

Single cell RNA sequencing (scRNAseq) platforms

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Erasmus MC, Group leader

2021 Single Cell Analysis Workshop, 2021/10/18

Single cell RNA sequencing (scRNAseq) platforms

Part I: Plate-based scRNAseq

Part II: Droplet-based scRNAseq

Outline of the Part I (Plate-based scRNAseq)

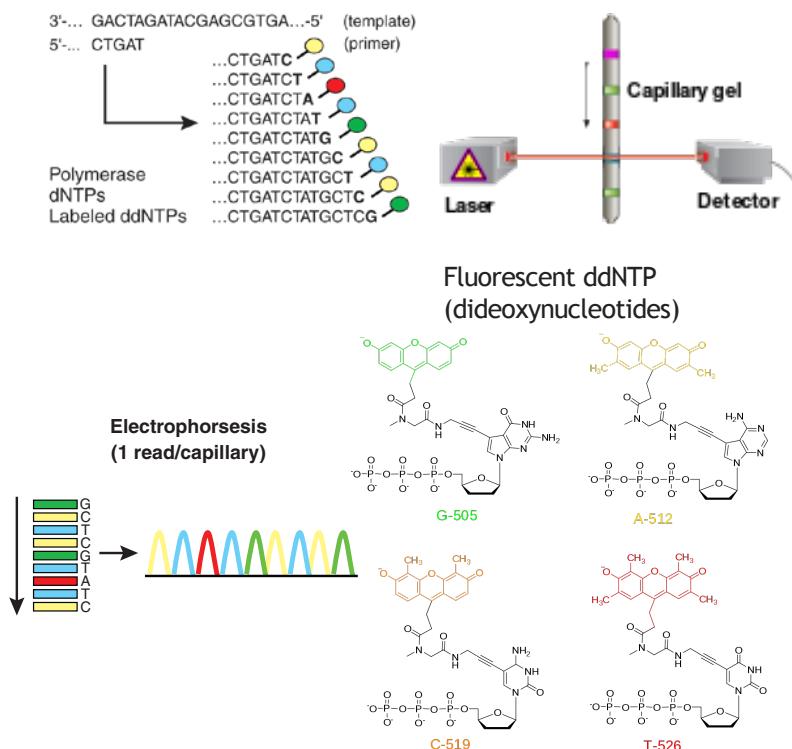
- General introduction about scRNAseq
- Different types of plate-based scRNAseq
- Workflow of different plate-based scRNAseq



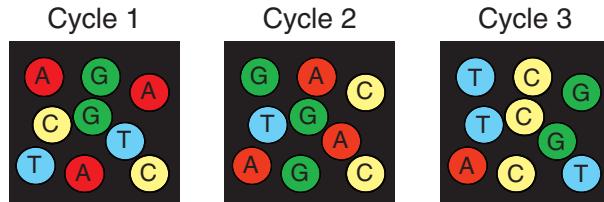
single cell sequencing **?** **next-generation sequencing**

Traditional sequencing vs next-generation sequencing

- From sanger sequencing to next-generation sequencing



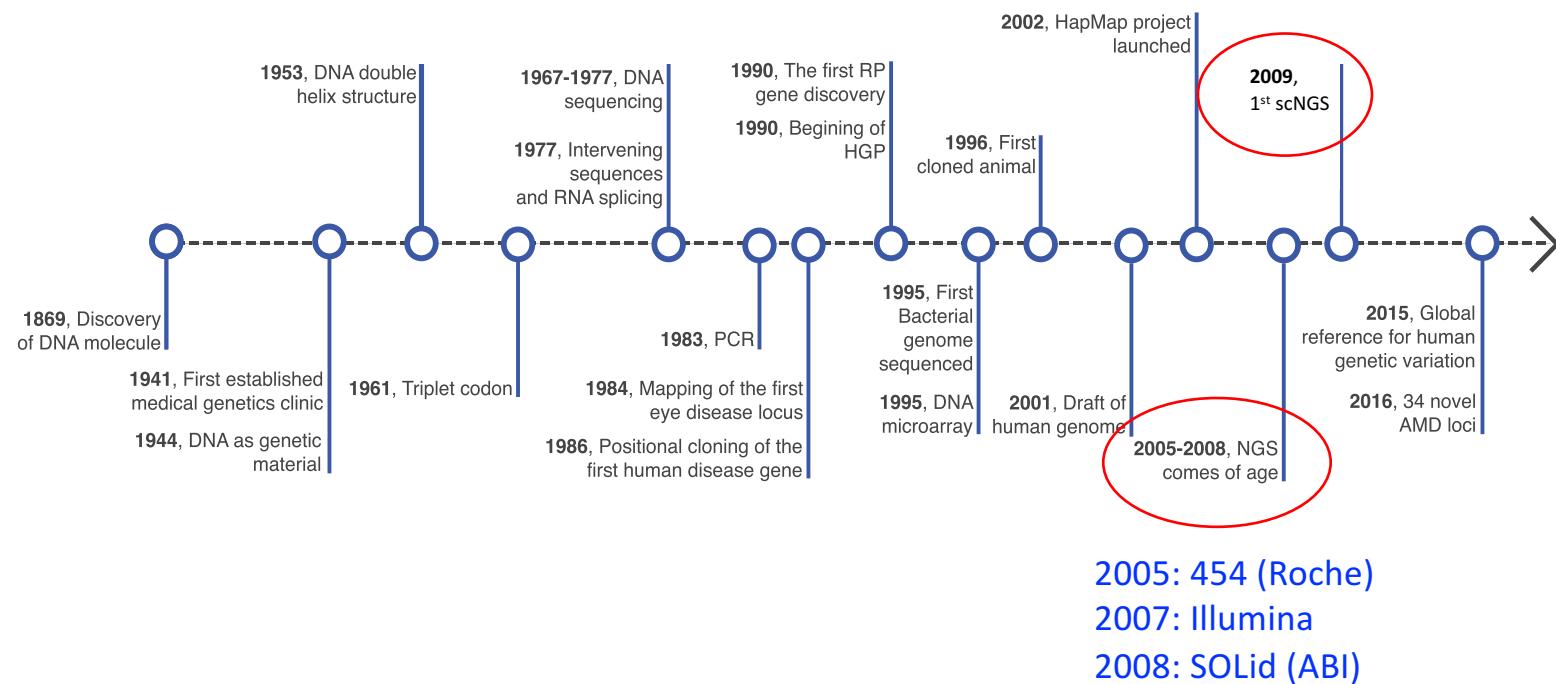
Cyclic array sequencing
($>10^6$ reads/array)



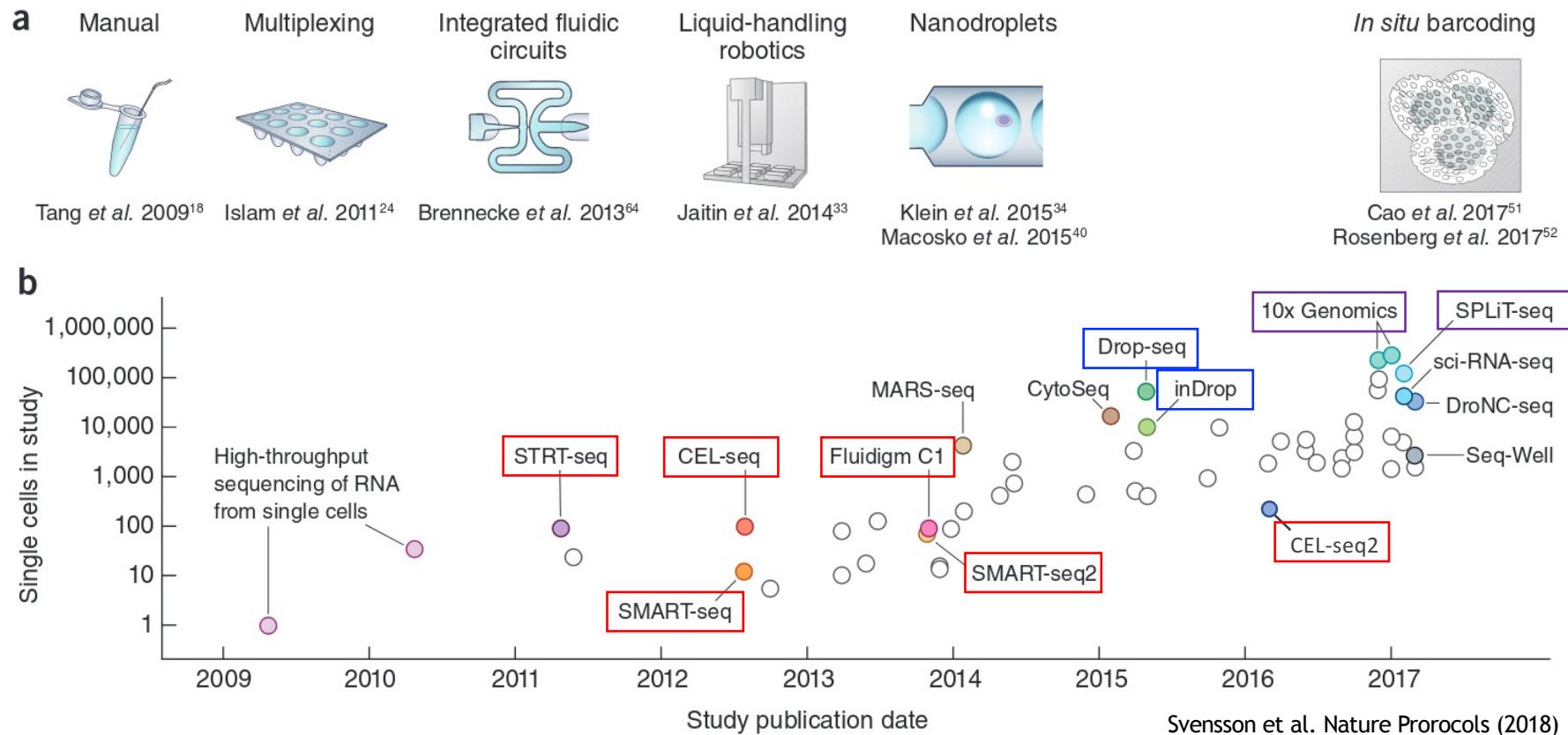
What is base 1? What is base 2? What is base 3?

- Illumina
- Roche 454
- ABI SOLiD
- ...

Next-generation sequencing & single cell sequencing



Evolution of scRNAseq techniques

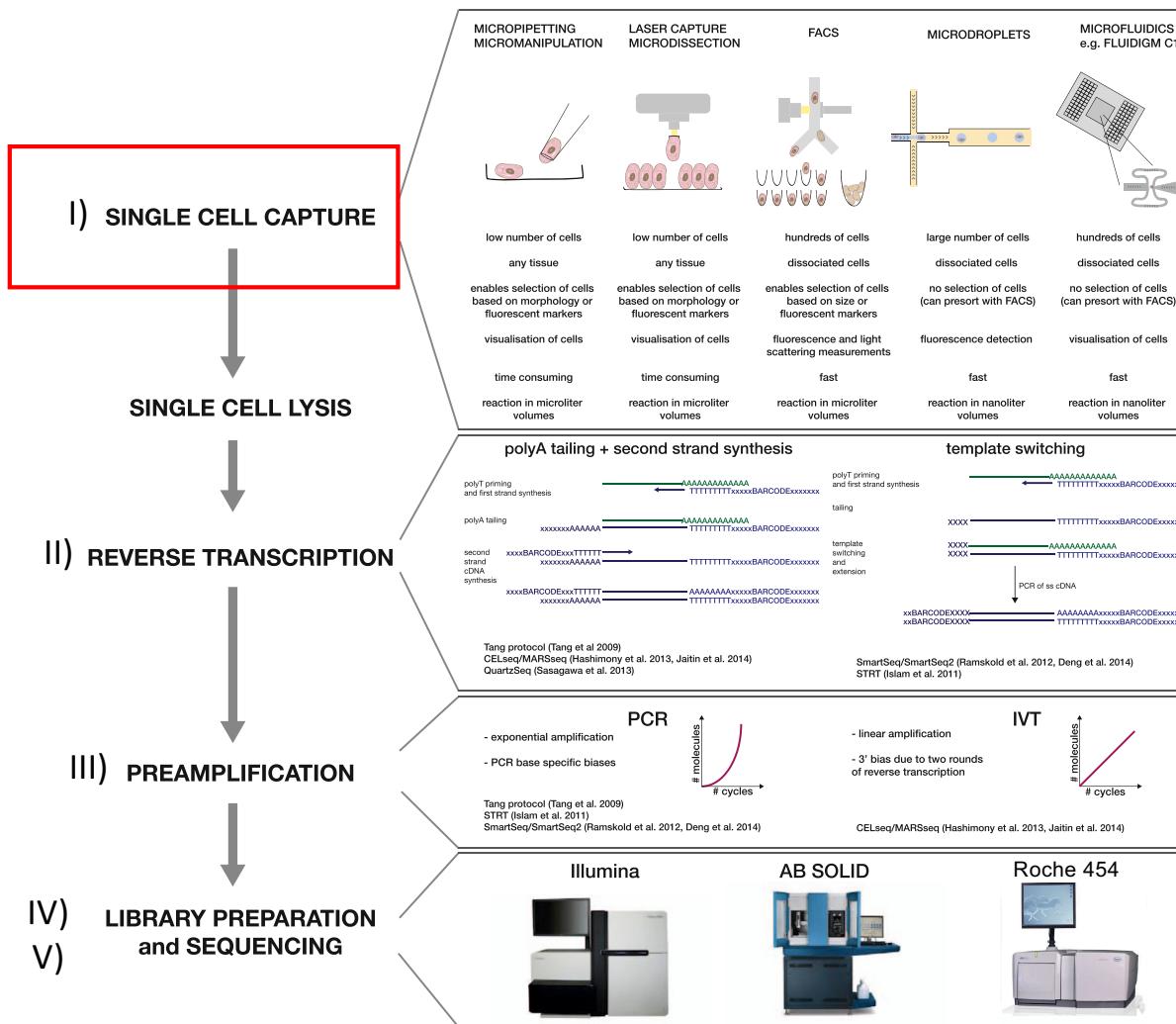


~100s cells thanks to **multiplexing (barcode)**

~10,000s cells thanks to random cell captures techniques with **nanodroplets (manual)**

~100K cells thanks to **10X Genomics** and ***In situ* barcoding**

Single-cell RNA sequencing experiment workflow



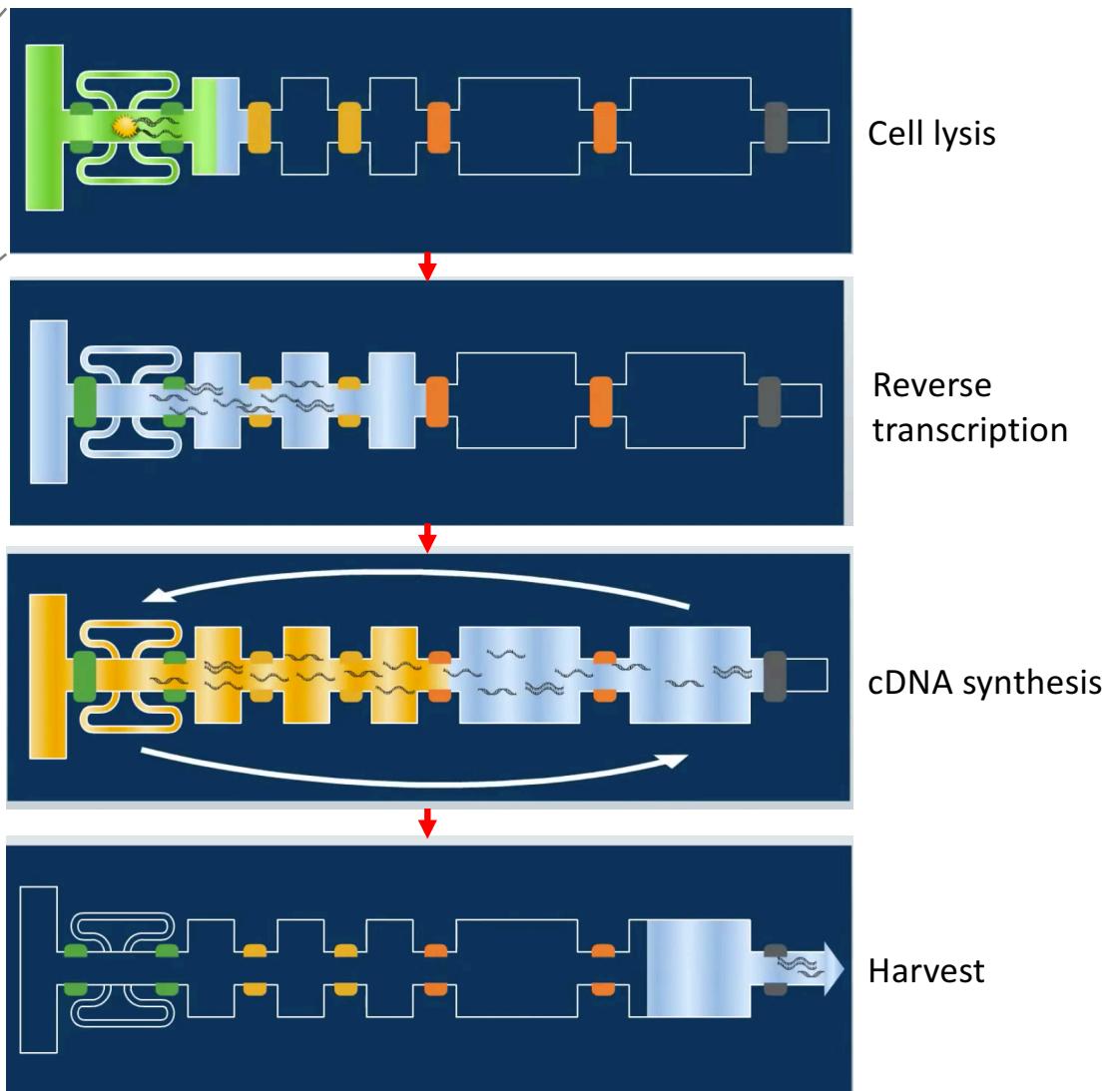
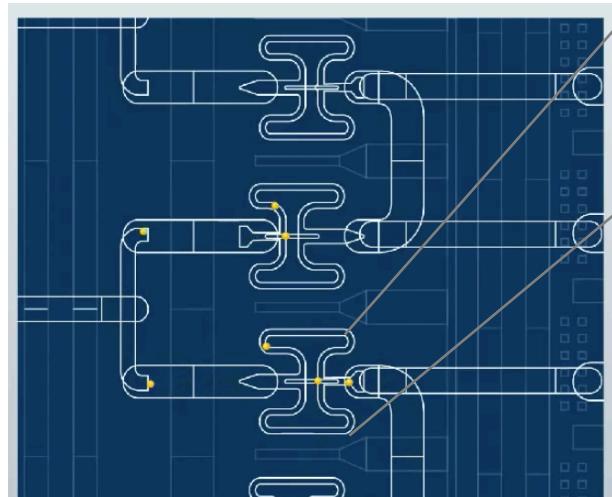
I) Single cell capture

MICROPIPETTING MICROMANIPULATION	LASER CAPTURE MICRODISSECTION	FACS	MICRODROPLETS	MICROFLUIDICS	Wafergen (iCell8), CellenOne
					& Liquid-handling robotics
low number of cells	low number of cells	hundreds of cells	large number of cells	hundreds of cells	medium-Large number of cells
any tissue	any tissue	dissociated cells	dissociated cells	dissociated cells	
enables selection of cells based on morphology or fluorescent markers	enables selection of cells based on morphology or fluorescent markers	enables selection of cells based on size or fluorescent markers	no selection of cells (can presort with FACS)	no selection of cells (can presort with FACS)	some selection criteria
visualisation of cells	visualisation of cells	fluorescence and light scattering measurements	fluorescence detection	visualisation of cells	
time consuming	time consuming	fast	fast	fast	fast
reaction in microliter volumes	reaction in microliter volumes	reaction in microliter volumes	reaction in nanoliter volumes	reaction in nanoliter volumes	

