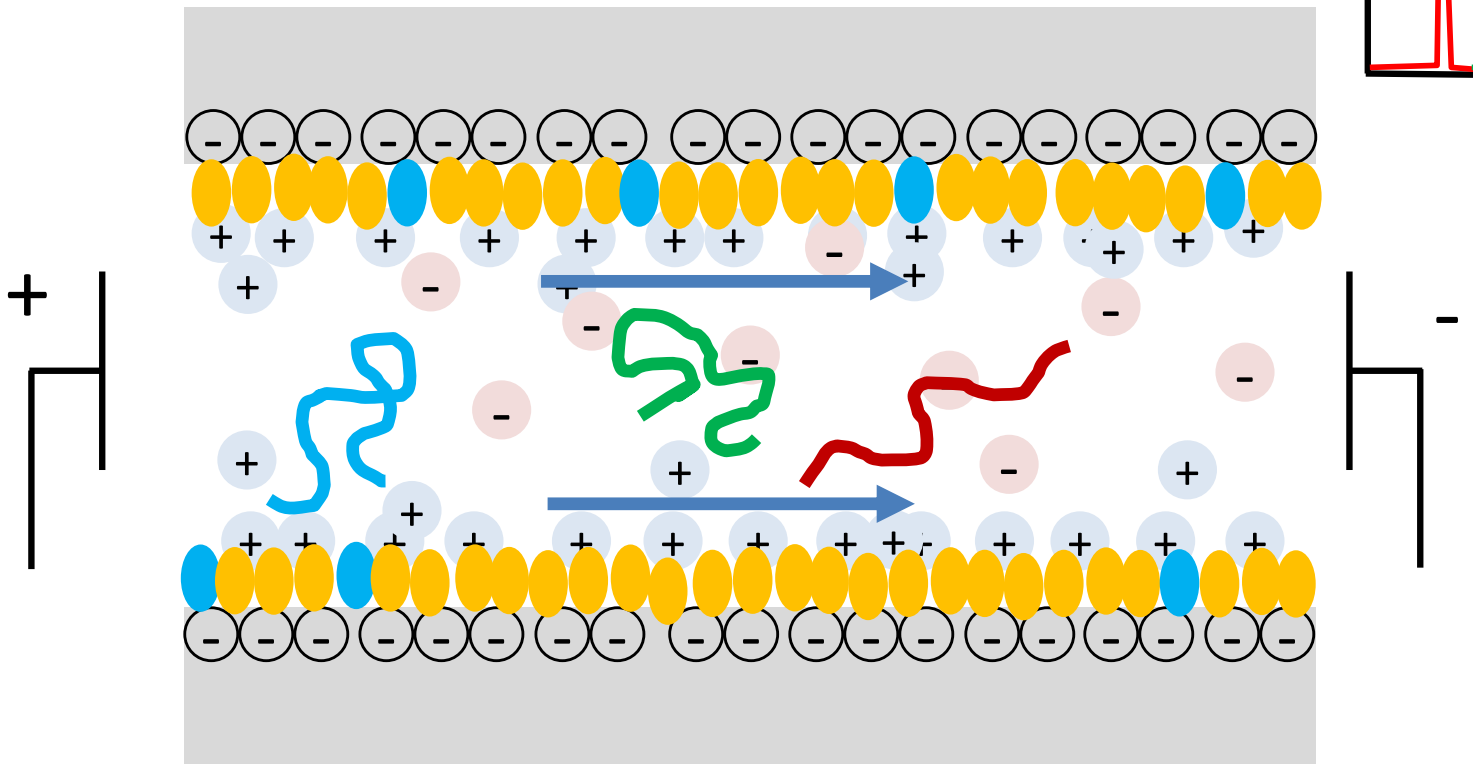


ionic
detergent mix

intensity



time

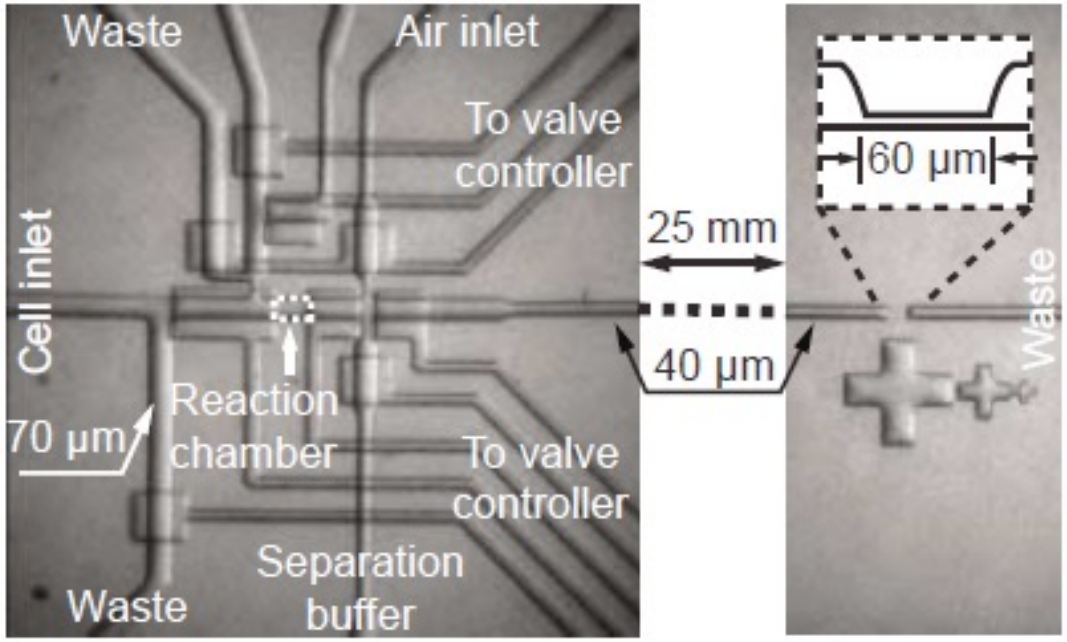


Surface passivation prevents channel fouling

Protein analysis: Low copy number proteins

- Low copy number proteins: less than 1000 copies per cell
  - Receptors
  - Transcription factors
  - Signaling proteins
- Below the detection limit of most techniques
- Low dilution in microfluidics: low detection limits
- Detection limit further lowered using single-molecule detection

Case study: Single cell protein analysis



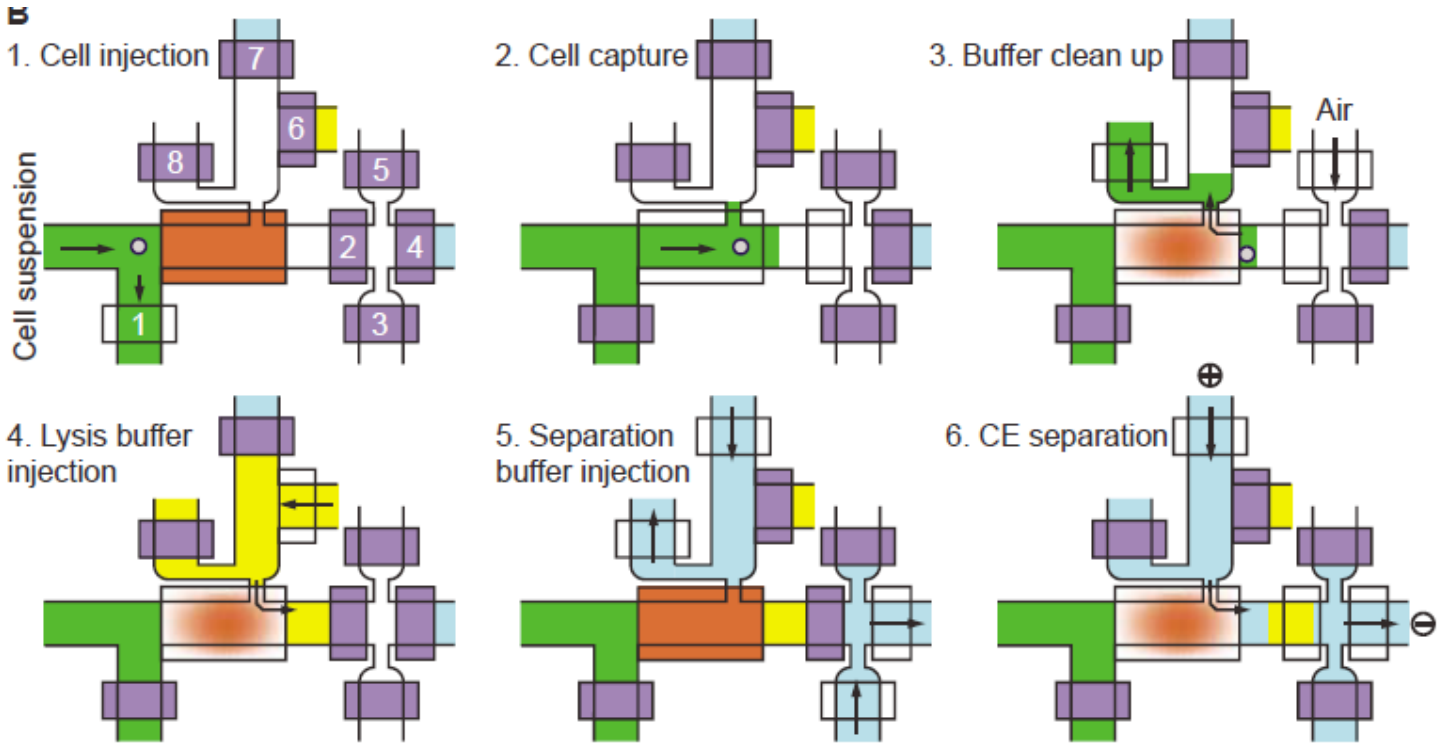
Single molecule detection

3-way valve cell capture & lysis (1% DDM)