I) Single cell capture (microfluidics)

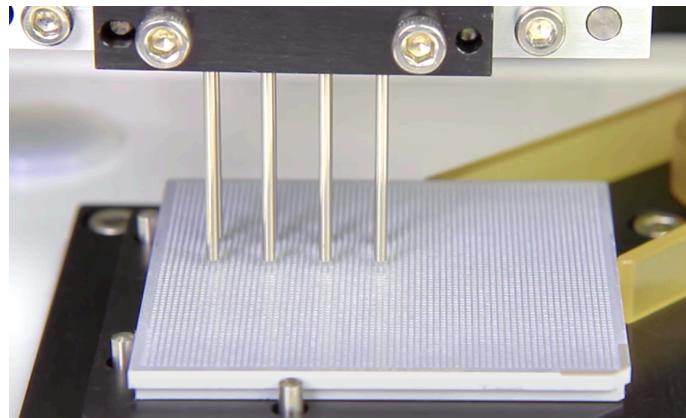
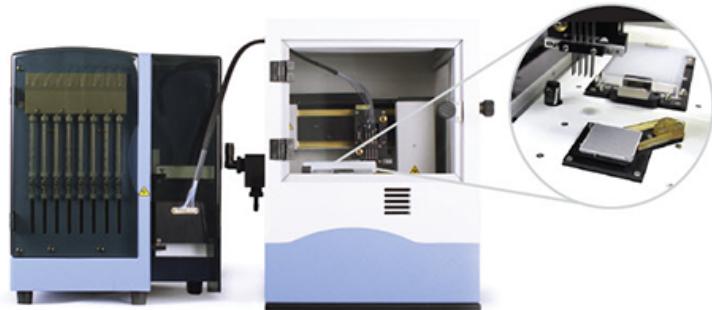
Fluidigm (C1):

Cell capture



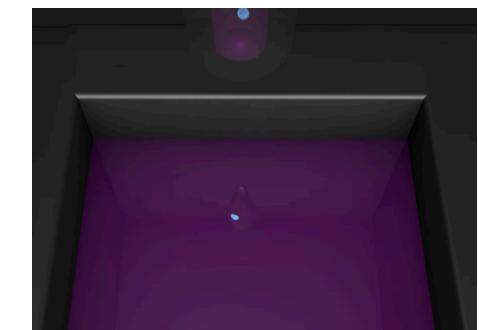
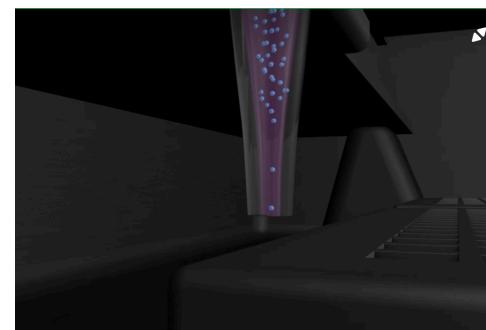
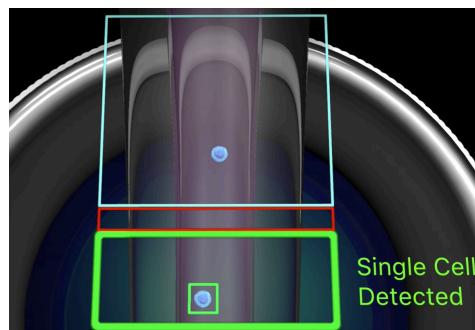
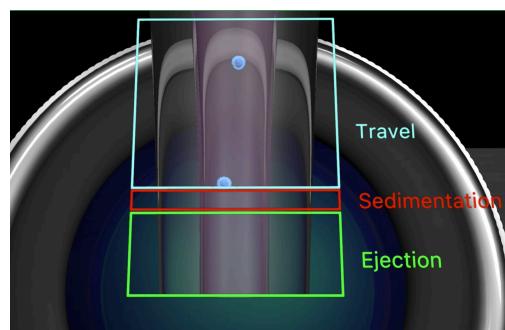
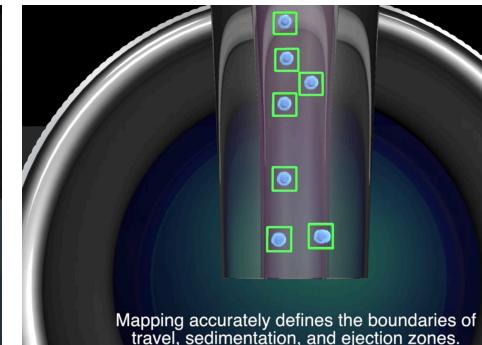
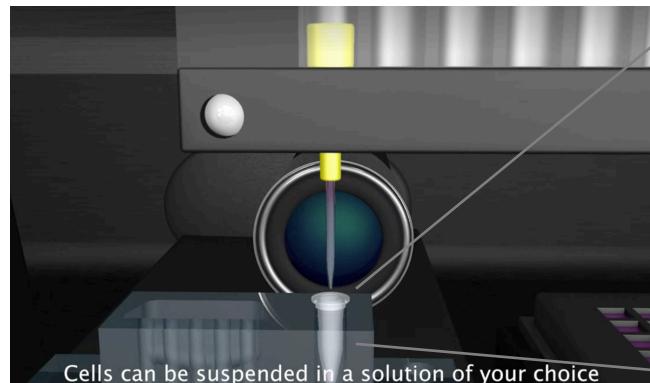
I) Single cell capture (Liquid-handling robotics)

Wafergen (iCell8)

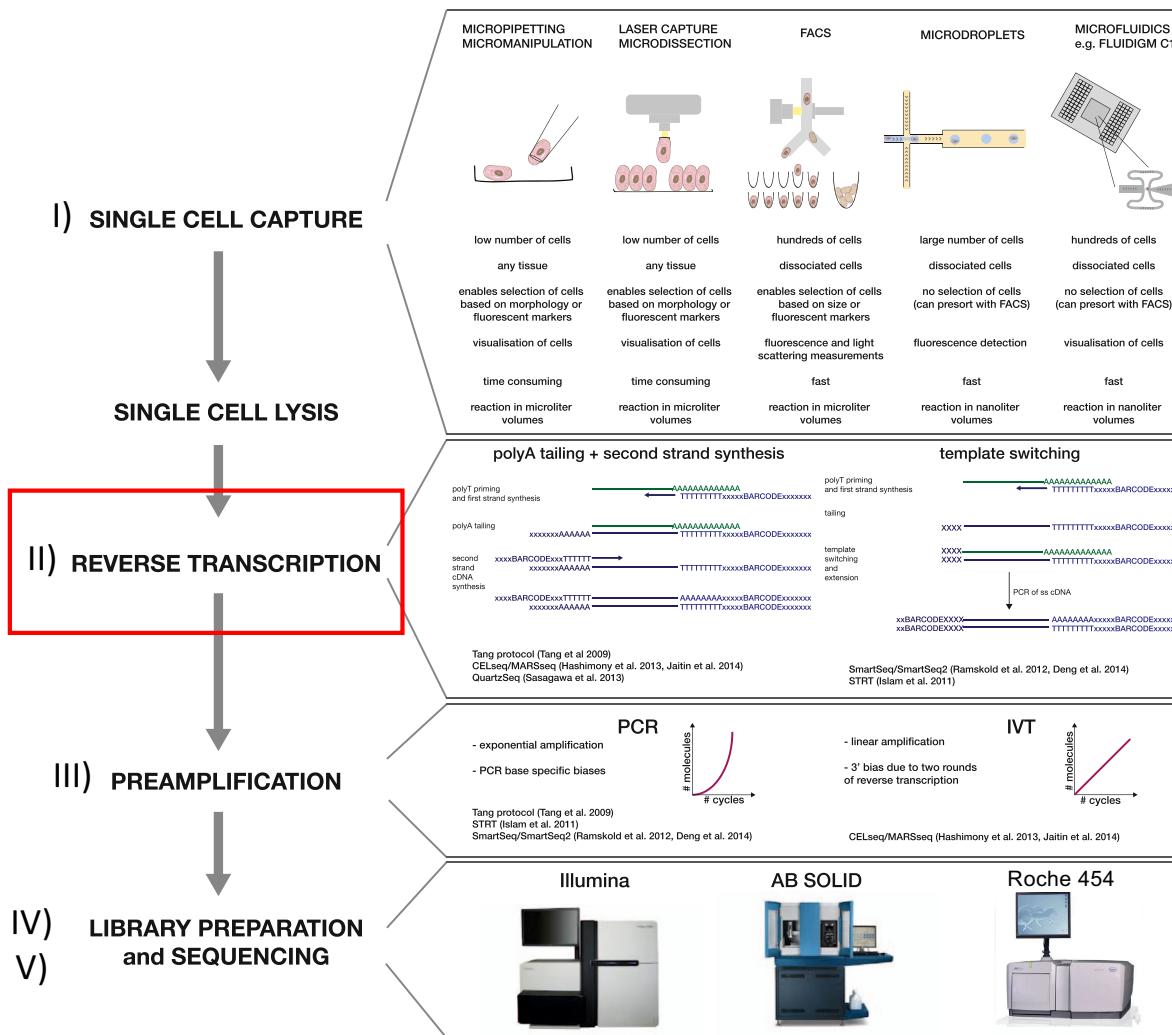


I) Single cell capture (Liquid-handling robotics)

Cellenion (CellenONE)



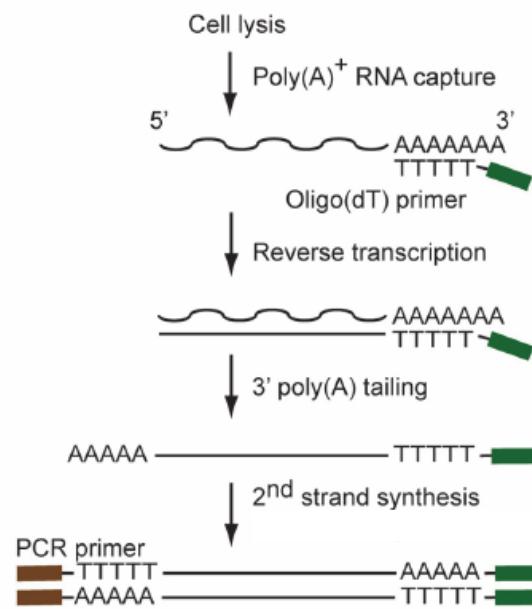
Single-cell RNA sequencing experiment workflow



II) Reverse transcription

Cel-Seq(2), (InDrop)

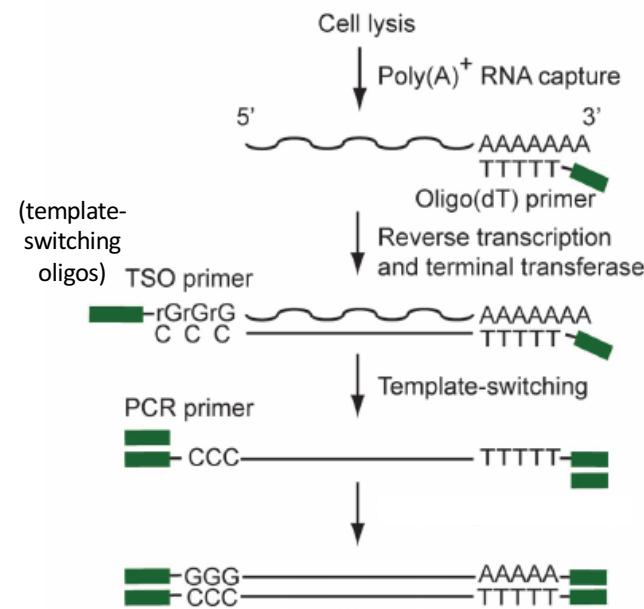
1) PolyA tailing + 2nd strand synthesis



PolyA tailing: added by template-free terminal transferase (in the presence of dATP)

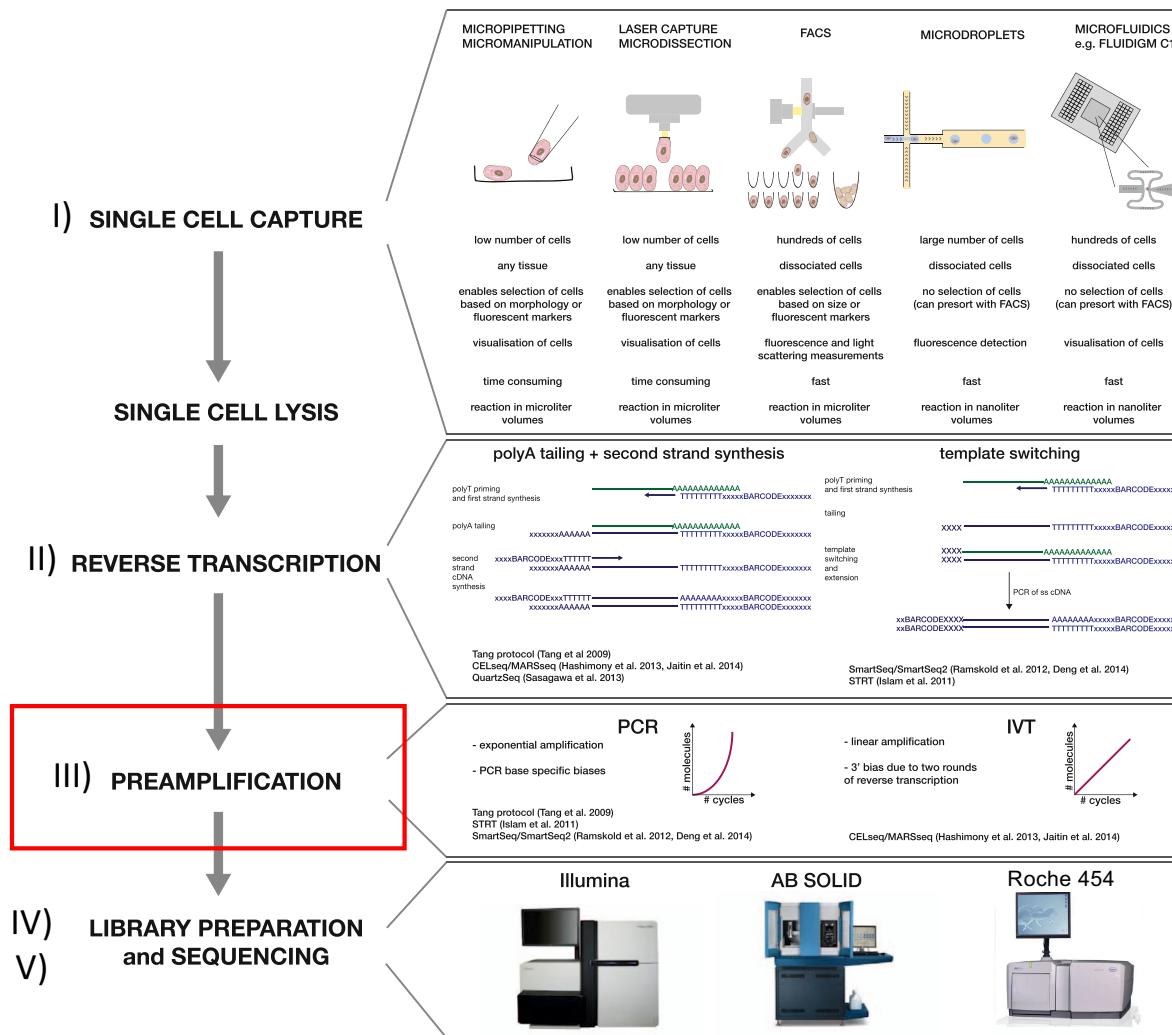
Smart-Seq(2), STRT-Seq, (Drop-seq, 10X)

2) Template switching



Template switching: added a few nucleotides in the 3'-end (usually "C") by MMLV reverse transcriptase

Single-cell RNA sequencing experiment workflow

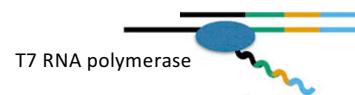


III) Preamplification

Cel-Seq(2), (InDrop)

(In vitro transcription)
IVT

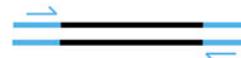
- linear amplification (slow)
- less error



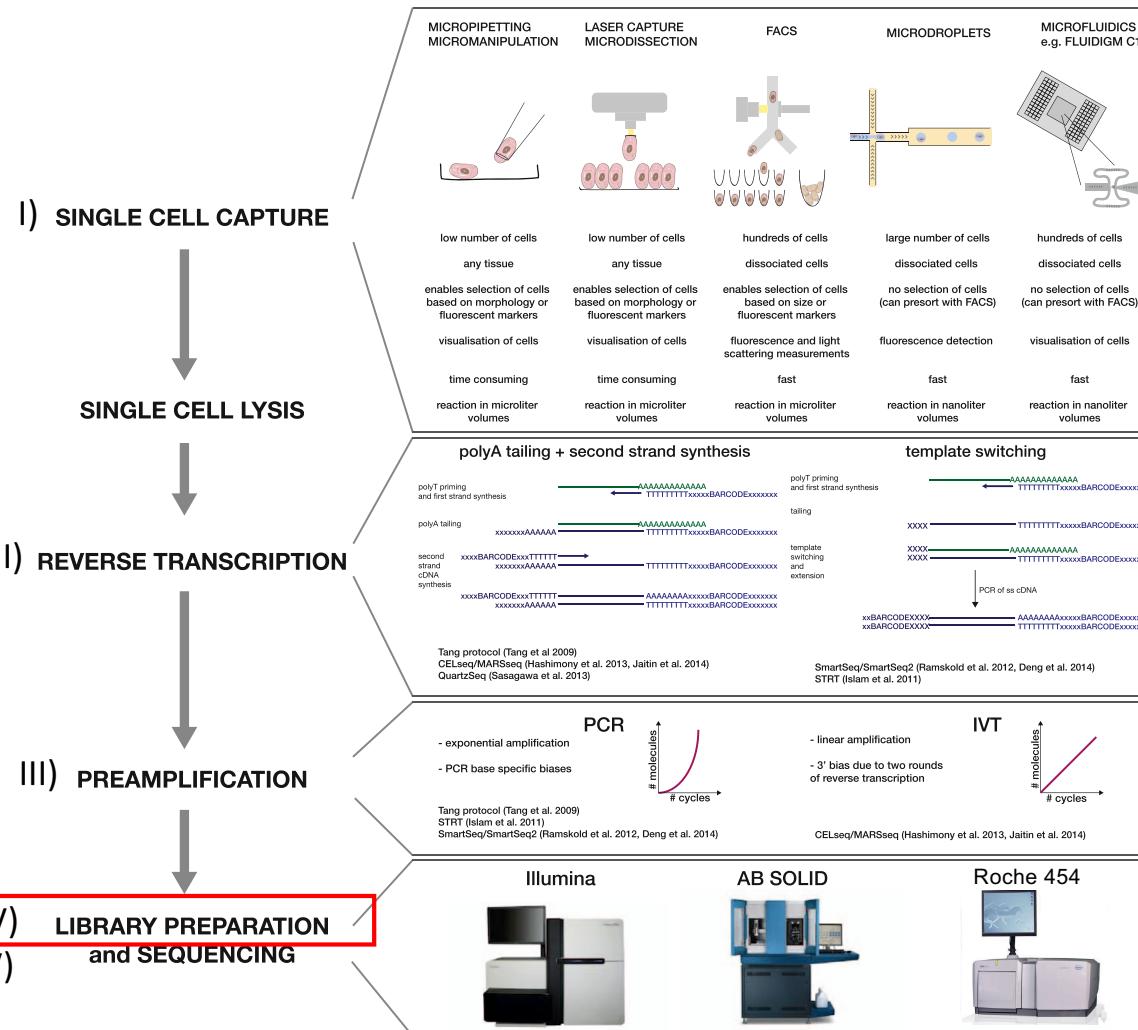
Smart-Seq(2), STRT-Seq, (Drop-seq, 10X)