CE separation

Huang et al. Science 2007

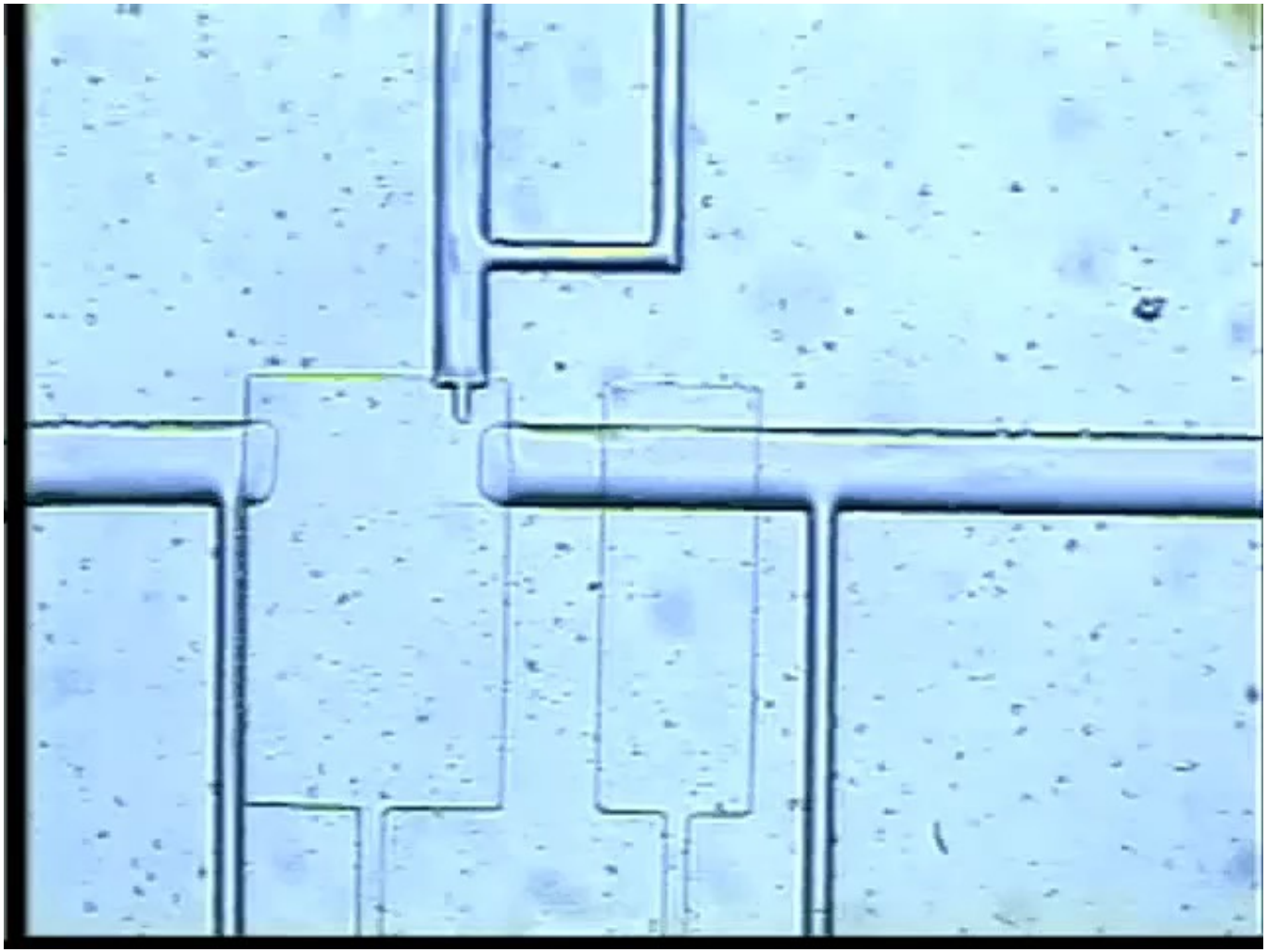
Cell lysis: Chemical Lysis



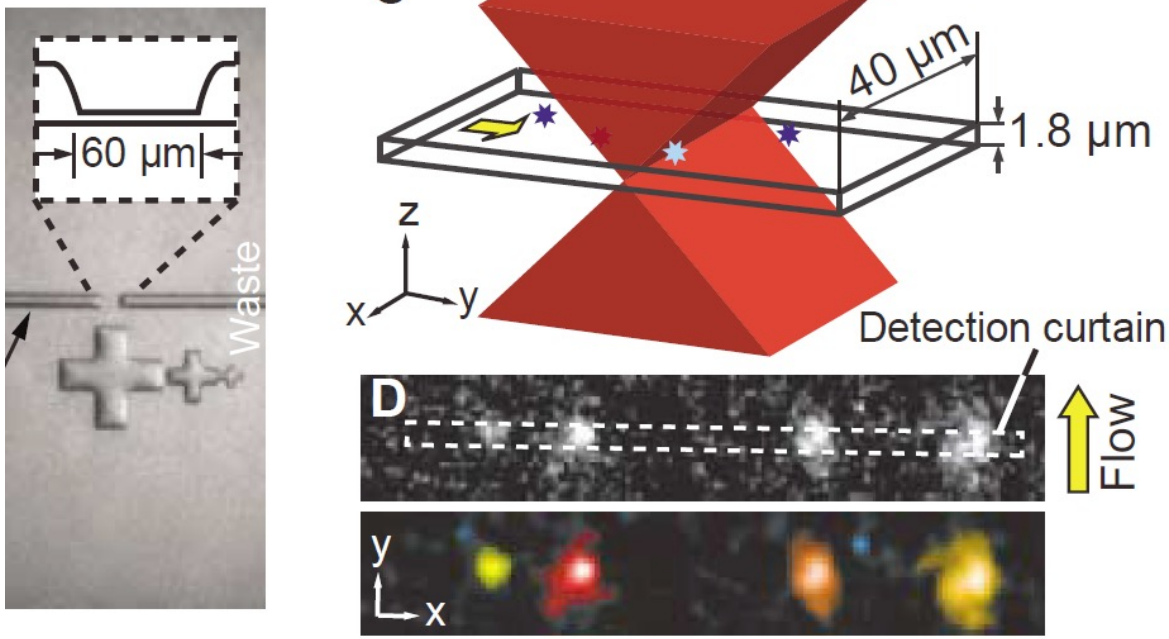
- Cell lysis with detergent solution
- Picoliter volume capture chamber using 3-way valves
- Consider: Does lysis solution alter cell contents (protein denaturation)

Huang et al., Science 2007

System in action



Fluorescence detection



Detection

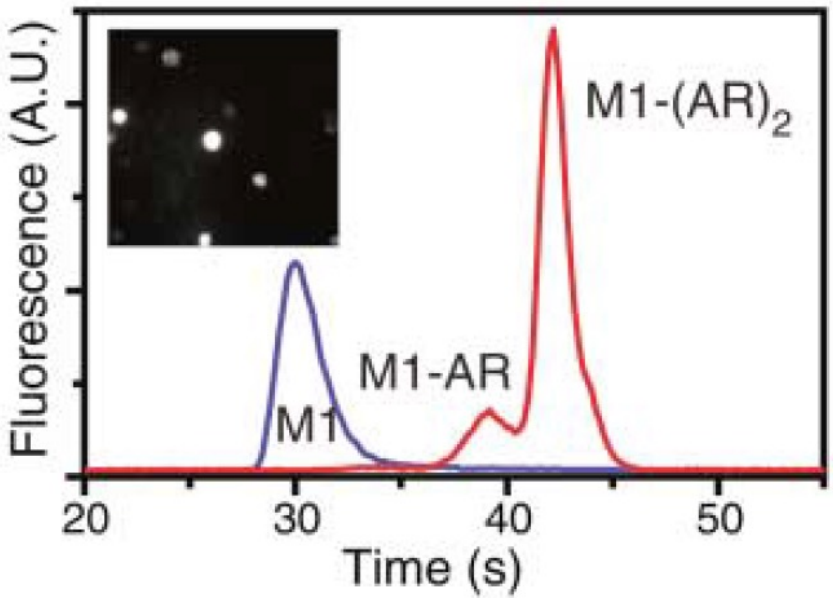
Flat channel with low aspect ratio

Widened laser excitation beam

Recording using a microcope/CCD camera

Huang et al. Science 2007

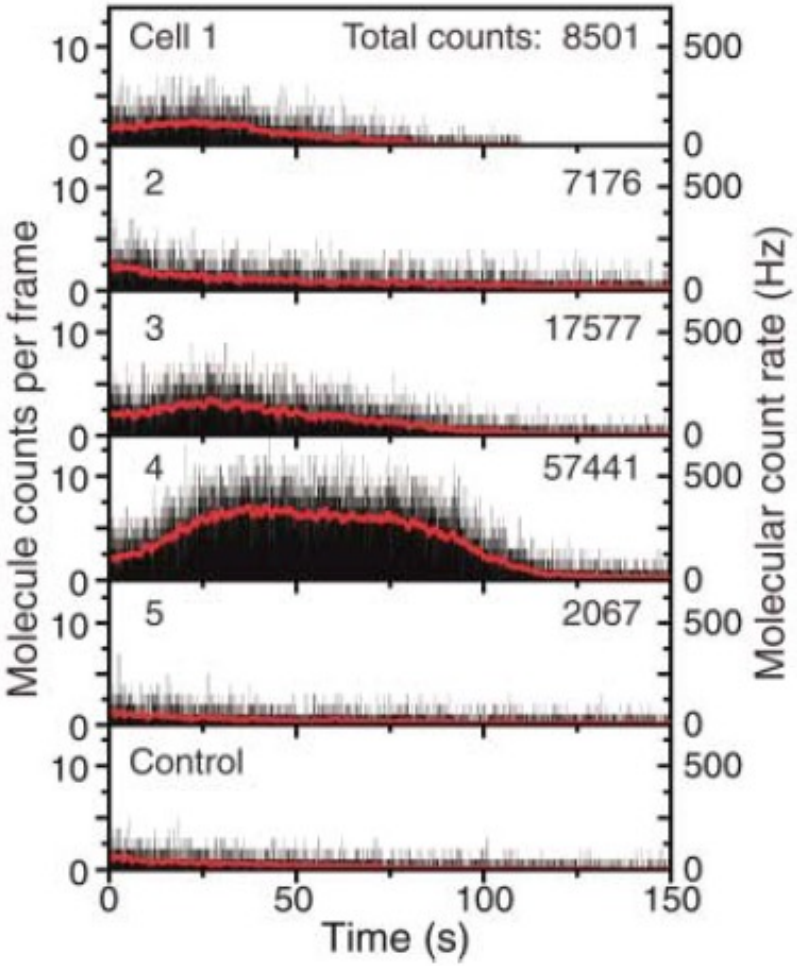
Characterization of the AB complex



- β_2AR (human, expressed in insect cells)
- Reaction of cells with Cy5 – labeled antibody
- FLAG-epitope for antibody binding
- Capillary electrophoresis separation of unbound antibodies

- Unbound antibody elutes first as a clearly separated peak

Protein counting from single cell (sf9)

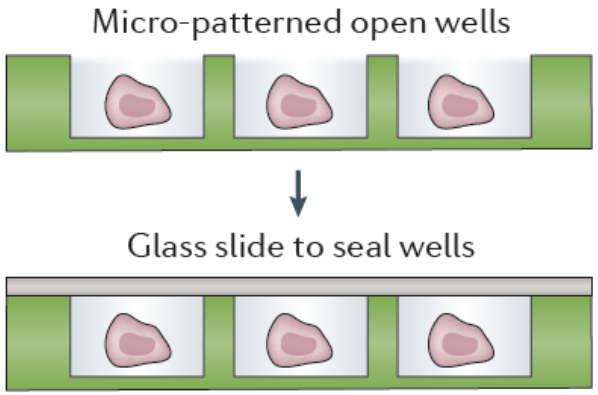


Targets:

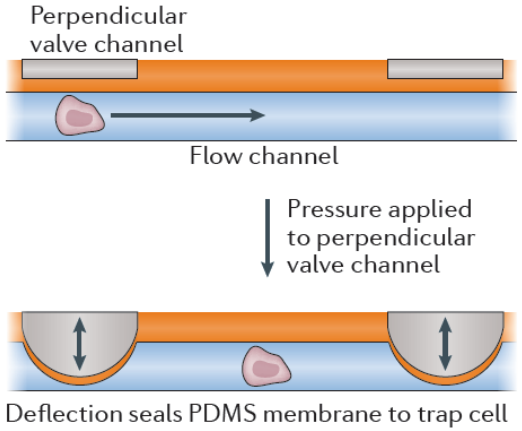
- antibody peak: not shown, comes before!
- varying copy number between 2000 – 60000
- cell-to-cell heterogeneity: here possibly due to uneven infection with expression virus