PCR

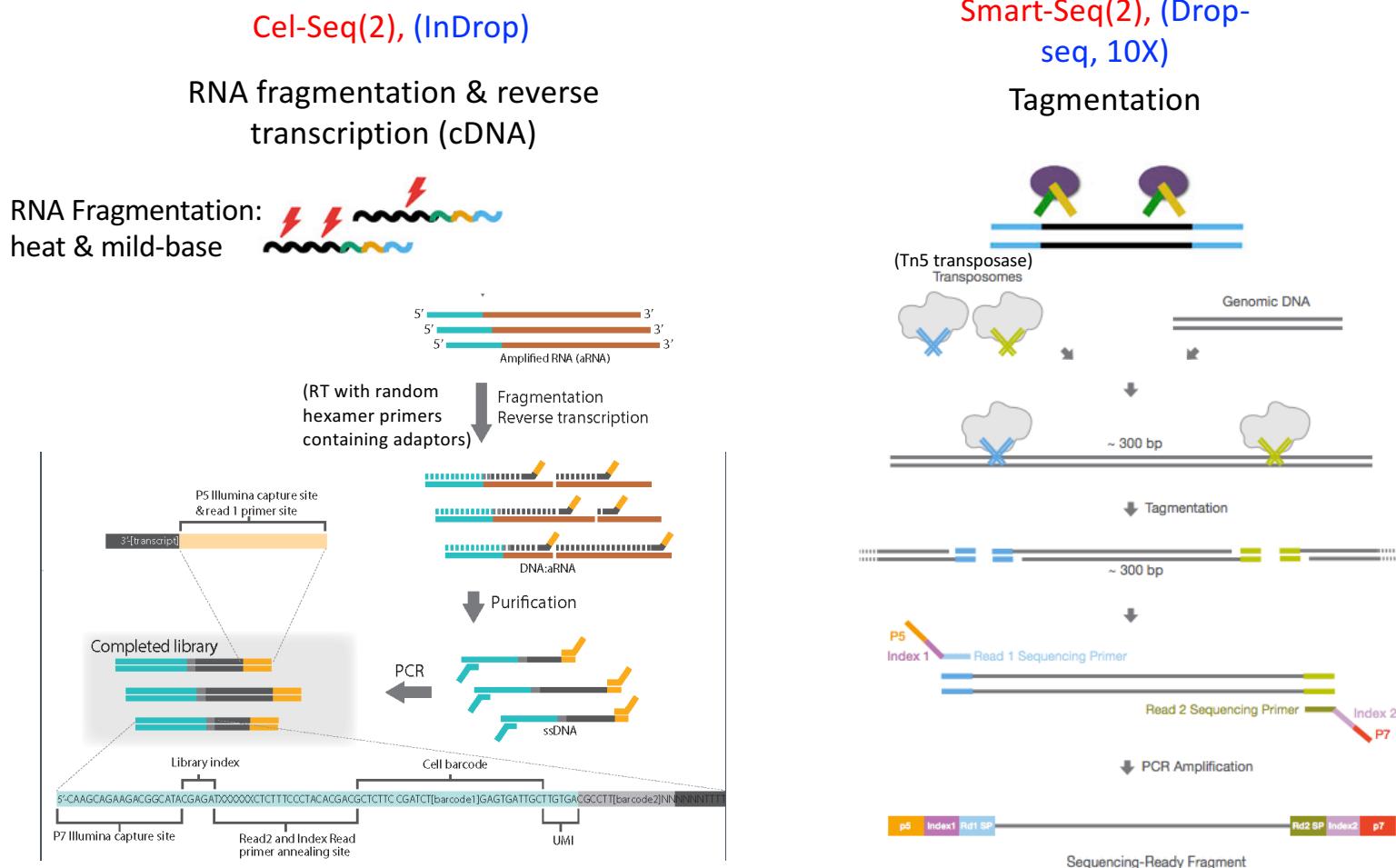
- exponential amplification (fast)
- error prone



Single-cell RNA sequencing experiment workflow



IV) Library preparation



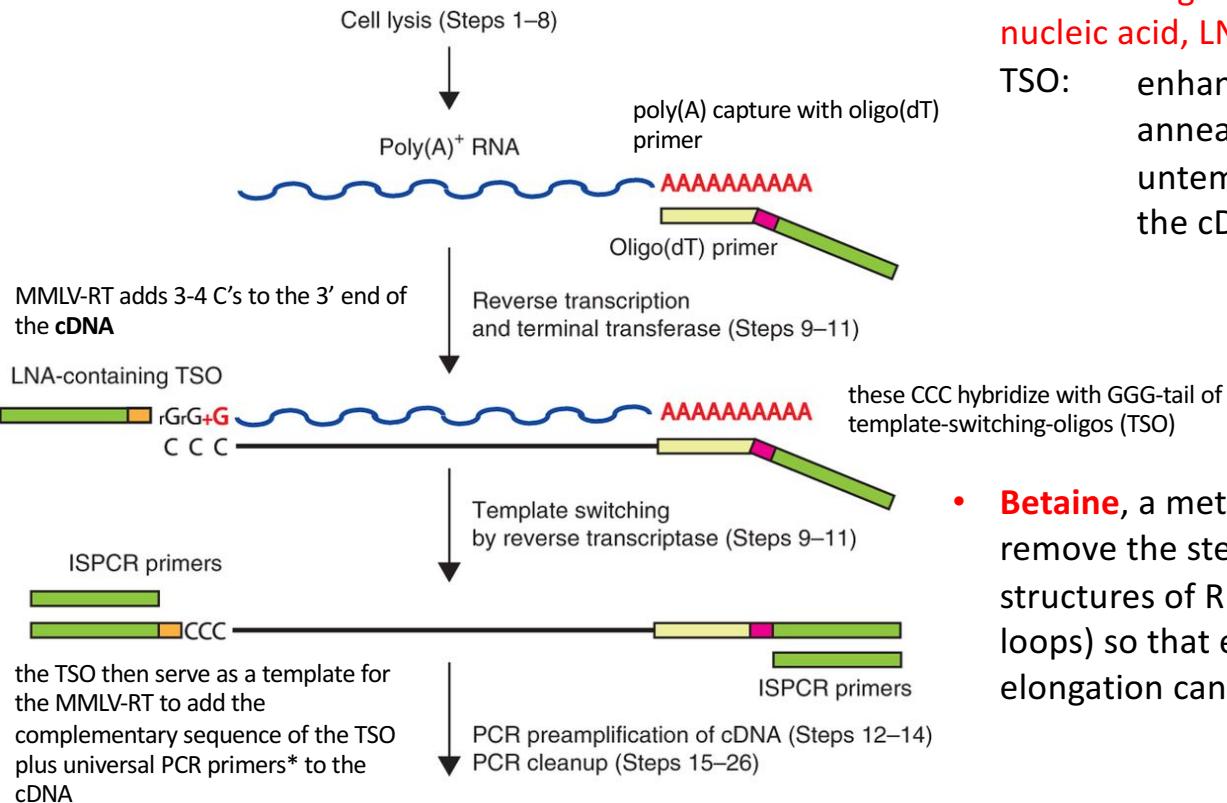
Three most popular plate-based scRNaseq

- SMART-seq2
- CEL-seq2
- STRT-seq

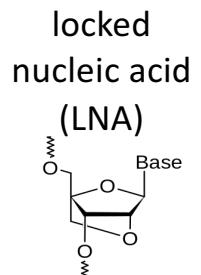
SMART-seq2

“SMART”: Switching Mechanism At the 5' end of the RNA Transcript

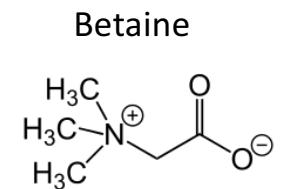
RNA capture and cDNA synthesis



- A modified guanosine (a locked nucleic acid, LNA) is incorporated in TSO: enhance thermal stability & anneal strongly to the untemplated 3' extension of the cDNA

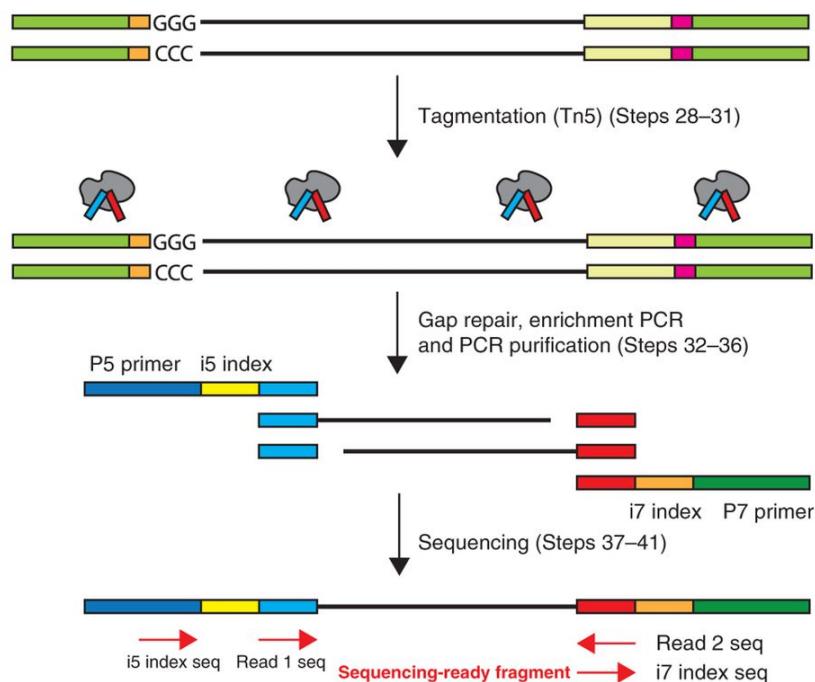


- **Betaine**, a methyl group donor, can remove the steric hindrance of secondary structures of RNAs (such as hairpins or loops) so that early termination of chain elongation can be blocked.



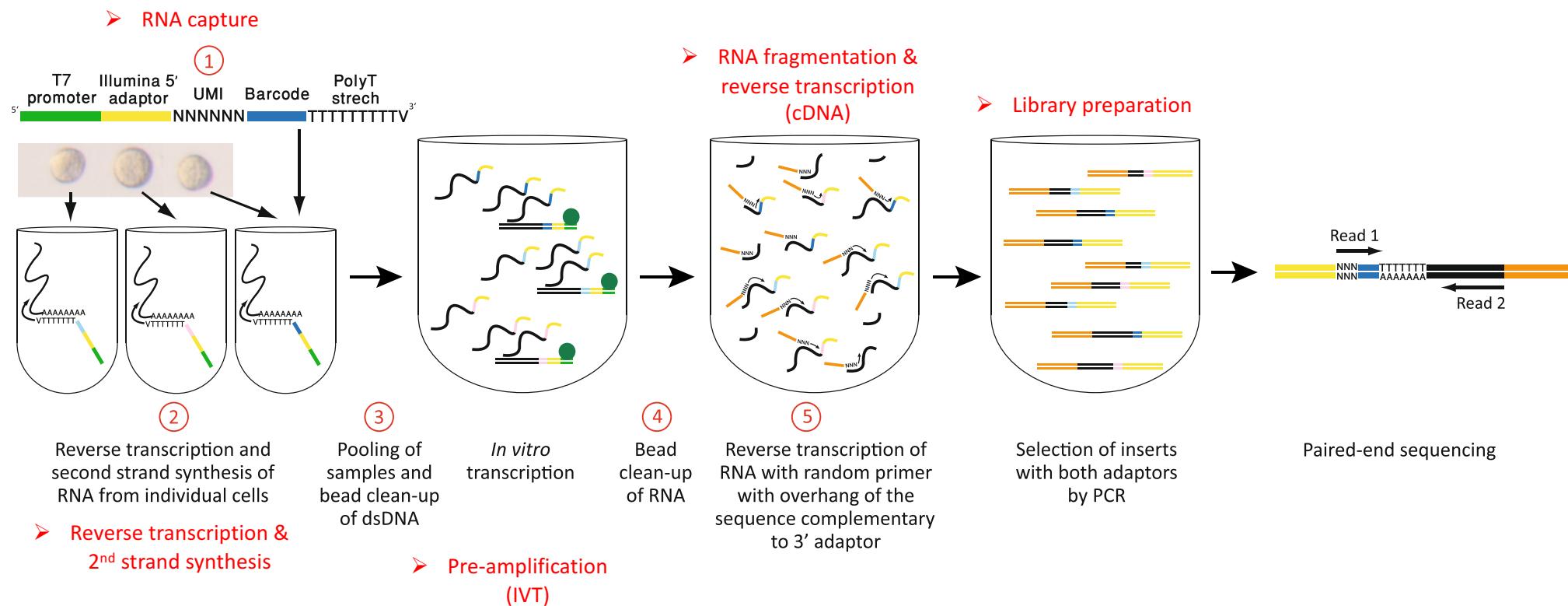
SMART-seq2

Library preparation

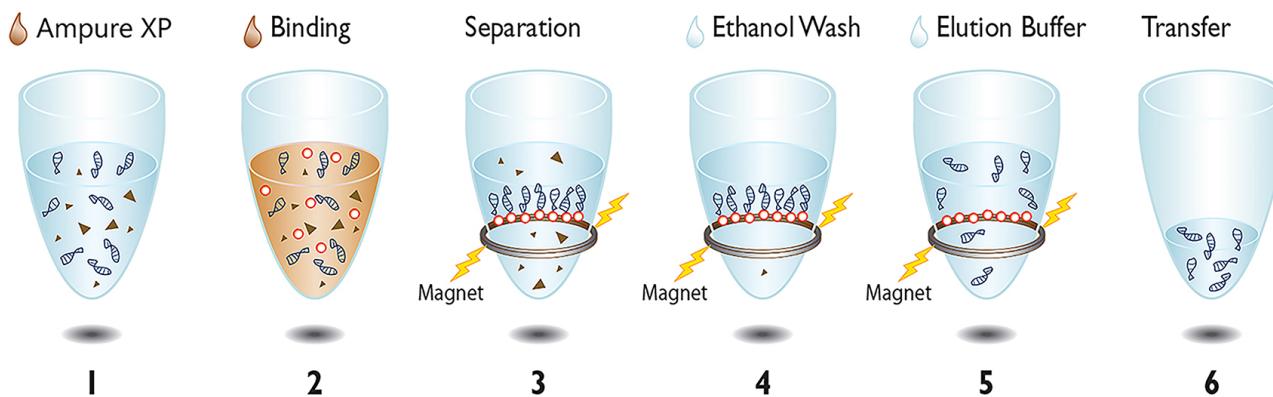


- amplification with few PCR cycles
- **tagmentation:** combining fragmentation and sequencing adapter integration
 - hyperactive derivative of the Tn5 transposase **cuts** the cDNA and **ligates** sequencing adapters

CEL-seq2



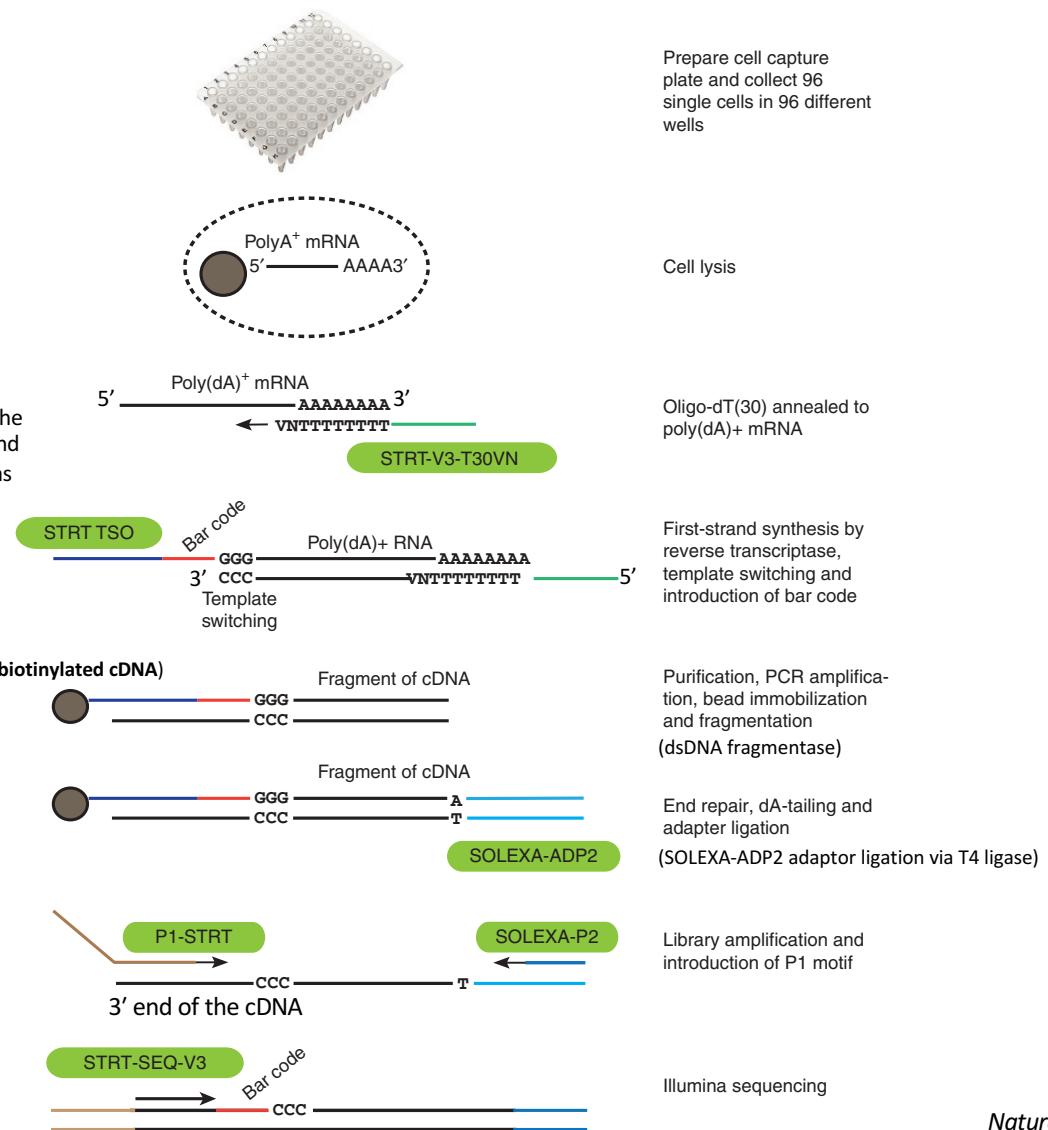
SPRI Bead technology



- **Solid Phase Reversible Immobilization**
- Carboxyl coated magnetic particles suspended in a solution of 10% PEG and 1.25M NaCl
- **Reversibly binds DNA**
 - Hawkins, et al. (1994) DNA purification and isolation using a solid-phase. Nucleic Acids Research, 22(21):4543-4544

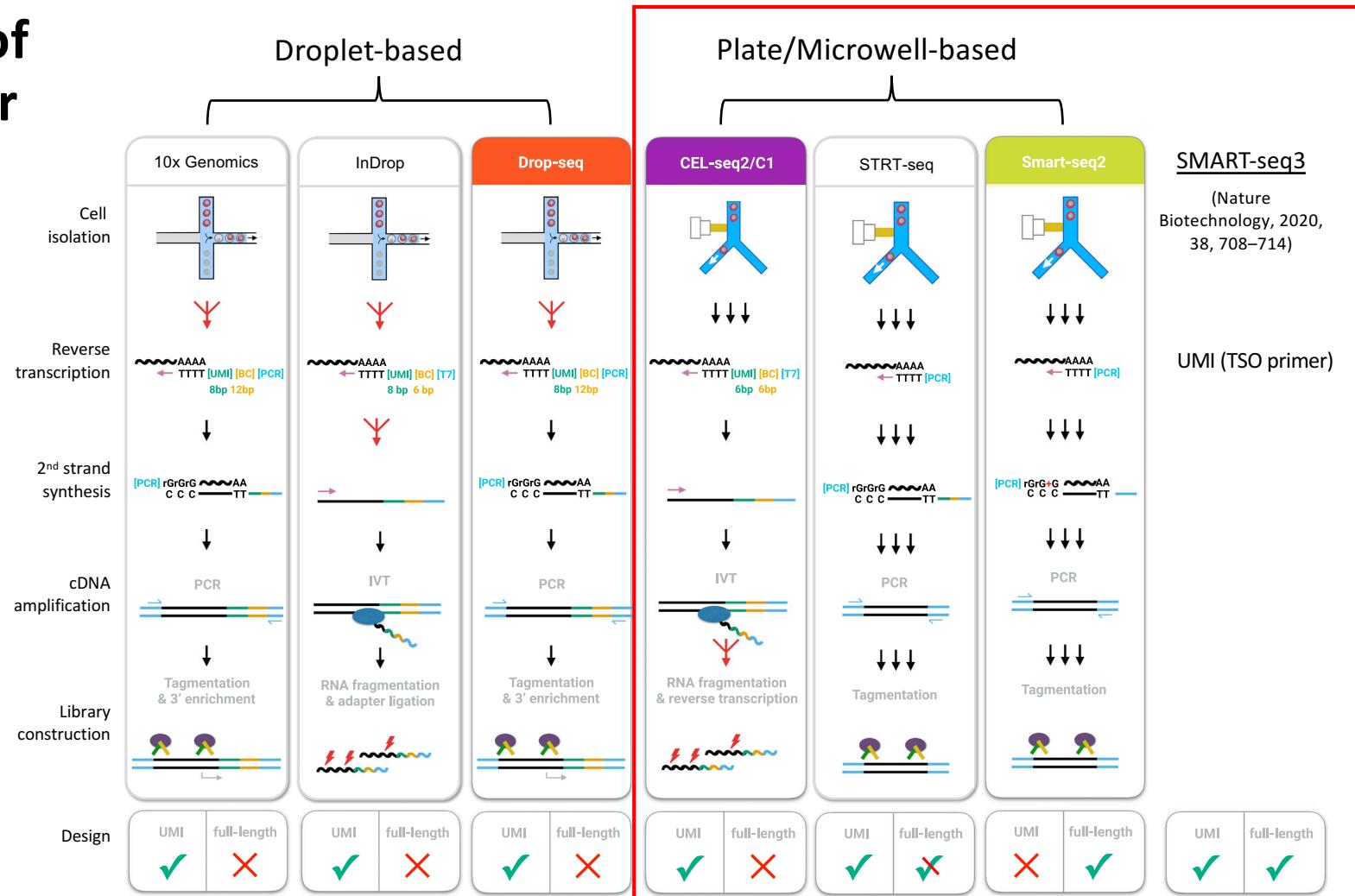
STRT-seq: single-cell tagged reverse transcription sequencing

An upstream sequence must be introduced at the 3' end of the cDNA (5' end of the mRNA) to serve as template for the amplification using a universal primer (**biotinylated primer**)

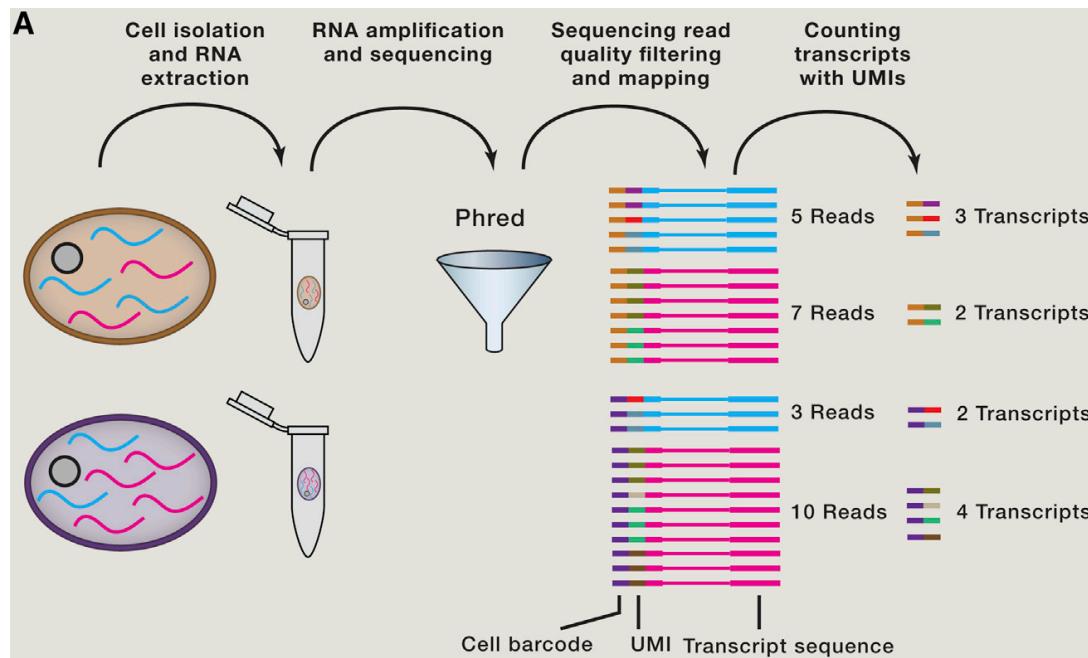


In this method, the sequenced fragments correspond to a template-switching site located preferentially at the **5' end of mRNA**, which can be used to analyze promoter usage in single cells, to characterize **transcription start sites** and to analyze enhancer elements.

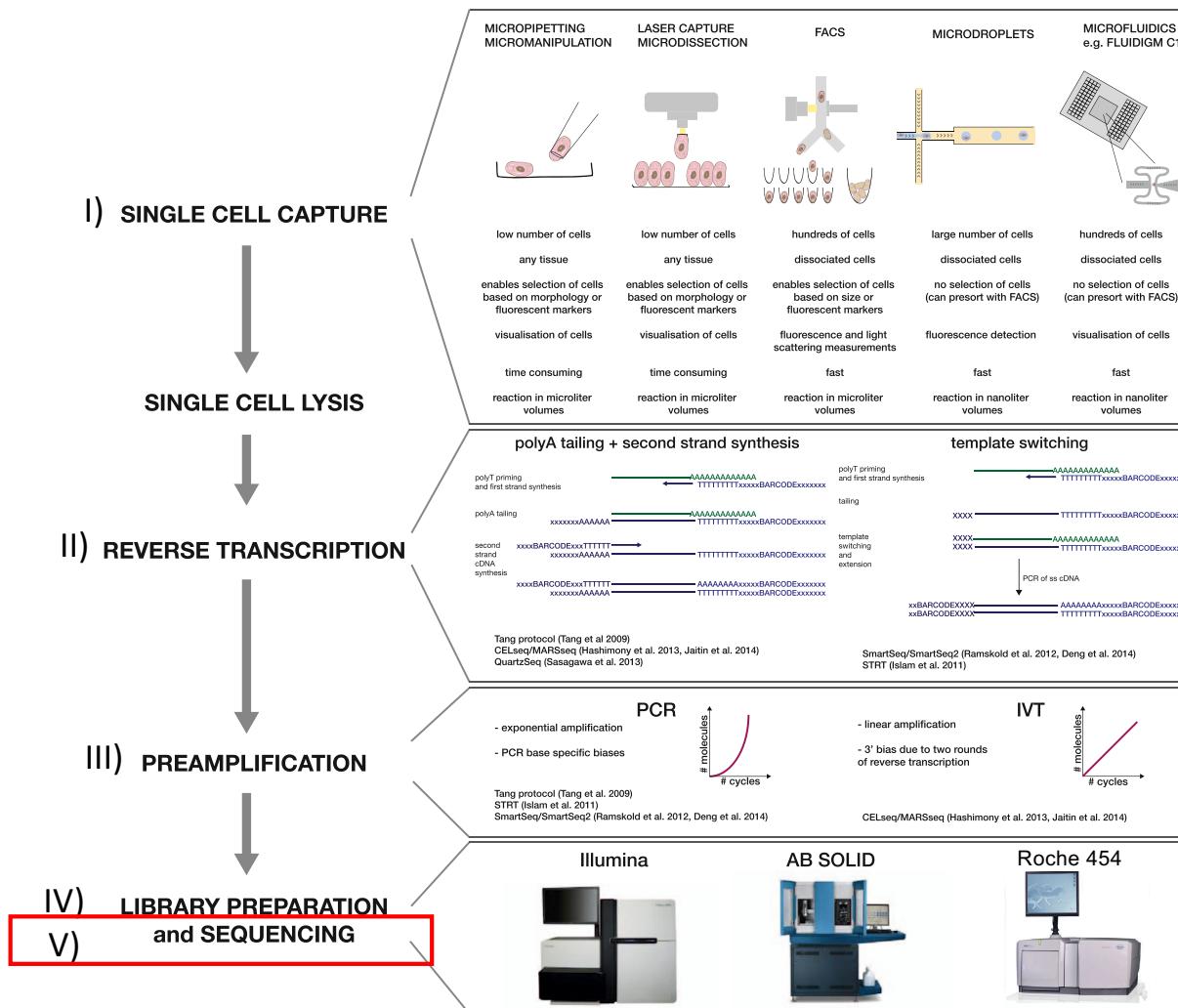
Summary of the popular scRNAseq methods



Quantification of mRNA Expression with unique molecular identifiers (UMIs)



Single-cell RNA sequencing experiment workflow



Conclusion for Part I (plate-based scRNAseq)

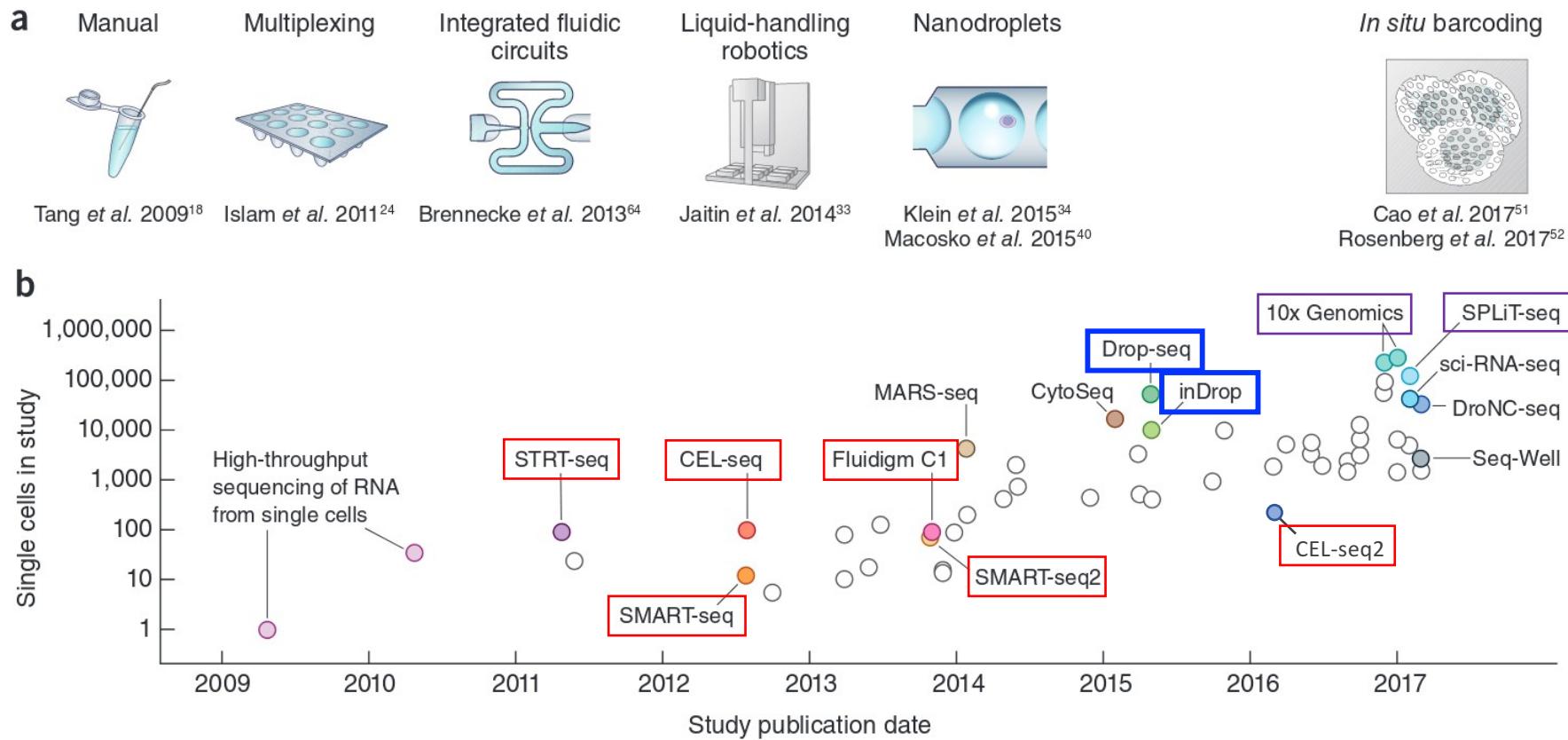
- **General introduction about scRNAseq**
 - scSeq vs NGS
- **Different types of plate-based scRNAseq**
 - SMART-seq2, CEL-seq2, STRT-seq
- **Workflow of different plate-based scRNAseq**
 - Single cell capture, cell lysis, reverse transcription, pre-amplification, library preparation, sequencing

Part II: Droplet-based single cell RNA sequencing (scRNAseq)

Outline of the Part II (Droplet-based scRNASeq)

- General introduction about droplet-based scRNASeq
- Different types of droplet-based scRNASeq
- Workflow of different droplet-based scRNASeq

Evolution of scRNAseq techniques



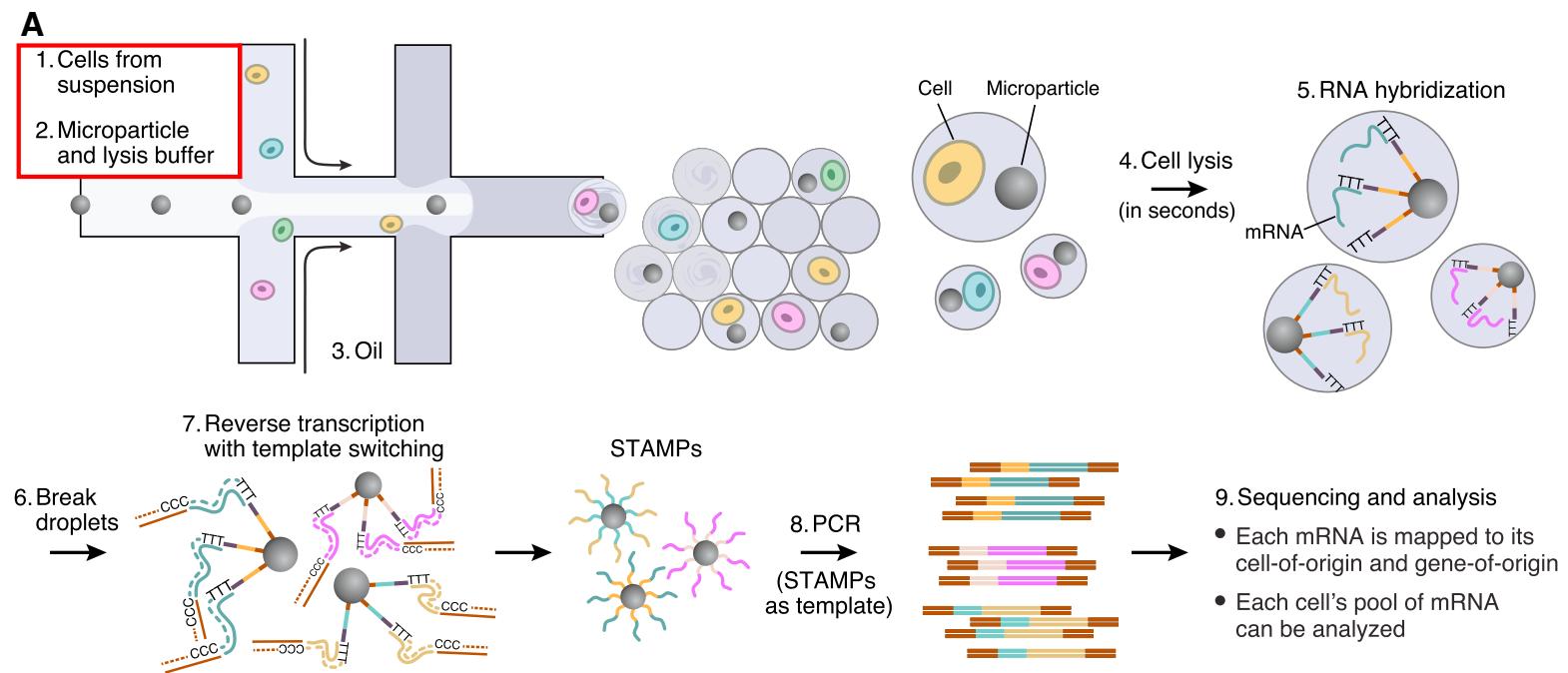
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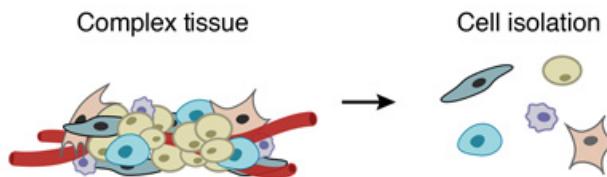
Svensson et al. *Nature Protocols* (2018)

DropSeq overview

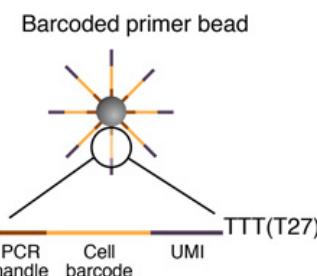


DropSeq overview

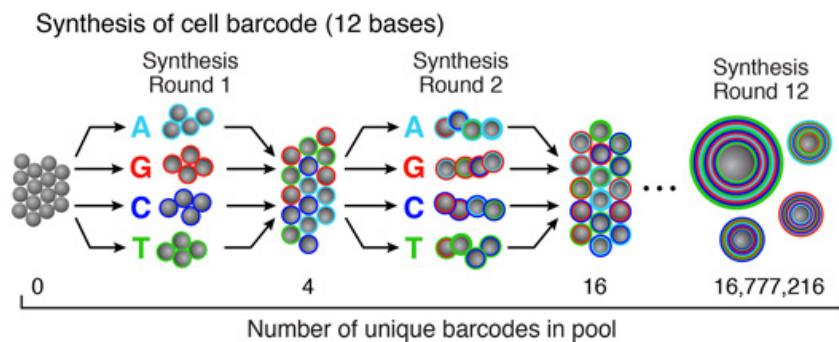
1. PREPARE A SINGLE-CELL SUSPENSION FROM A COMPLEX TISSUE



2. MICROPARTICLE PREPARATION

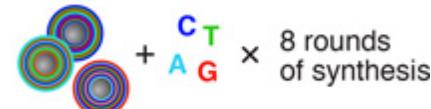


Split-and-pool synthesis of the cell barcode



Synthesis of a unique molecular identifier (UMI)