Single-cell analysis

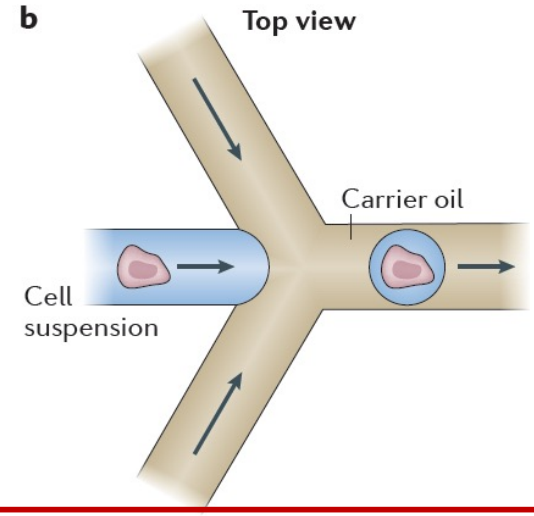
Based on nanowells



Based on valves

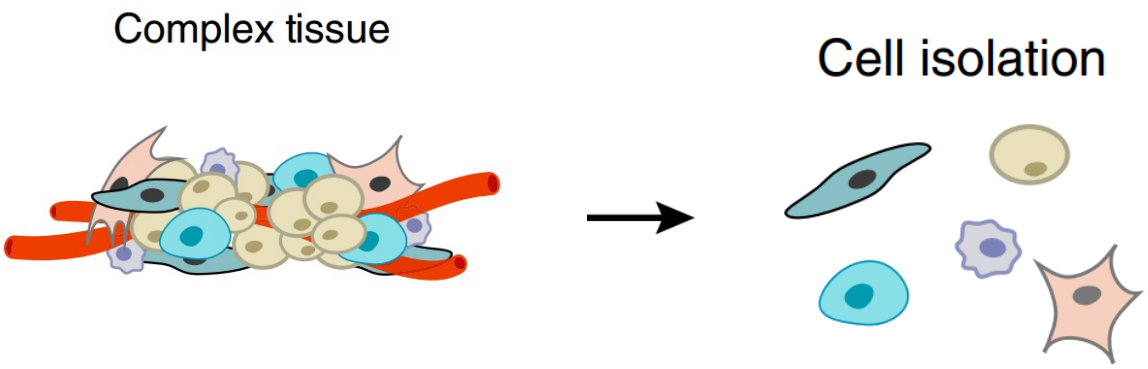


Based on droplets



Prakadan, Nat Rev Genet 2017

Single-cell transcriptome sequencing



what are the differences in gene expression across the cell types?

Solution: Single cell transcriptome sequencing.

Question: How to process the single cells to not "mix" up the individual transcriptomes?

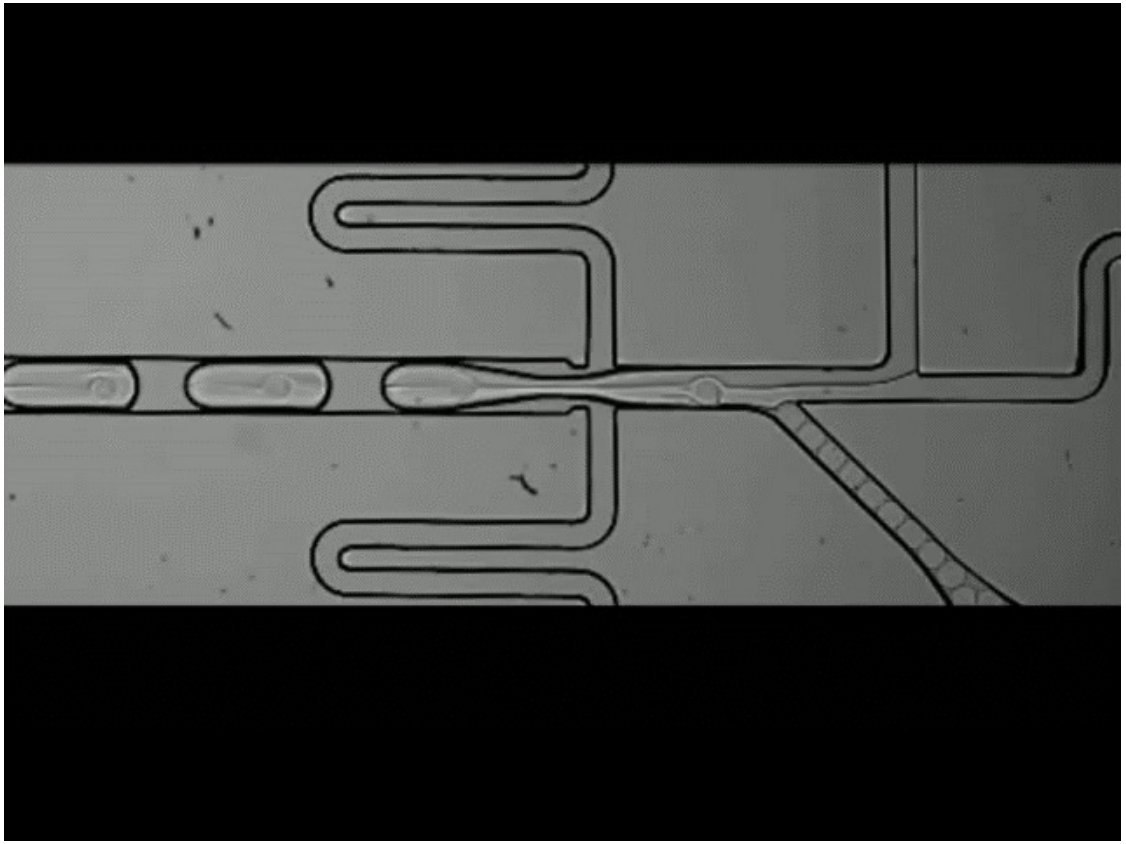
Droplet microfluidics – Basics

Two immiscible phases:

- oil
- aqueous solution

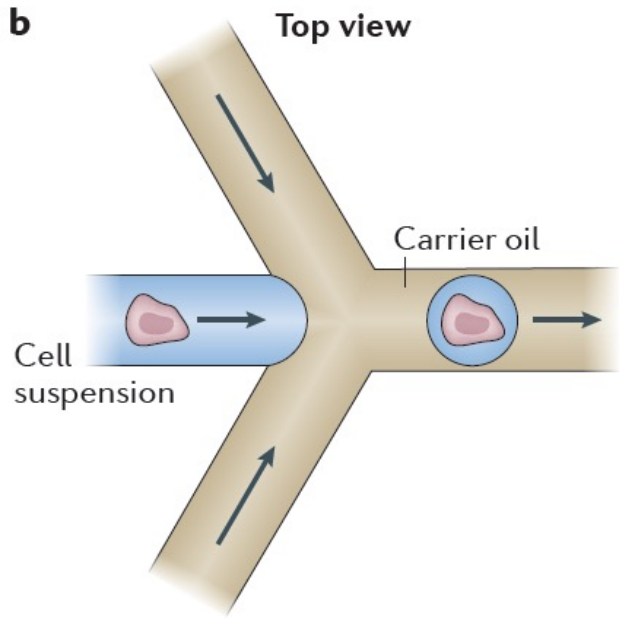
flow to create :