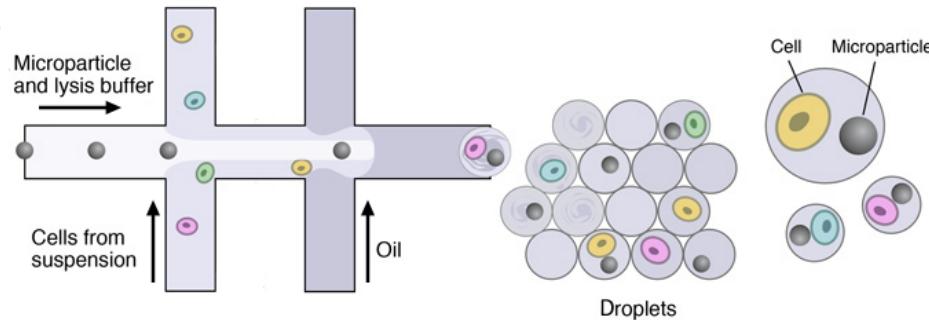
Synthesis of UMI (8 bases)



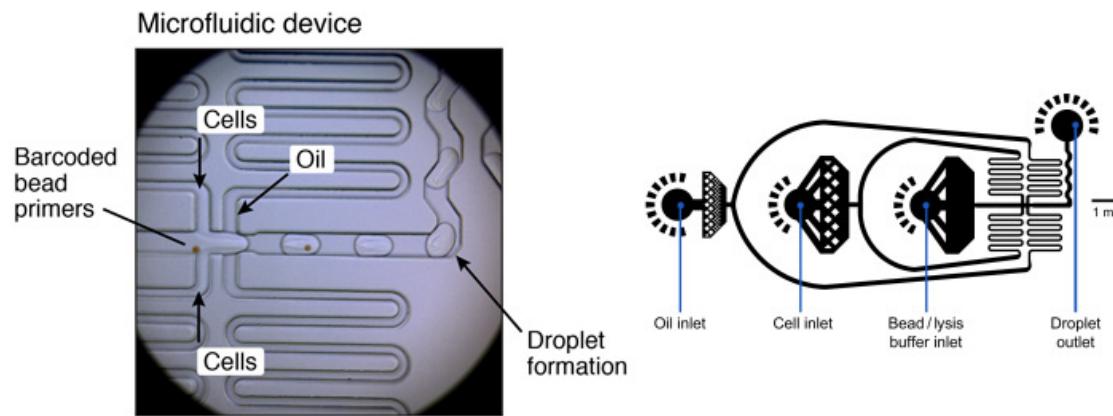
- Millions of the same cell barcode per bead
- 4^8 different molecular barcodes (UMIs) per bead

DropSeq workflow

3. MICROFLUIDIC DEVICE



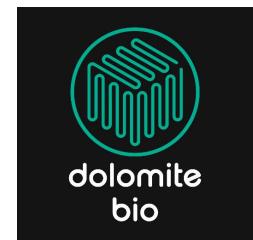
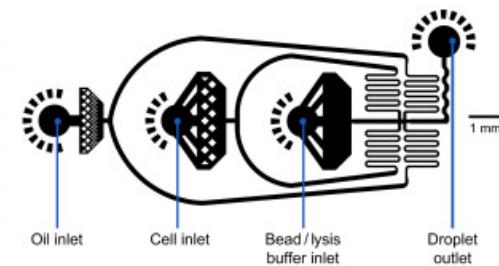
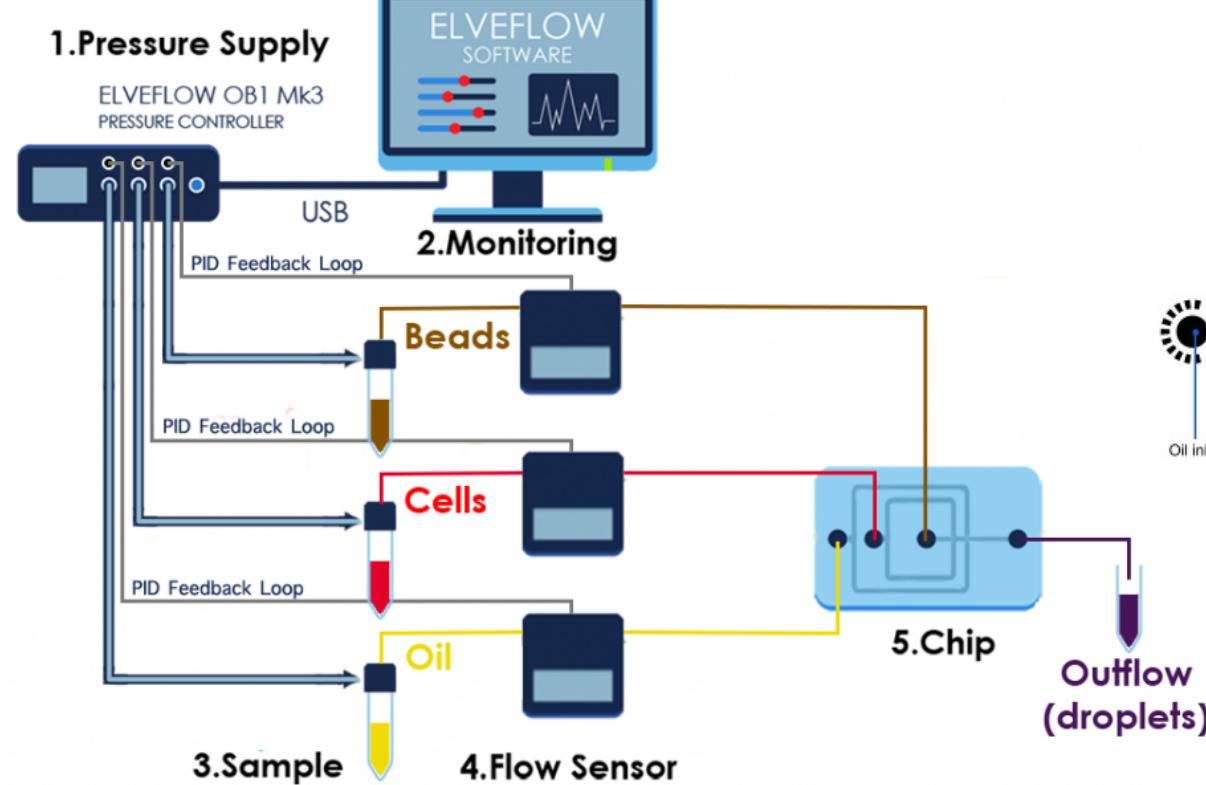
Microfluidic setup



DropSeq workflow

3. MICROFLUIDIC DEVICE

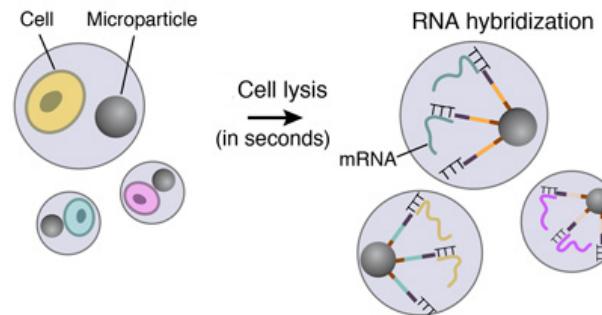
Set-up Diagram



Cell, 2015, 161, 1202–1214

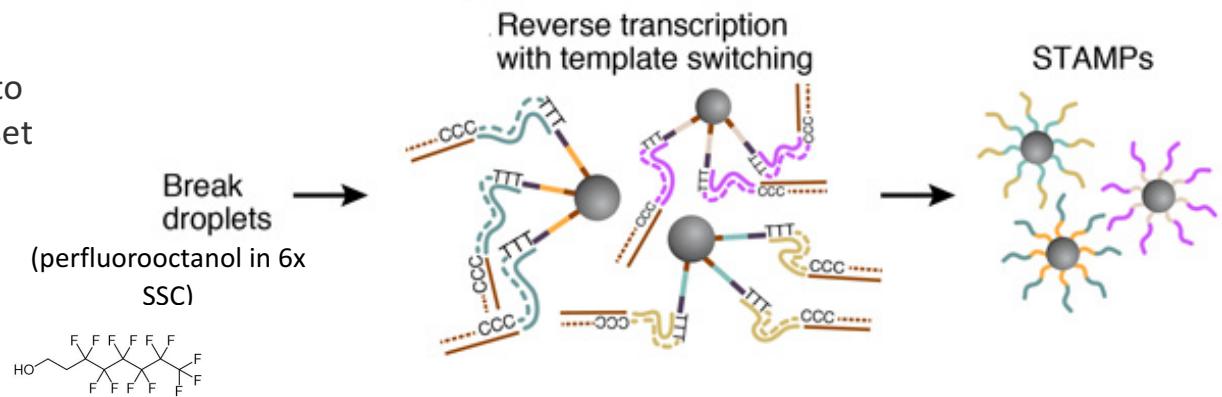
DropSeq workflow

4. CELL LYSIS AND RNA HYBRIDIZATION



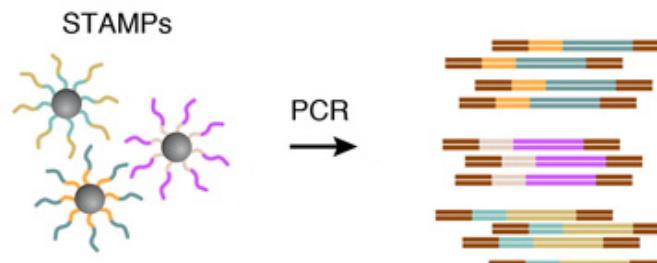
5. STAMPS GENERATION

The mRNAs are then reverse-transcribed into cDNAs together in one reaction, forming a set of beads called “single-cell transcriptomes” attached to microparticles” (STAMPs).



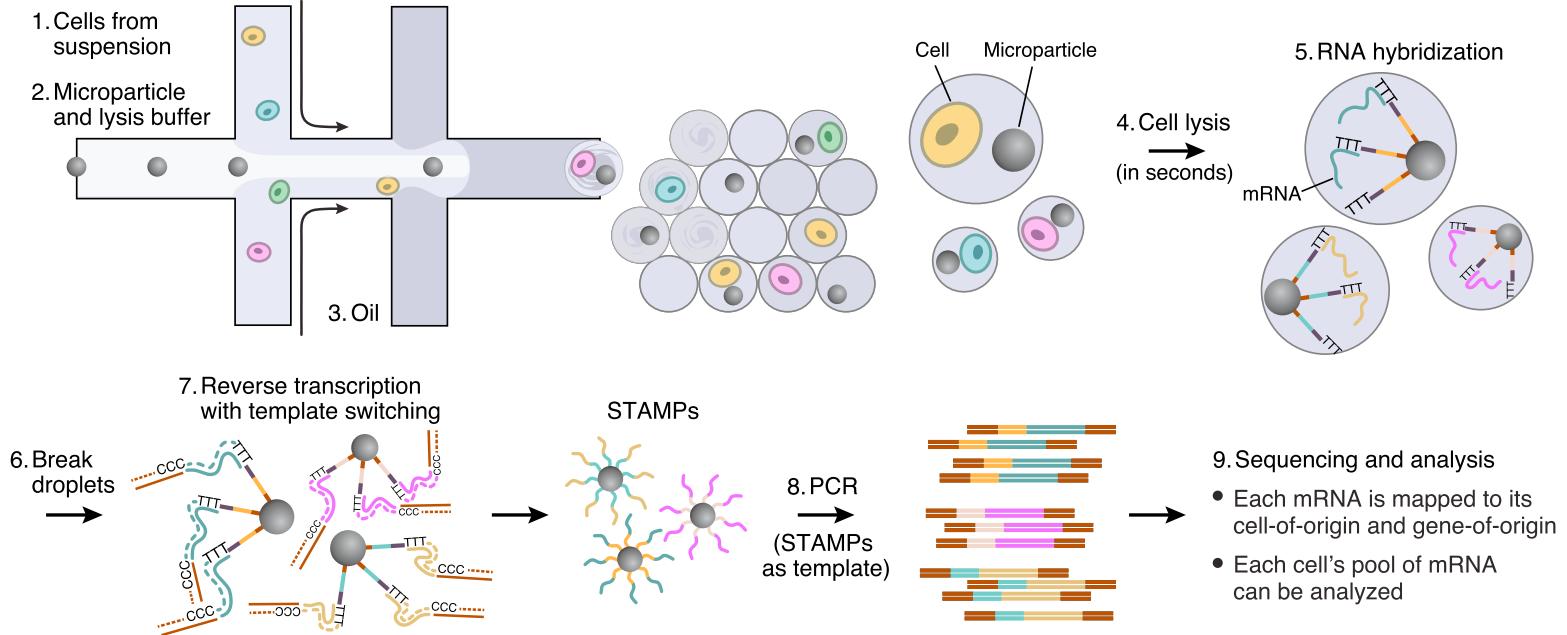
6. AMPLIFICATION OF STAMPS

The barcoded STAMPs can then be amplified in pools by PCR reaction for high-throughput mRNA-sequencing, to analyze any desired number of individual cells.

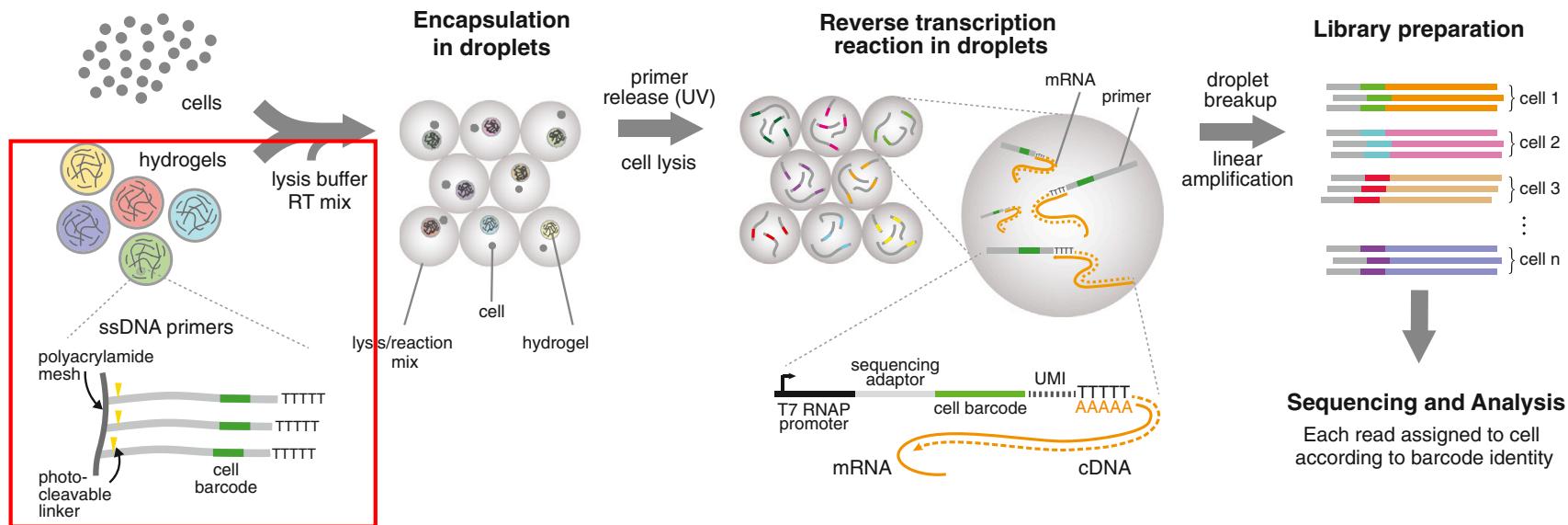


DropSeq overview

A



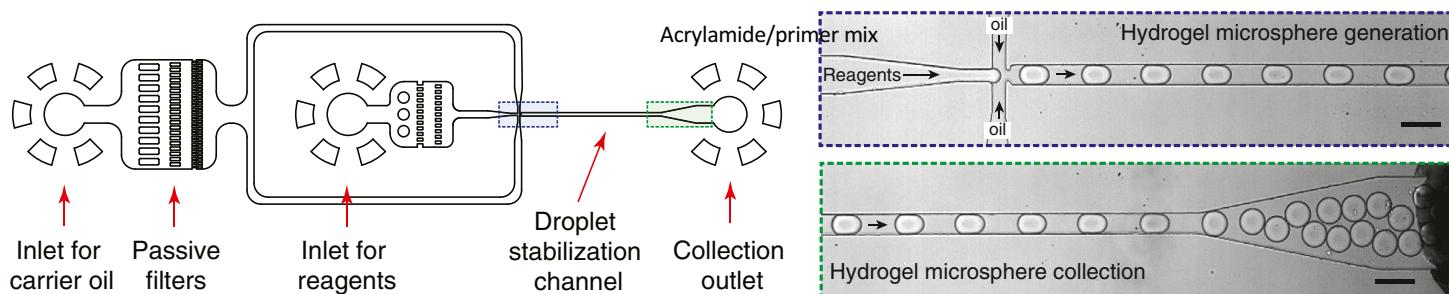
InDrop overview



InDrop workflow

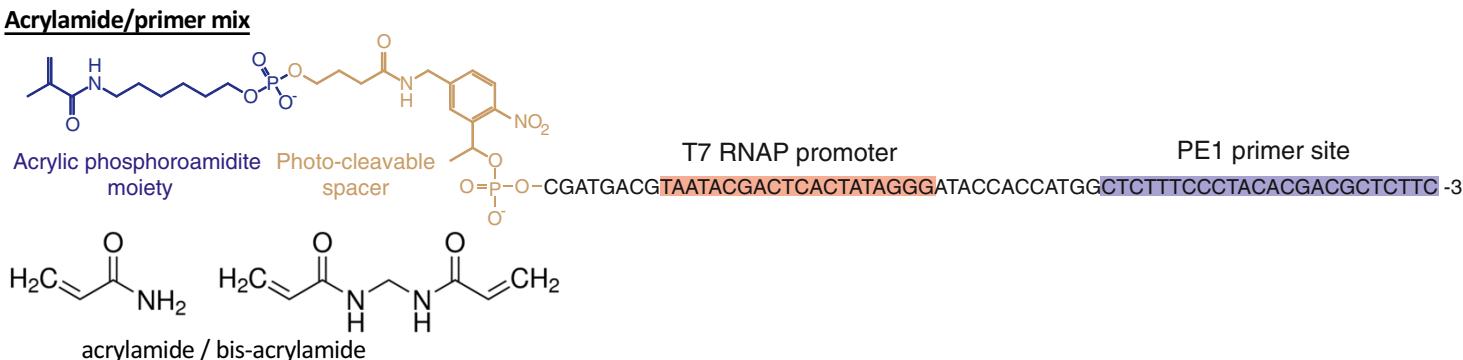
I) Synthesis of barcoded hydrogel beads

A



Droplet collection
and off-chip
polymerization

B



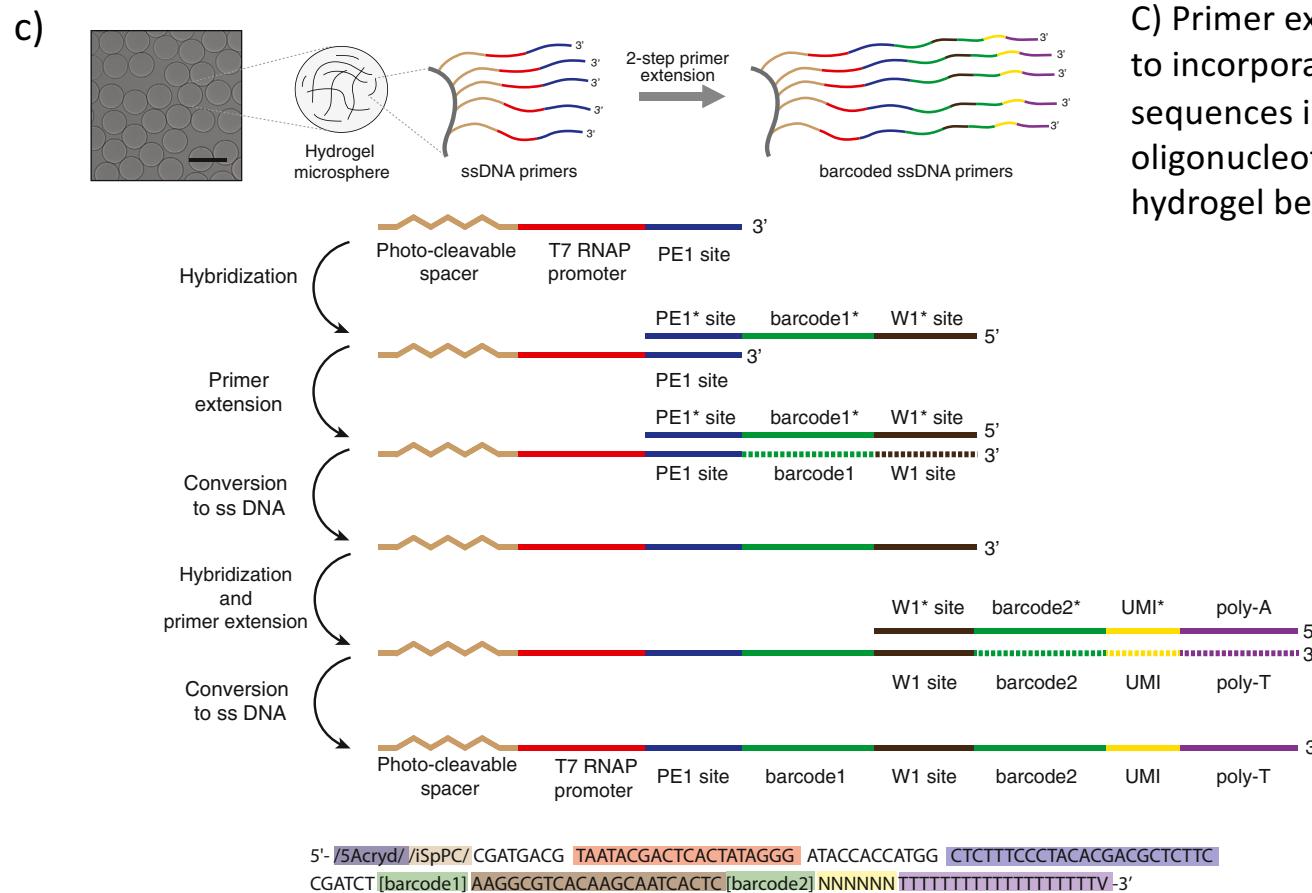
(A-B) Hydrogel bead generation and collection. An aqueous acrylamide/bis-acrylamide solution carrying acrydite-modified DNA oligonucleotide is emulsified using a microfluidic device to yield highly monodispersed droplets, which are collected off-chip and polymerized into hydrogel beads. Scale bars, 100 μ m.

1CELLBIO

Cell, 2015, 161, 1187–1201

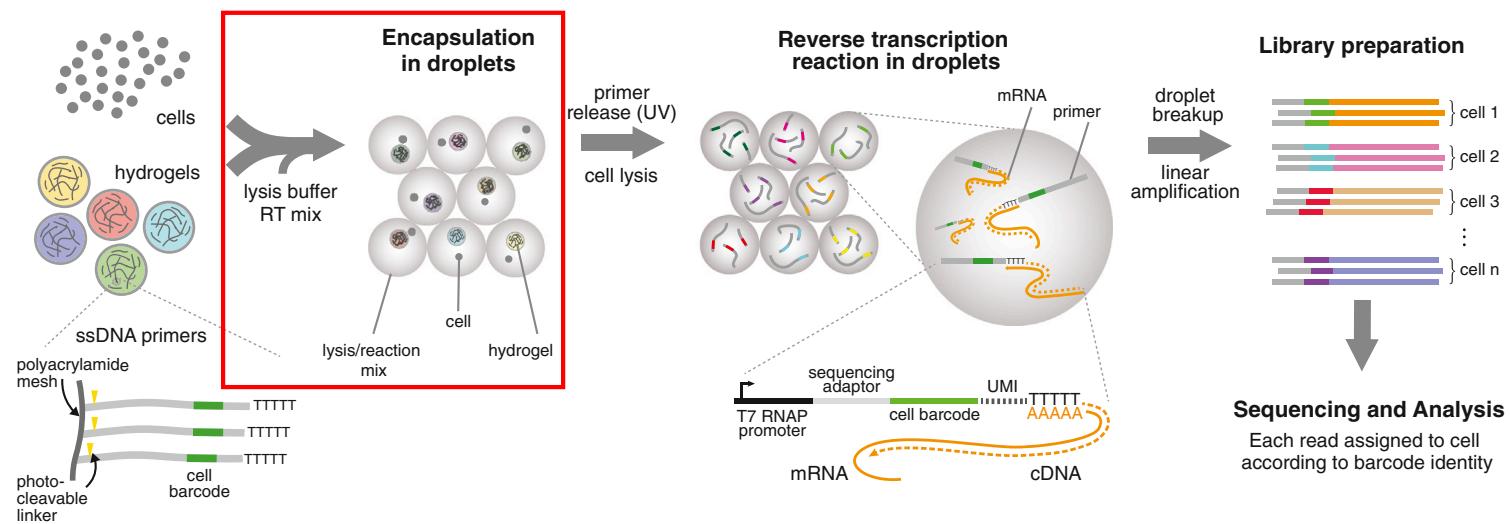
InDrop workflow

I) Synthesis of barcoded hydrogel beads



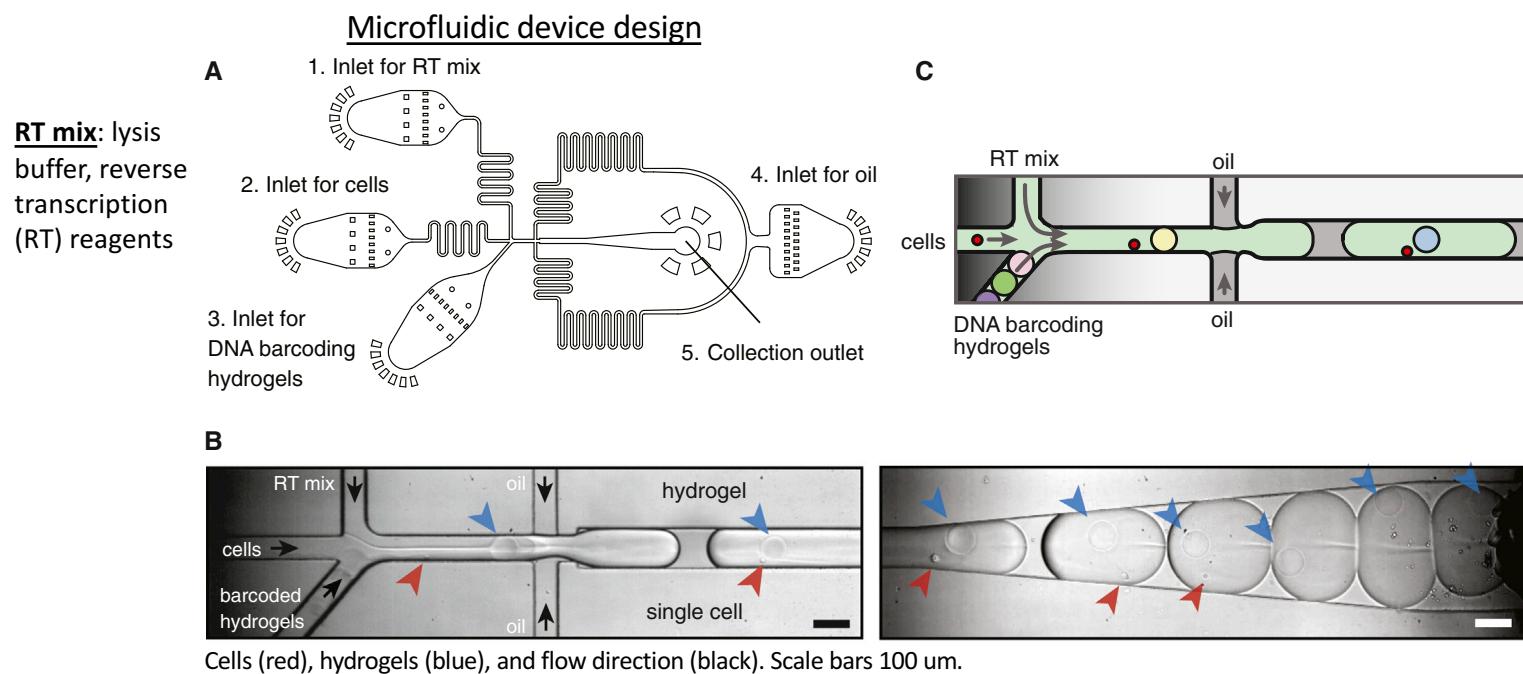
C) Primer extension reaction to incorporate barcode sequences into DNA oligonucleotides attached to hydrogel beads

InDrop overview

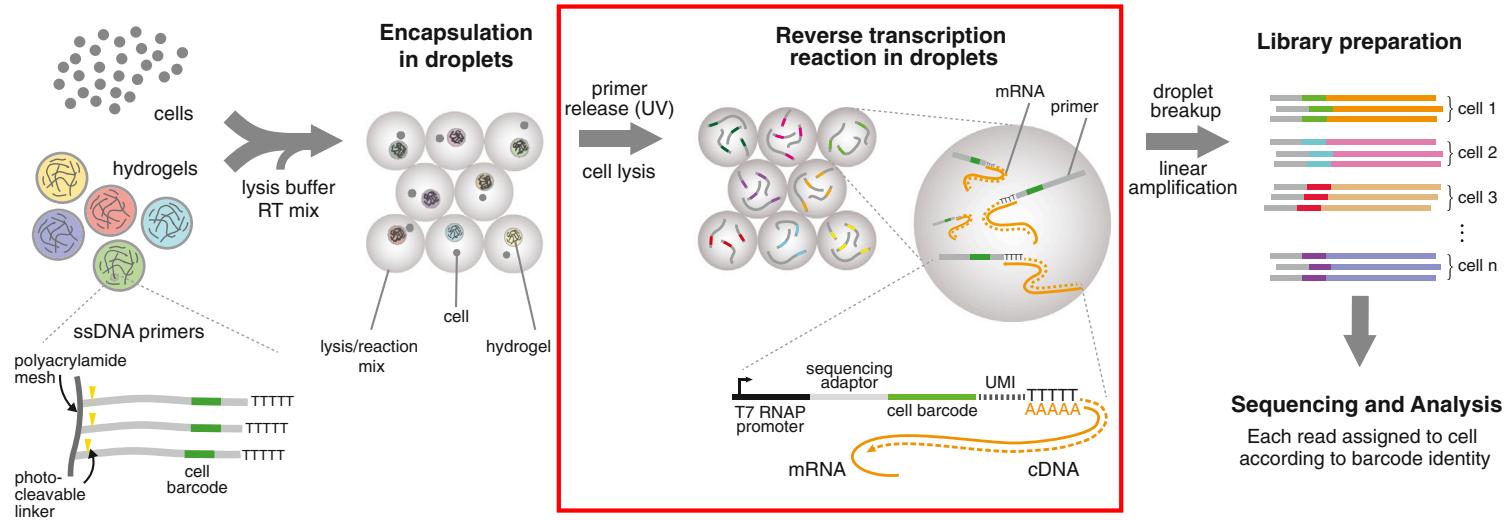


InDrop workflow

II) Encapsulation in droplets



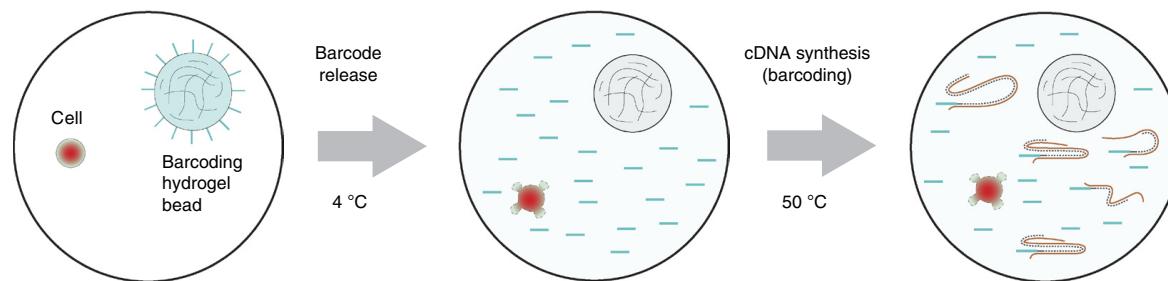
InDrop overview



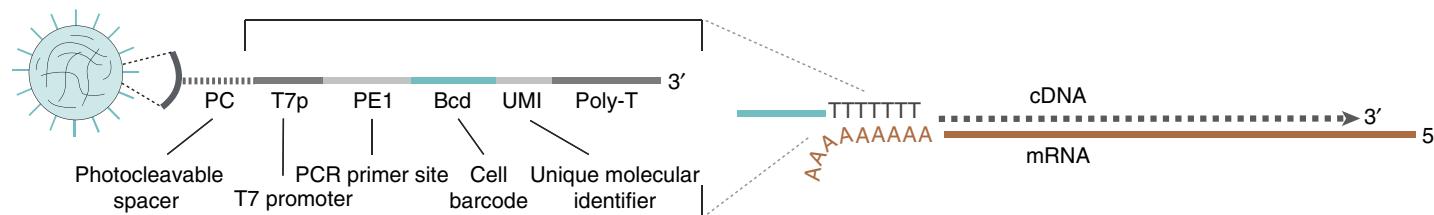
InDrop workflow

III) Reverse transcription in droplets

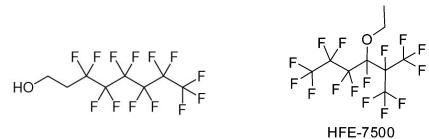
- After cell and hydrogel bead encapsulation, the barcoded cDNA primers are released from the beads using **365 nm UV light** ($\sim 10 \text{ mW/cm}^2$; which is not damaging to DNA/RNA), followed by mRNA capture and reverse transcription



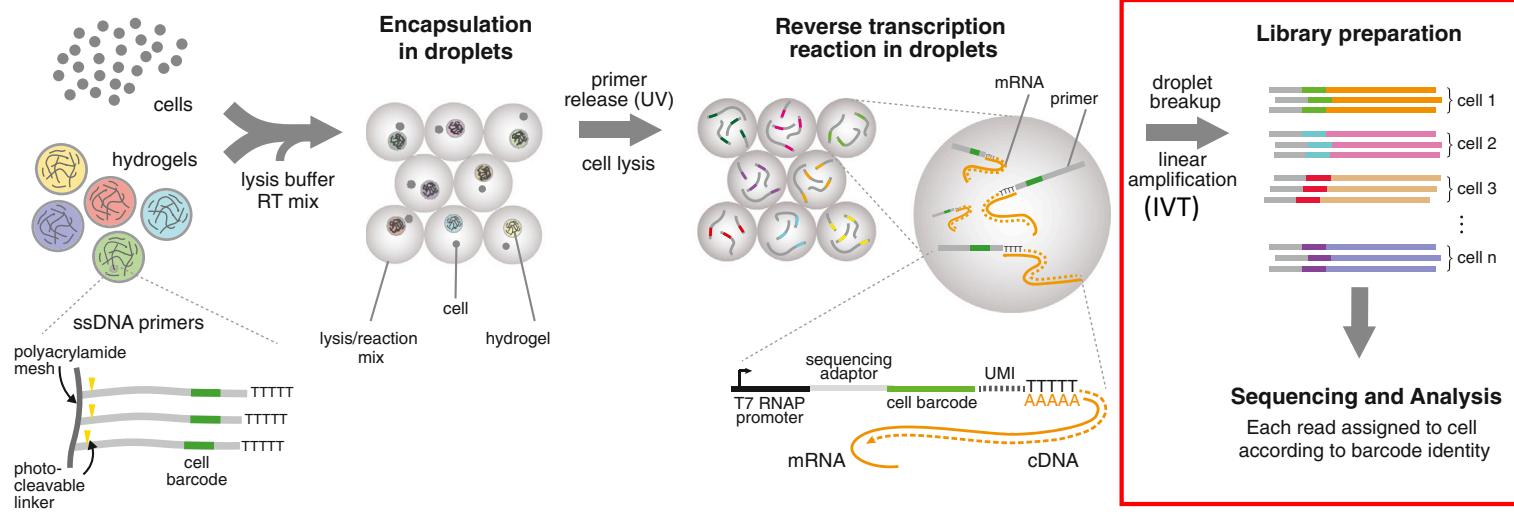
- mRNA capture and reverse transcription



InDrop overview



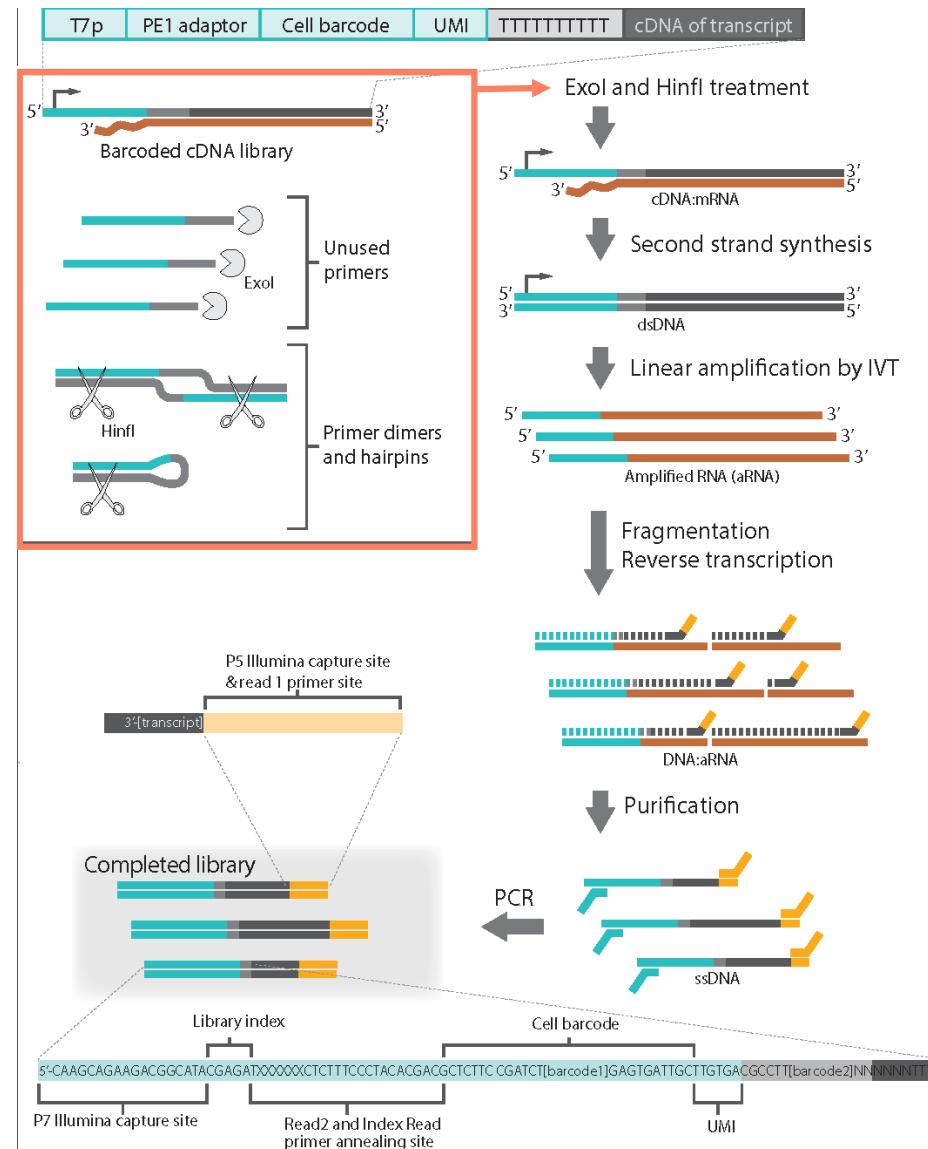
20% (v/v) perfluorooctanol + 80% (v/v) HFE-7500