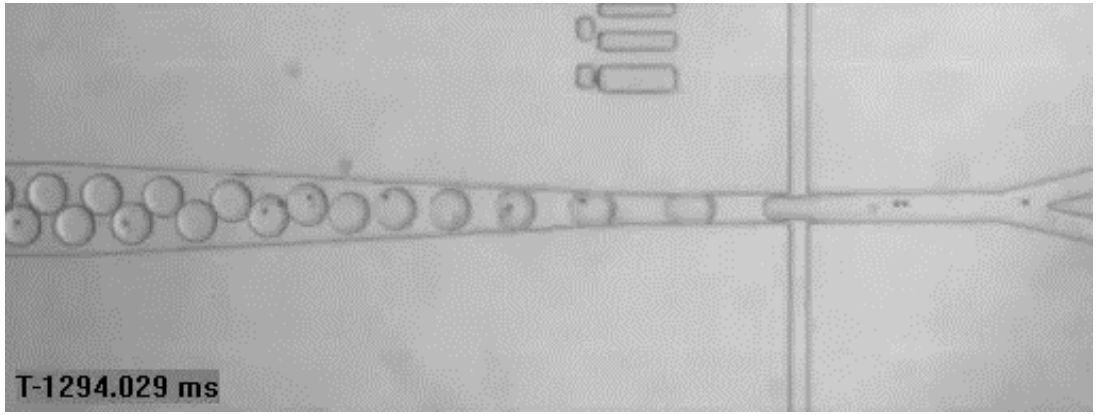
- droplets
- slugs
- discrete volumes



→ allows to perform **complex operations** without increasing device size and complexity

Cell sorting and analysis by droplet microfluidics





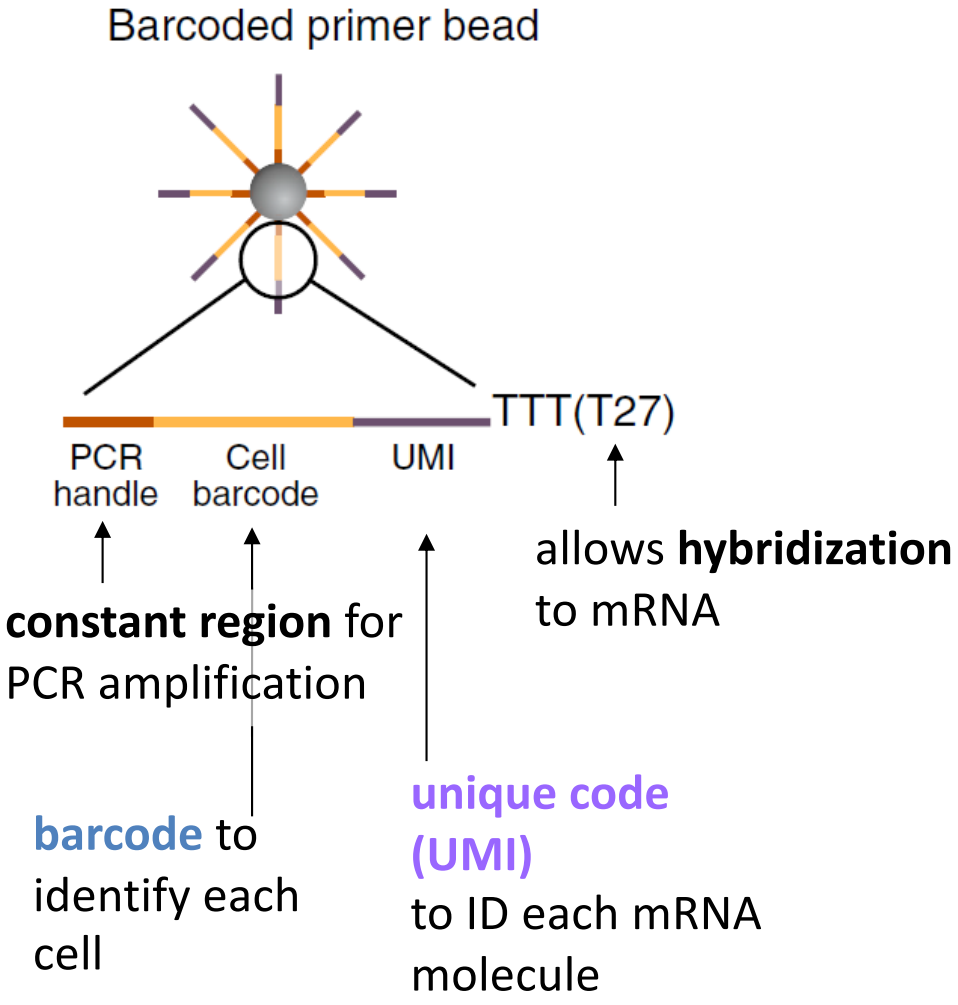
High flow rate carrier oil
low flow rate cell suspension

-> capture of cells in single droplets with nL volume

Droplets act as nanoreactor

can be collected at the end by breaking the emulsion

How to identify each cell?



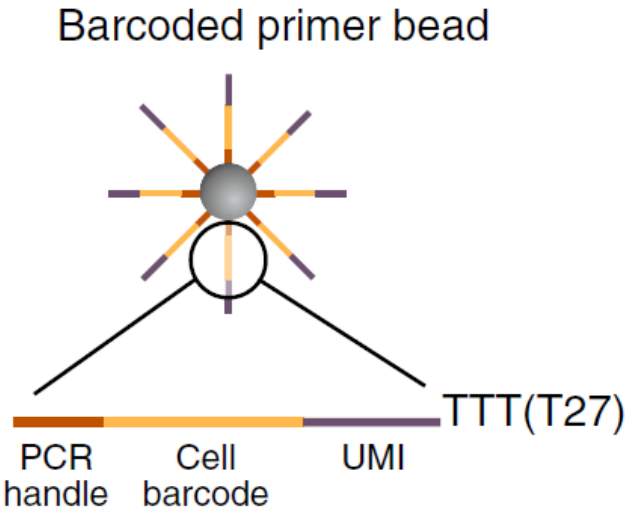
Each cell is paired with a **barcoded primer bead**

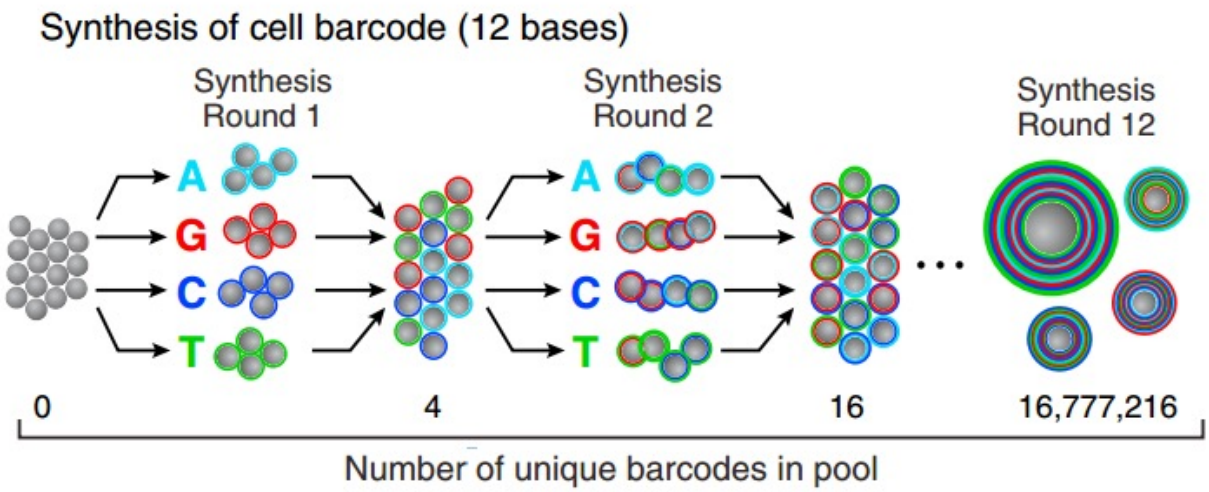
Primer bead:

- high number of DNA primers attached
- each primer has 4 elements:
  - PCR handle
  - barcode
  - UMI (unique molecular identifier)
  - polyT tract

Each primer bead is paired with a cell. The **barcode** identifies each cell.
The **UMI** marks each mRNA molecule.

Synthesis of primer beads: Barcode





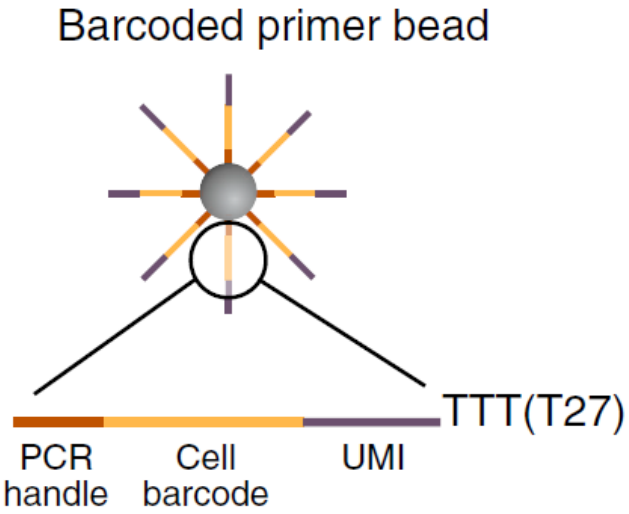
Barcode:
Used to ID **each cell**, and all **mRNA molecules** originating from it.

Split-pool synthesis: Add one nucleotide to each bead in 4 pools. Then pool beads, and redistribute into 4 wells. Then, add the next nucleotide & repeat

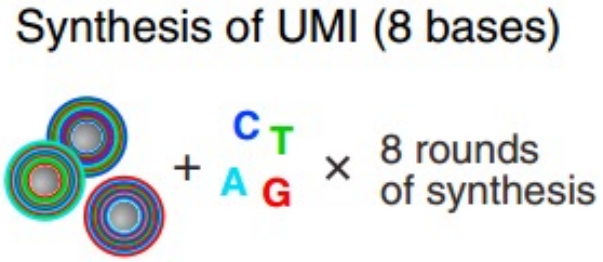
→ Results in 4^N sequences (N: number of synthesis steps).
→ Each bead carries the same unique sequence.

e.g. 12 bp Cell barcode:
17 millions of the **same** cell barcode per bead

Synthesis of primer beads: UMI



Used to ID **each mRNA molecule** (thousands per bead)
→ allows to count multiple copies of the same mRNA after a PCR amplification step