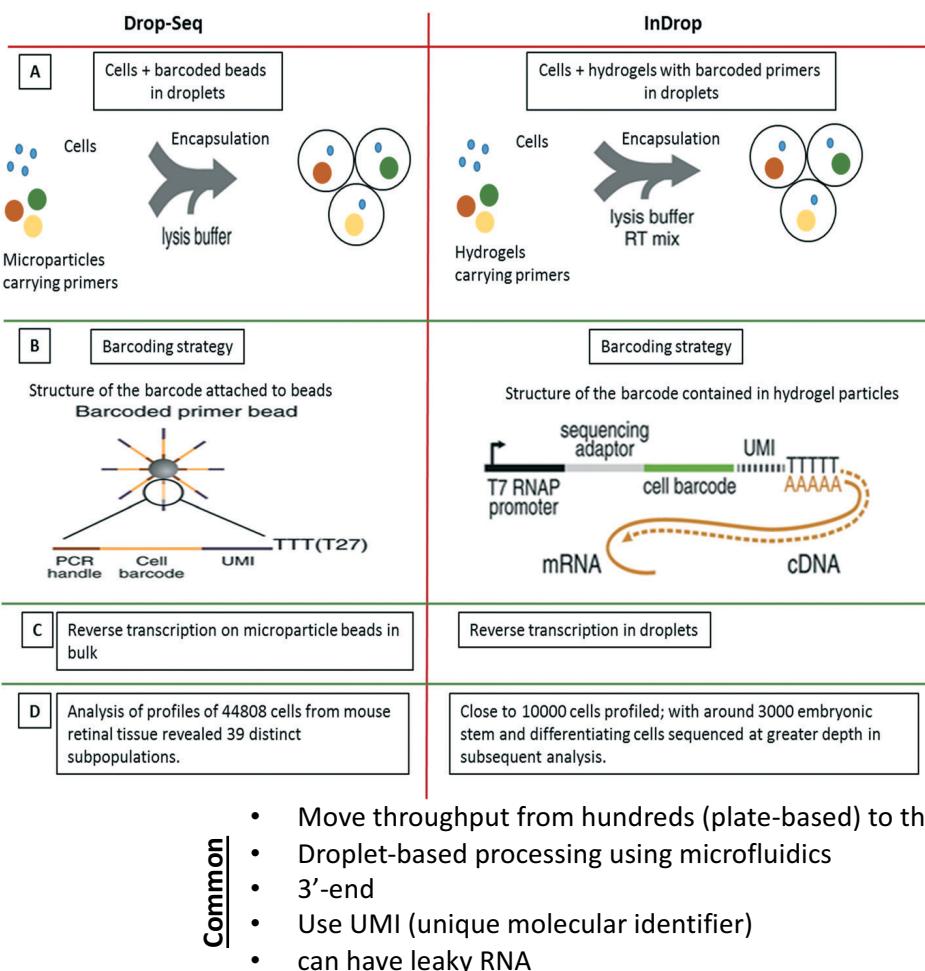
InDrop workflow

IV) InDrop library prep

- 2nd strand synthesis to make full length dsDNA
- In vitro transcription (IVT): using the T7 promoter from the primer
- Fragmentation of RNAs (heat/base)
- RT with random hexamer primers containing adaptors
- PCR the adaptors to add indexes and illumina adaptors



DropSeq vs InDrop



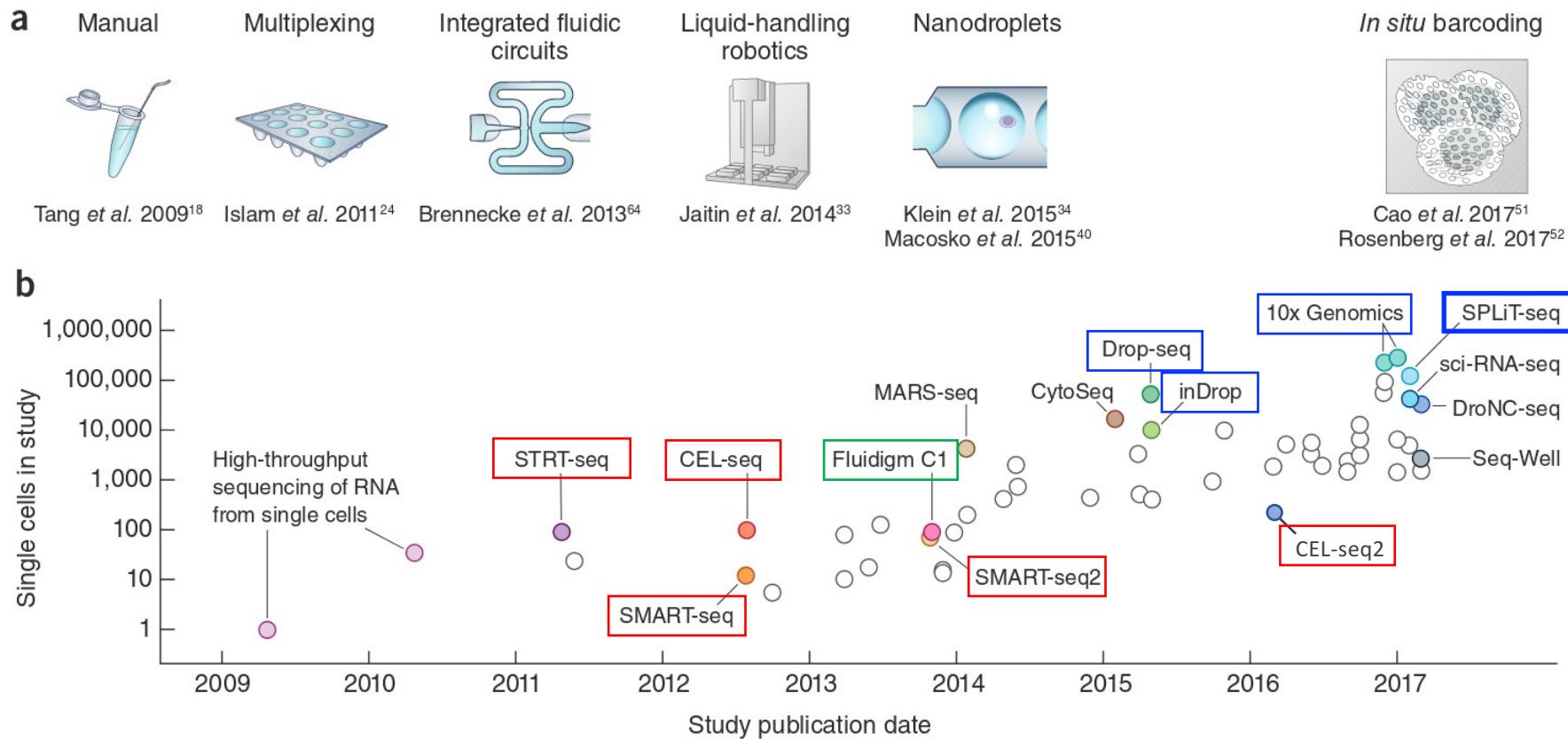
DropSeq

- Droplets are broken and reverse transcription (RT)/template switching occurs on beads in pool
- STAMP: single cell transcriptomes attached to microparticles
- more leaky RNAs (than InDrop)

InDrop

- Reverse transcription (RT) in droplets (not in pool)

Evolution of scRNAseq techniques



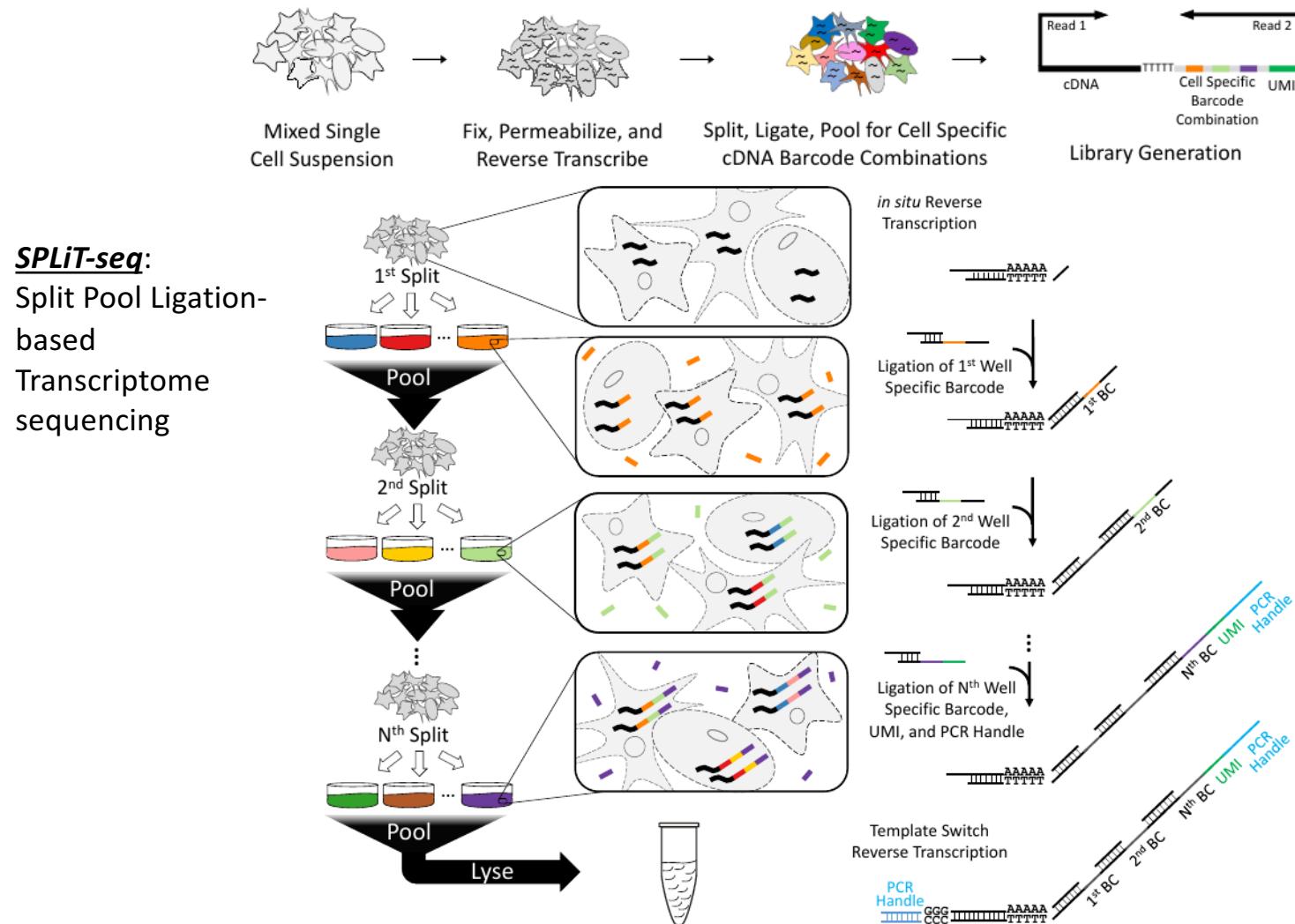
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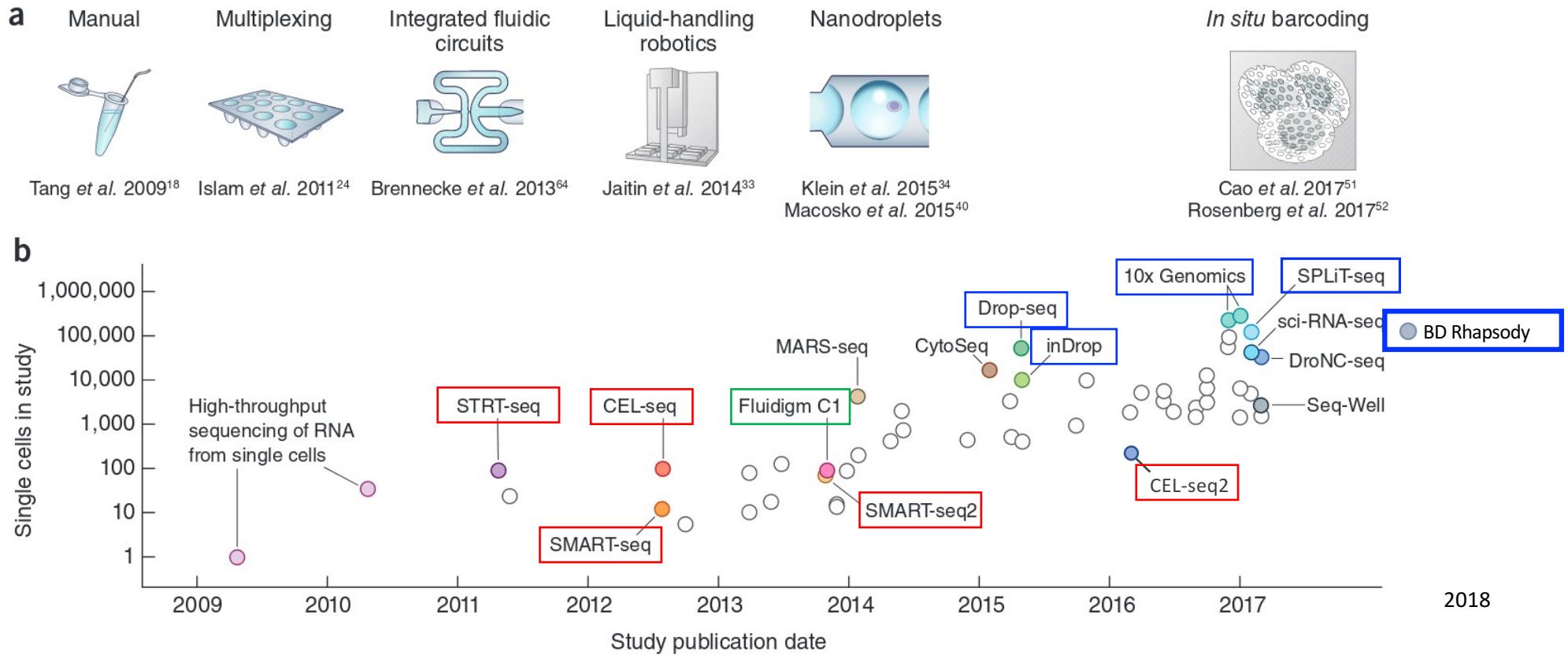
Svensson et al. *Nature Protocols* (2018)

In situ barcoding



Rosenburg et al, 2017, BioRxiv

Evolution of scRNAseq techniques



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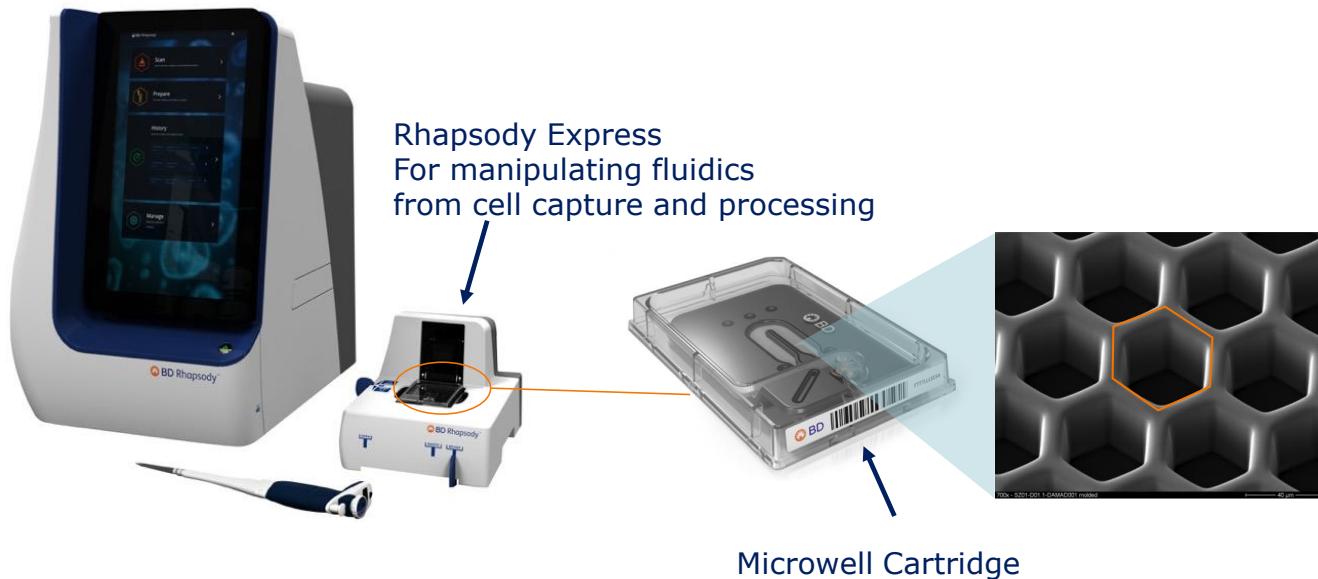
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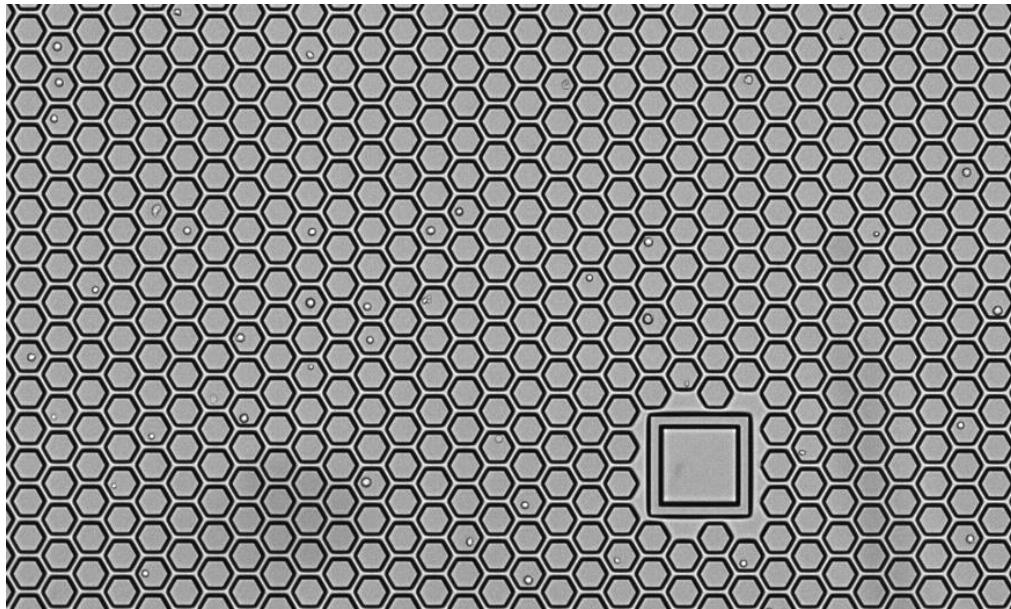
BD Rhapsody™ Instrument

Rhapsody imaging
Scanner for QC
Measurements

One system for analyzing
both protein and RNA at a
single cell level



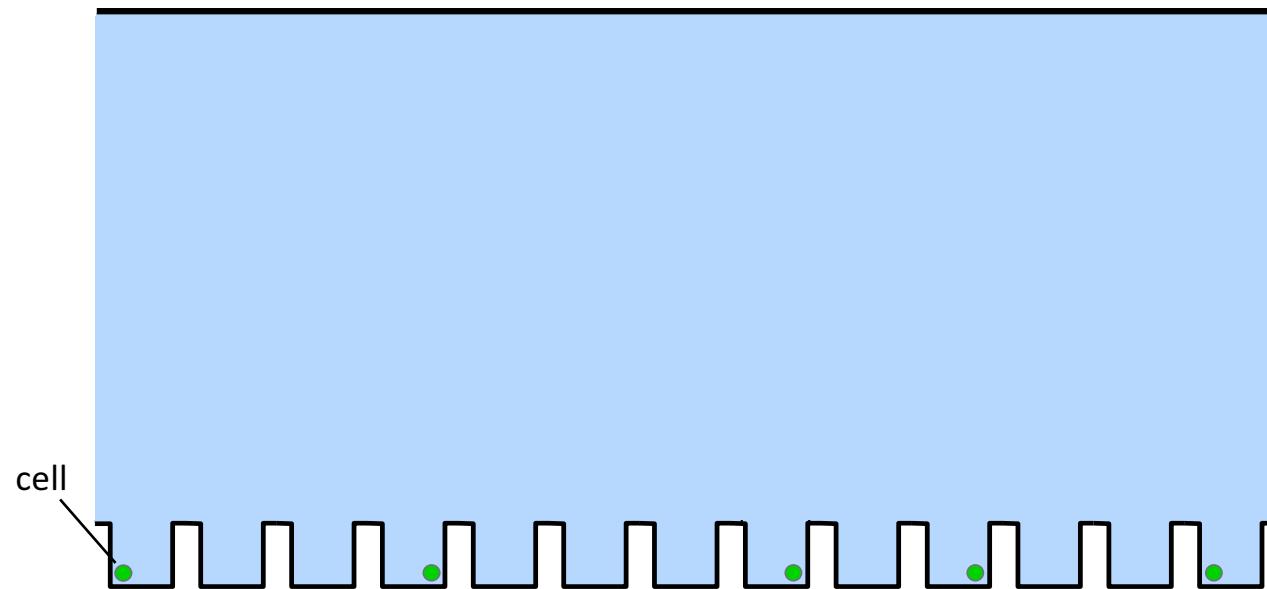
BD Rhapsody – microwell cartridge



- Sparse cell loading
- Multiplet rate predicted by Poisson distribution
 - ~ 2% multiplets in 10k cells
 - ~ 4-5% multiplets in 20k cells
 - ~ 10% multiplets in 40k cells

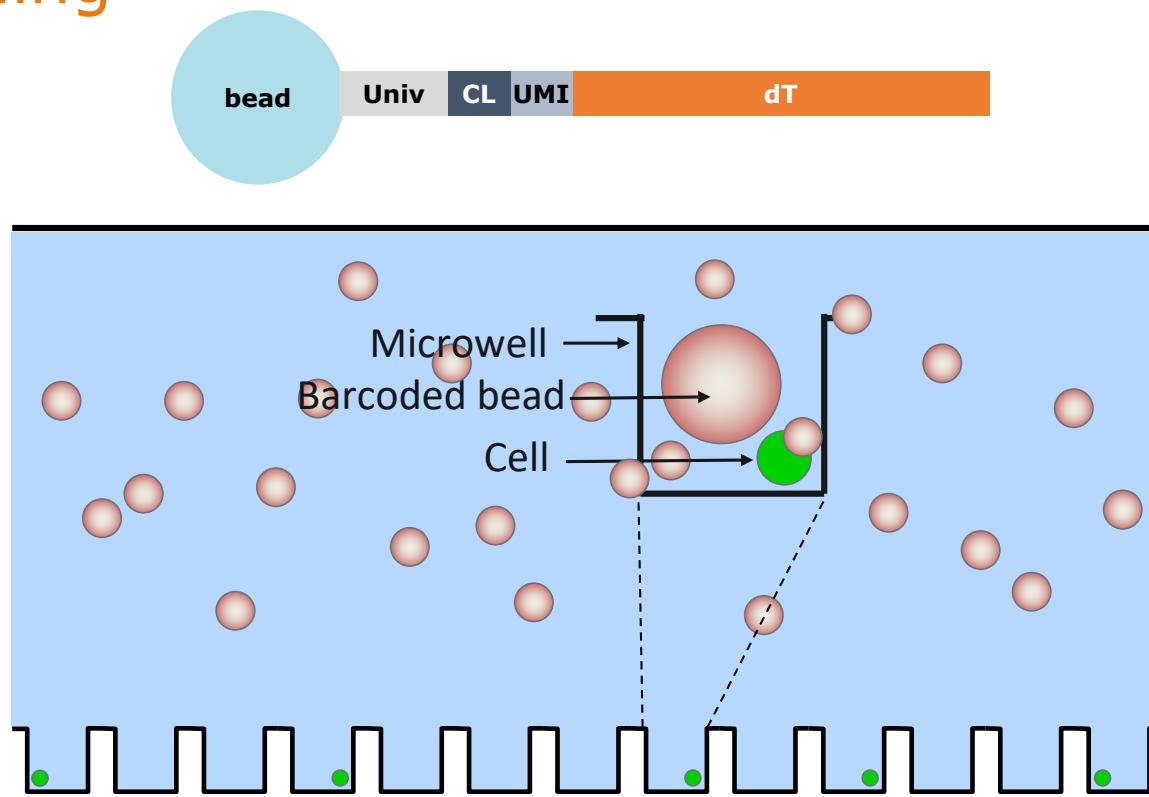
BD Rhapsody – microwell cartridge workflow

Cell loading



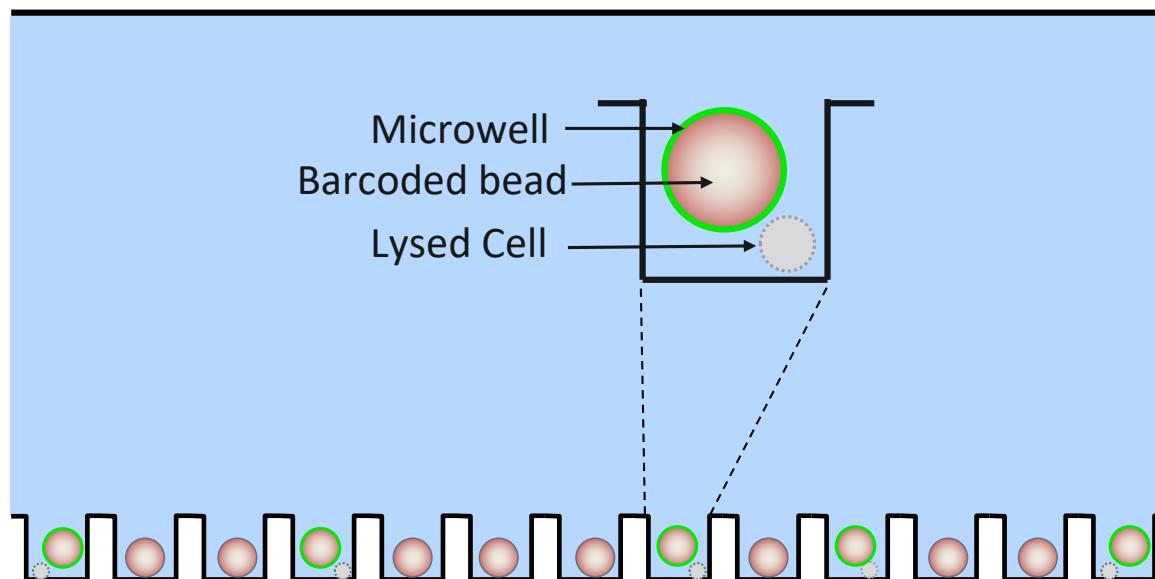
BD Rhapsody – microwell cartridge workflow

Bead loading



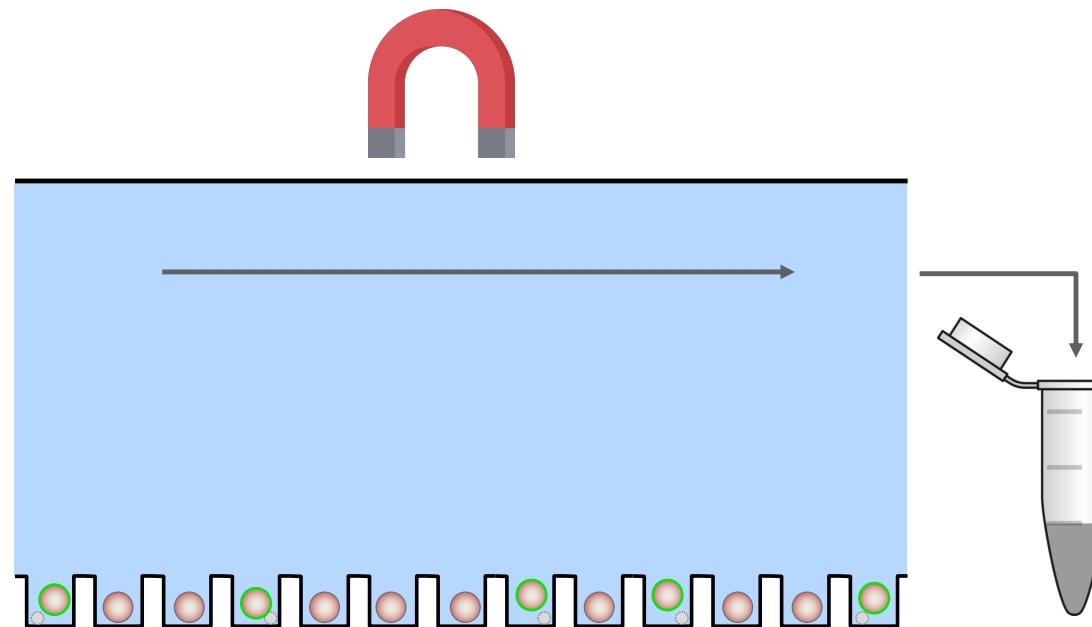
BD Rhapsody – microwell cartridge workflow

Lysis and mRNA hybridization

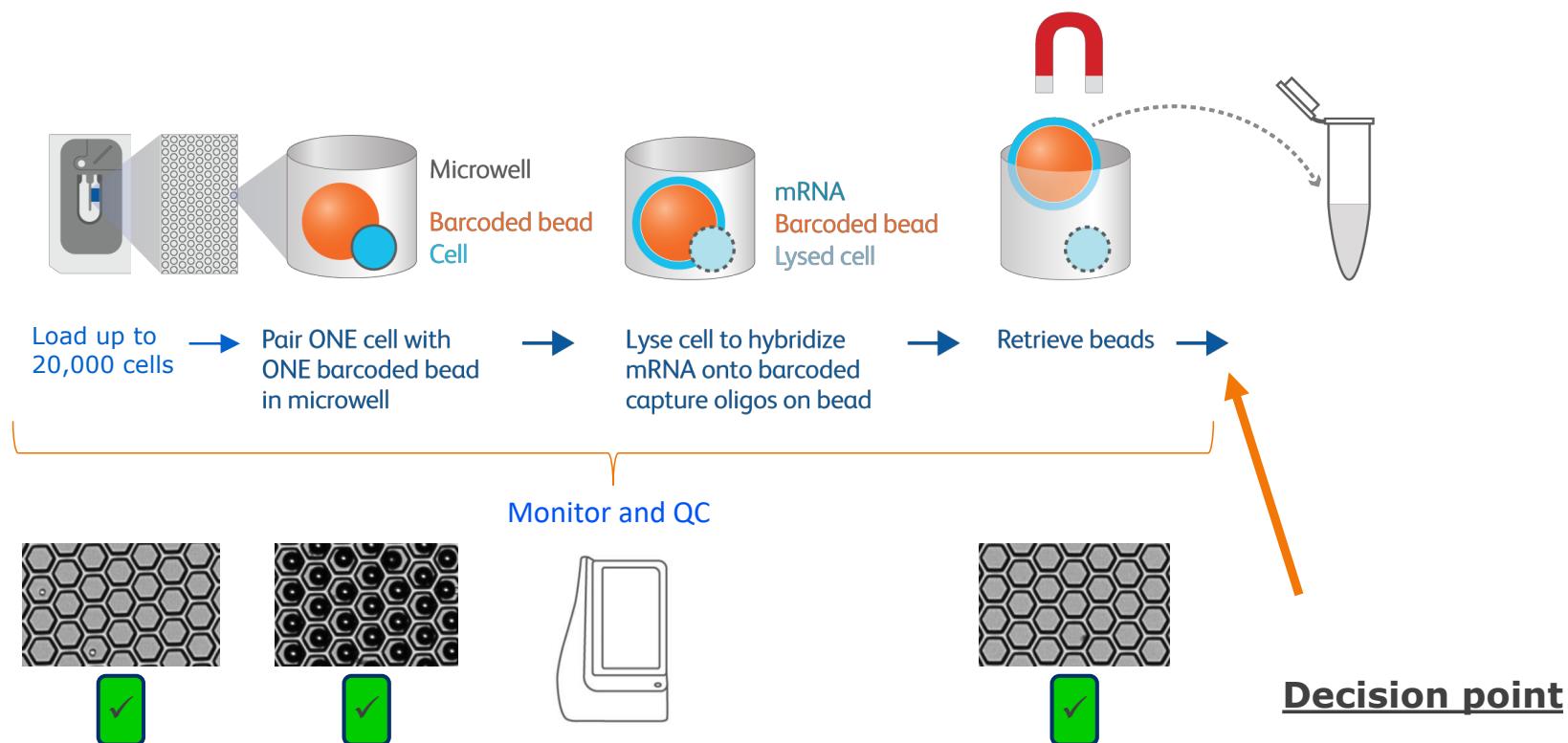


BD Rhapsody – micowell cartridge workflow

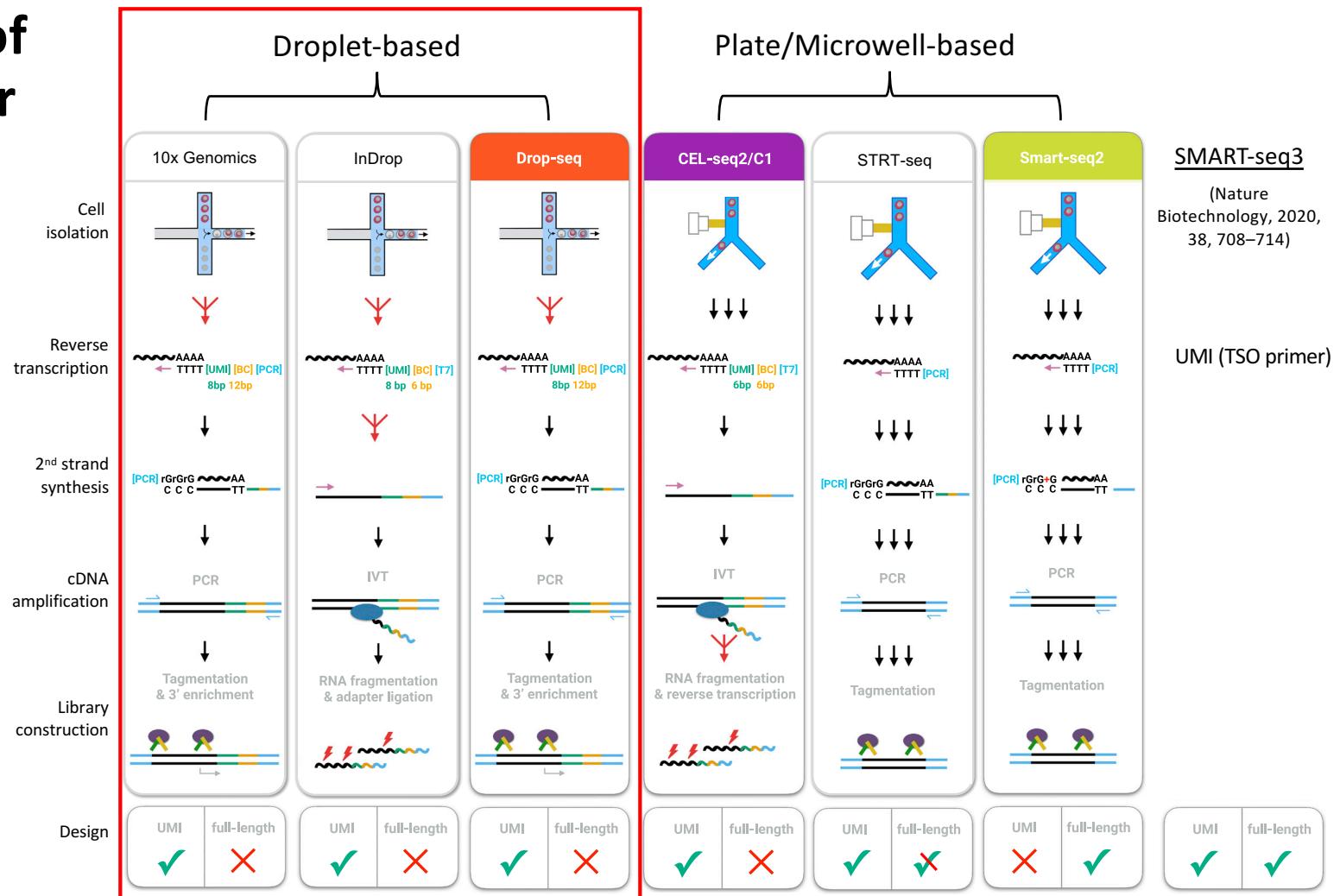
Retrieval of beads



BD Rhapsody Cartridge workflow



Summary of the popular scRNAseq methods



Molecular Cell, 2017 65, 631–643

Conclusion for Part II (Droplet-based methods)

- **General introduction about droplet-based scRNASeq**
 - Timeline of scRNASeq
- **Different types of droplet-based scRNASeq**
 - Drop-seq, InDrop, BD Rhapsody, (10X Genomics)
- **Workflow of different droplet-based scRNASeq**
 - Single cell suspension, Primer synthesis (barcode, UMI, SP), Microfluidic setup, Cell lysis, RNA capture, cDNA amplification, Library preparation

Which method should I use?



- If you want to study overall variability in transcription of cells within or across different tissues? Many cells (hundreds to thousands); droplet-based methods
- If you want to look at a few genes associated with a specific process, such as cell death? Plate-based methods (deeper sequencing depth); panel-based methods
- To capture low-abundance gene? Plate-based methods (deeper sequencing depth)
- To get a full-length coverage of transcriptomes from single cells (study splice variant)? SMART-seq2/3 (STRT-seq?)
- To have fewer errors? IVT-based amplification method (CEL-seq2, InDrop)
- To study transcription start sites? STRT-seq, SMART-seq2/3, 10X Genomics 5' method