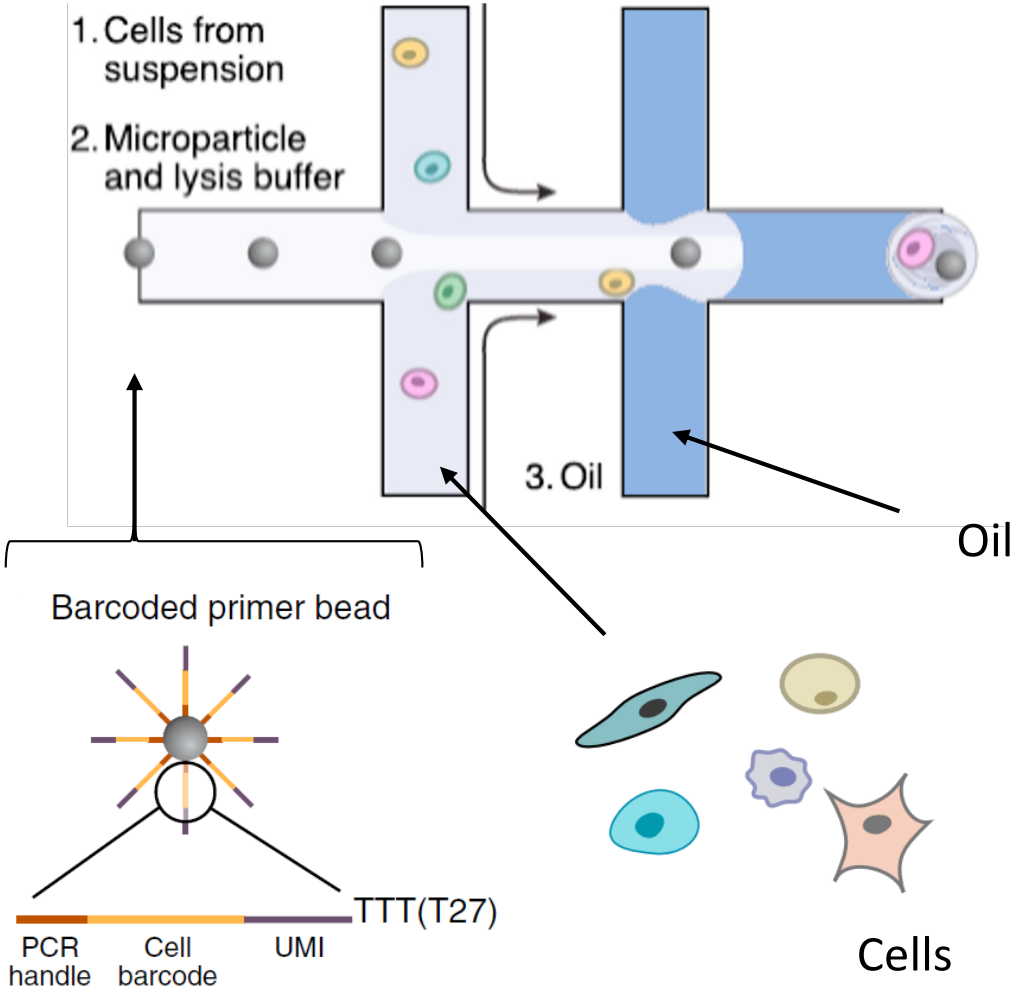
Random synthesis, 8bp UMI

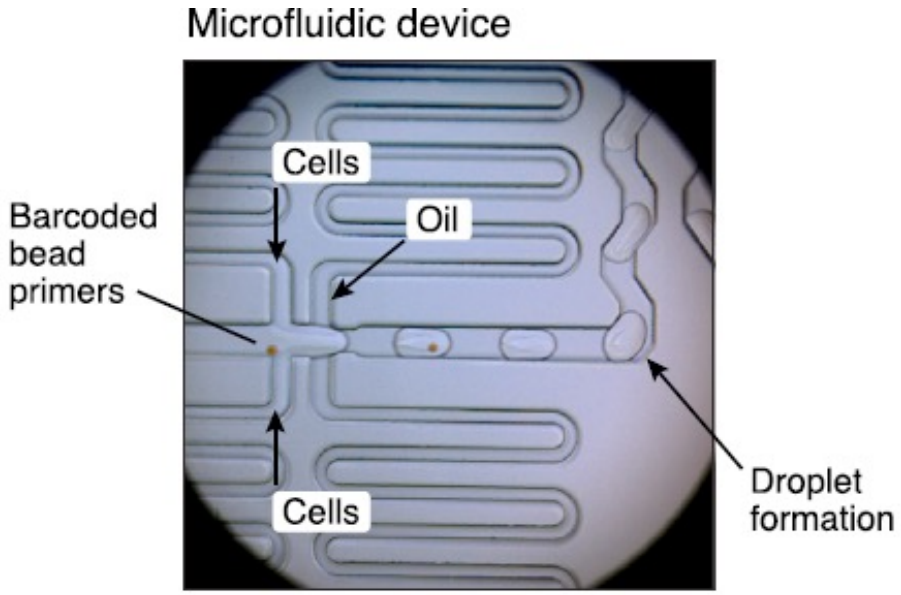
each synthesis step is performed using a 1:1:1:1 mixture of all 4 nucleotides

For an 8 bp UMI, this results in 4^8 different unique molecular identifiers per bead.

Droplet microfluidics with DNA barcoding

Macosko et al. Cell 2015



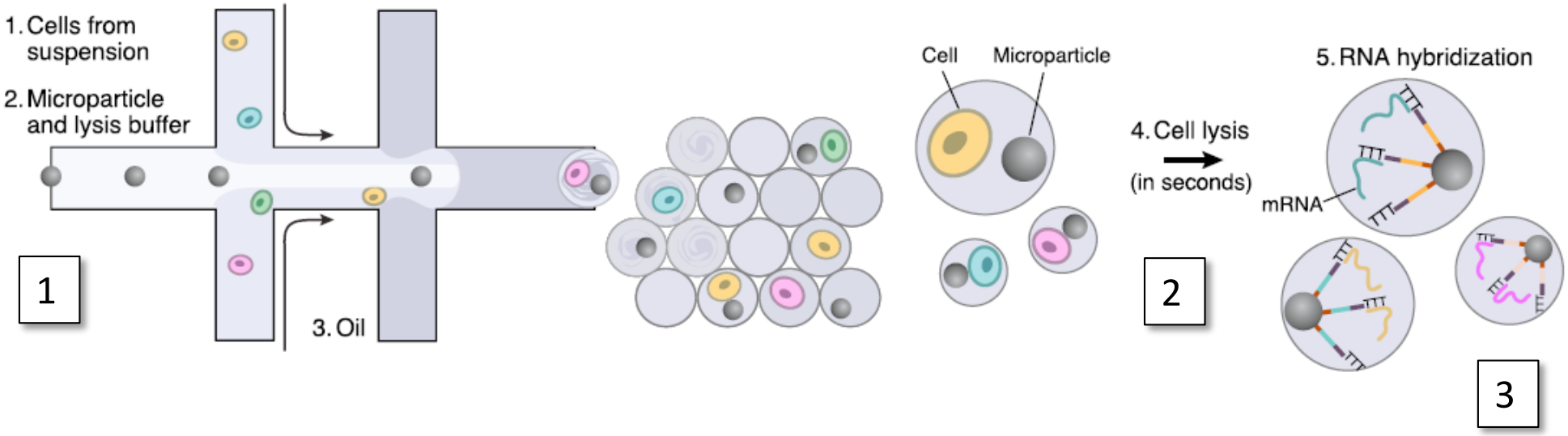


Cells are encapsulated in droplets. Each droplet contains:

- **one cell**
- **lysis buffer**
- **one bead** decorated with **DNA barcodes**

Single-cell Drop-seq Transcriptomics

Macosko et al. Cell 2015

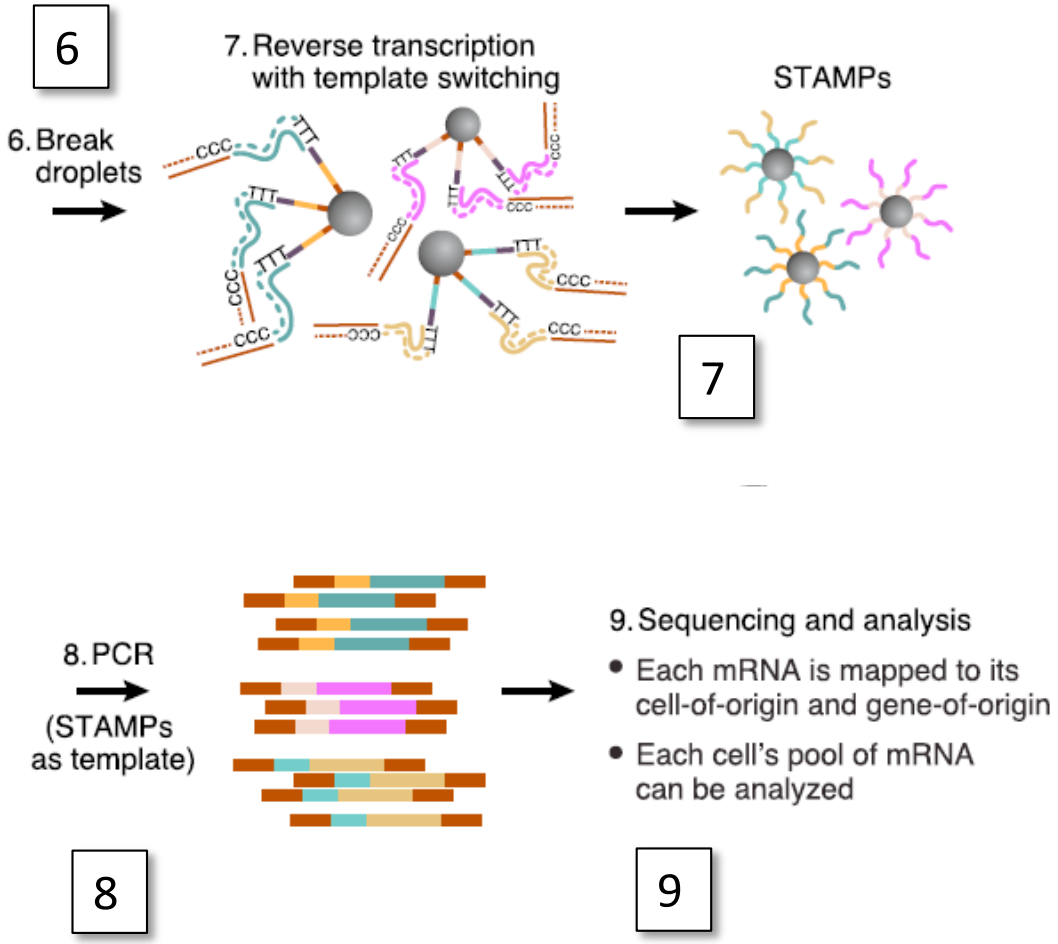


1. Capture of single cells with barcoded beads in droplet

2. Cell lysis within the droplet

3. RNA capture / hybridization

Single-cell Drop-seq Transcriptomics



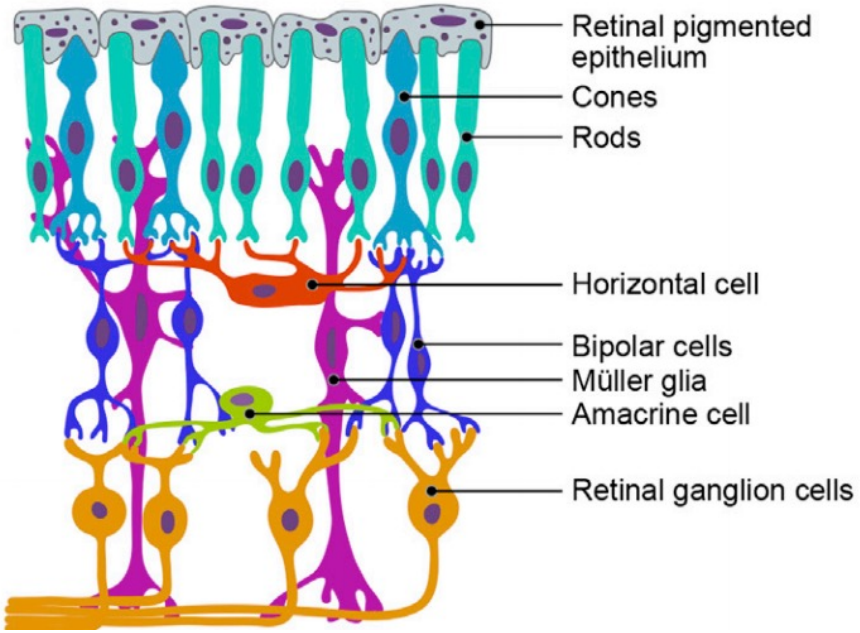
6. Droplets are collected, the emulsion is broken and all beads are pooled

7. Reverse transcription on the barcoded beads using the immobilized **DNA barcodes as primers**.
"single-cell transcriptomes attached to microparticles" (STAMPs) are generated

8. PCR amplification, using STAMPs as templates.
→ each mRNA gives rise to thousands of DNA molecules. Each carries encoded information on:
1. the cell of origin
2. the mRNA molecule of origin

9. the libraries are sequenced by Illumina technology.

Transcriptome profiling of retinal cells



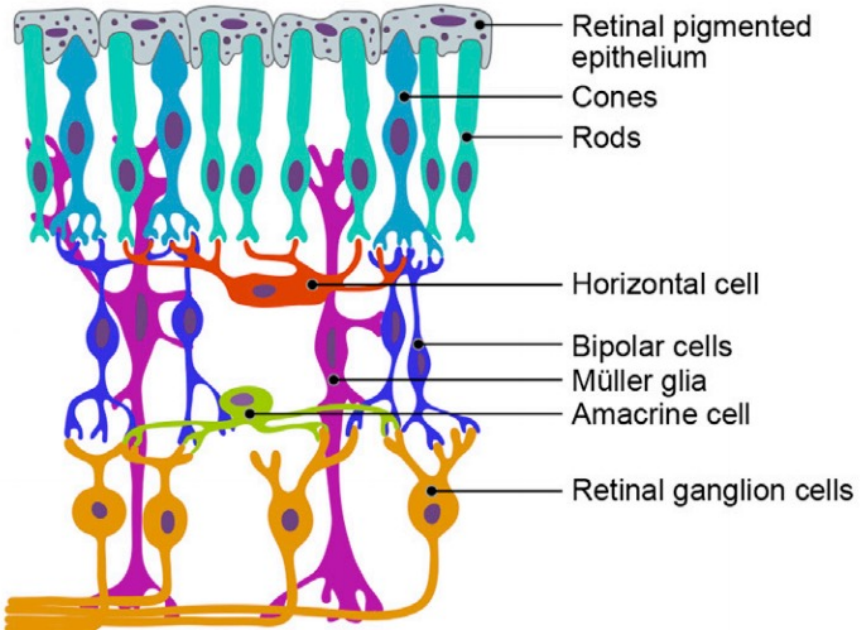
Retinal cells
solubilized and analyzed by Drop-Seq

Each cell reveals:

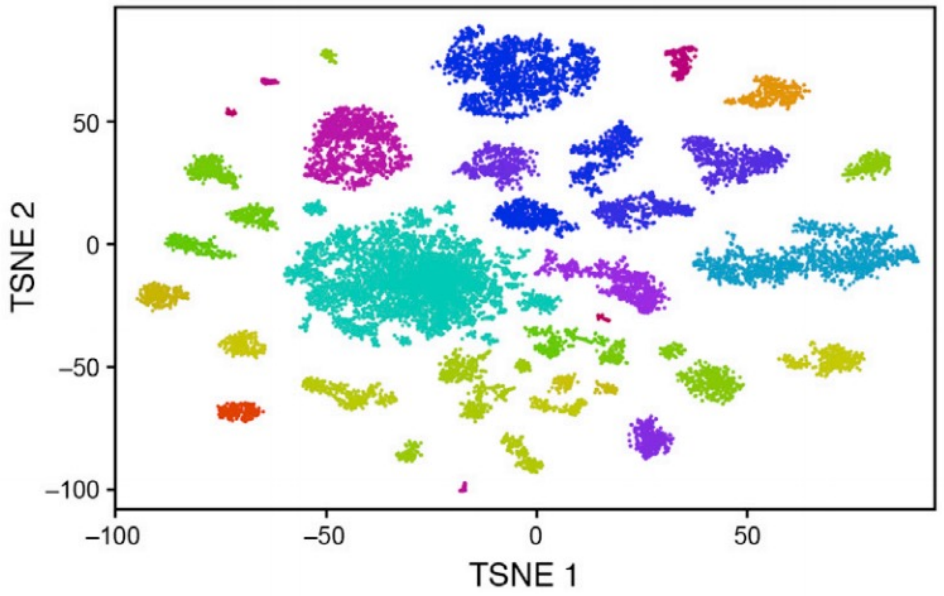
- transcriptional profile
- which genes are expressed
- what is the copy number of for each mRNA

Cell 1				**Cell 2**		
Gene	**Uniprot**	**Score**		**Gene**	**Uniprot**	**Score**
Mpg	Q04841	1		Mpg	Q04841	1
Cdc5l	Q6A068	14		Srcap	A0A087WQ44	21
Zmynd8	A2A484	8622		Zmynd8	A2A484	4053
Srsf3	P84104	3		Bptf	A2A654	191
Bptf	A2A654	290		Prdm2	A2A7B5	165
Rlf	A2A7F4	118		Gm6710	A2ART0	57
Gm13242	A2A9J5	1460		Baz2b	A2AUY4	16917
Qser1	A2BIE1	1185		Abcf1	Q6P542	1503
Abcf1	Q6P542	1111		Acaca	Q5SWU9	25
Acin1	Q9JIX8	14		Acin1	Q9JIX8	59

Transcriptome profiling of retinal cells



Retinal cells
solubilized and analyzed by **Drop-Seq**



Data analysis, e.g. by principal component analysis (PCA)

- transcriptome profiles cluster accoding to cell types
- identification of new cell types
- unique signatures

Summary: Microfluidoc single-cell analysis

- **Precise Manipulation:** Microfluidic channels enable precise manipulation of individual cells, allowing for controlled environments and optimized reactions.
- **High Throughput:** Microfluidic platforms automate and parallelize sample processing steps, facilitating high-throughput analysis of single cells.
- **Single-Cell Sequencing:** Microfluidic devices facilitate single-cell sequencing techniques, enabling comprehensive genomic and transcriptomic analysis.
- **Enhanced Sensitivity:** Microfluidics reduce sample consumption and increase sensitivity and elucidation of complex biological processes at the single-cell level.