

Single cell RNA sequencing (scRNASeq) platforms

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Erasmus MC, Assistant professor

2022 Single Cell Analysis Workshop, 2022/10/10

Single cell RNA sequencing (scRNASeq) platforms

Part I: Plate-based scRNASeq

Part II: Droplet-based scRNASeq

Outline of the Part I (Plate-based scRNASeq)

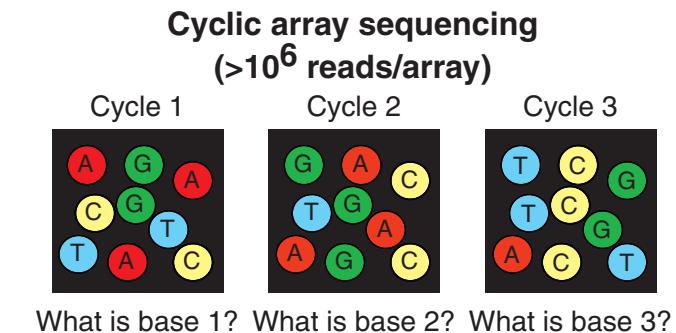
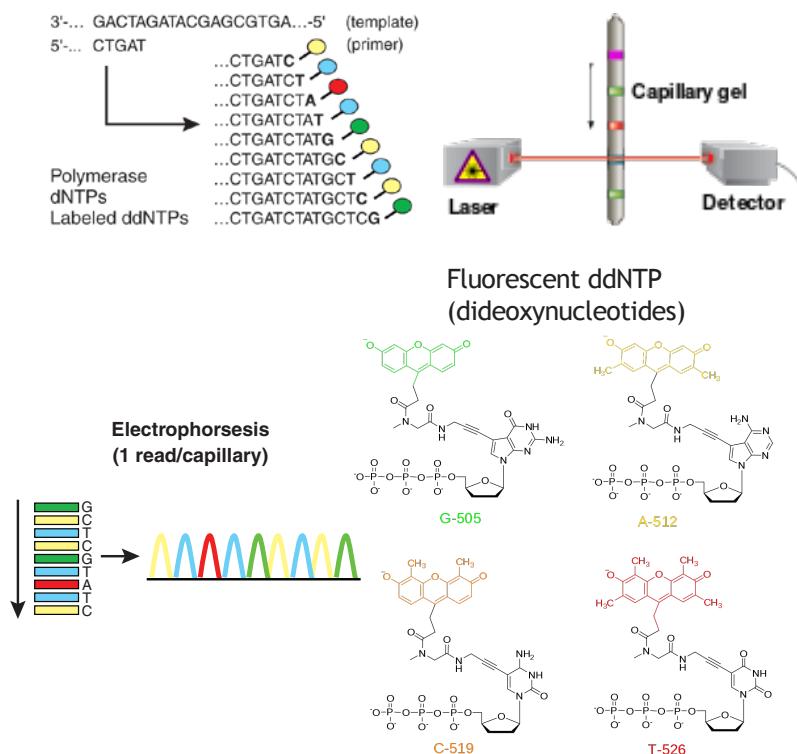
- General introduction about scRNASeq
- Different types of plate-based scRNASeq
- Workflow of different plate-based scRNASeq



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

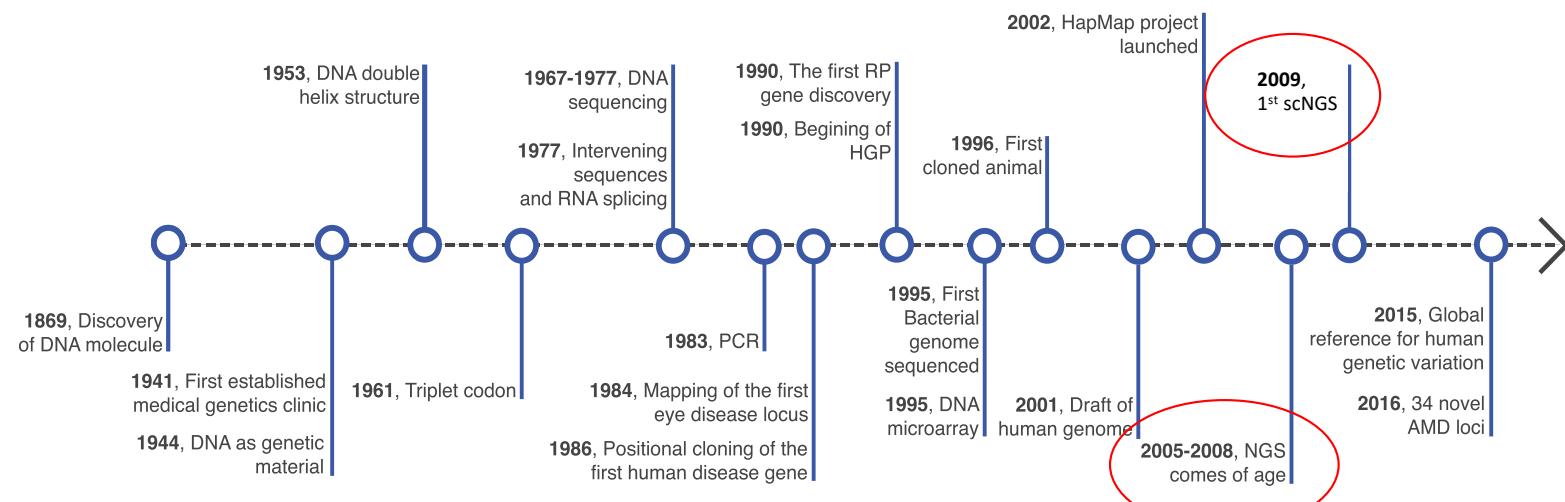
Traditional sequencing vs next-generation sequencing

- From sanger sequencing to next-generation sequencing



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

Next-generation sequencing & single cell sequencing

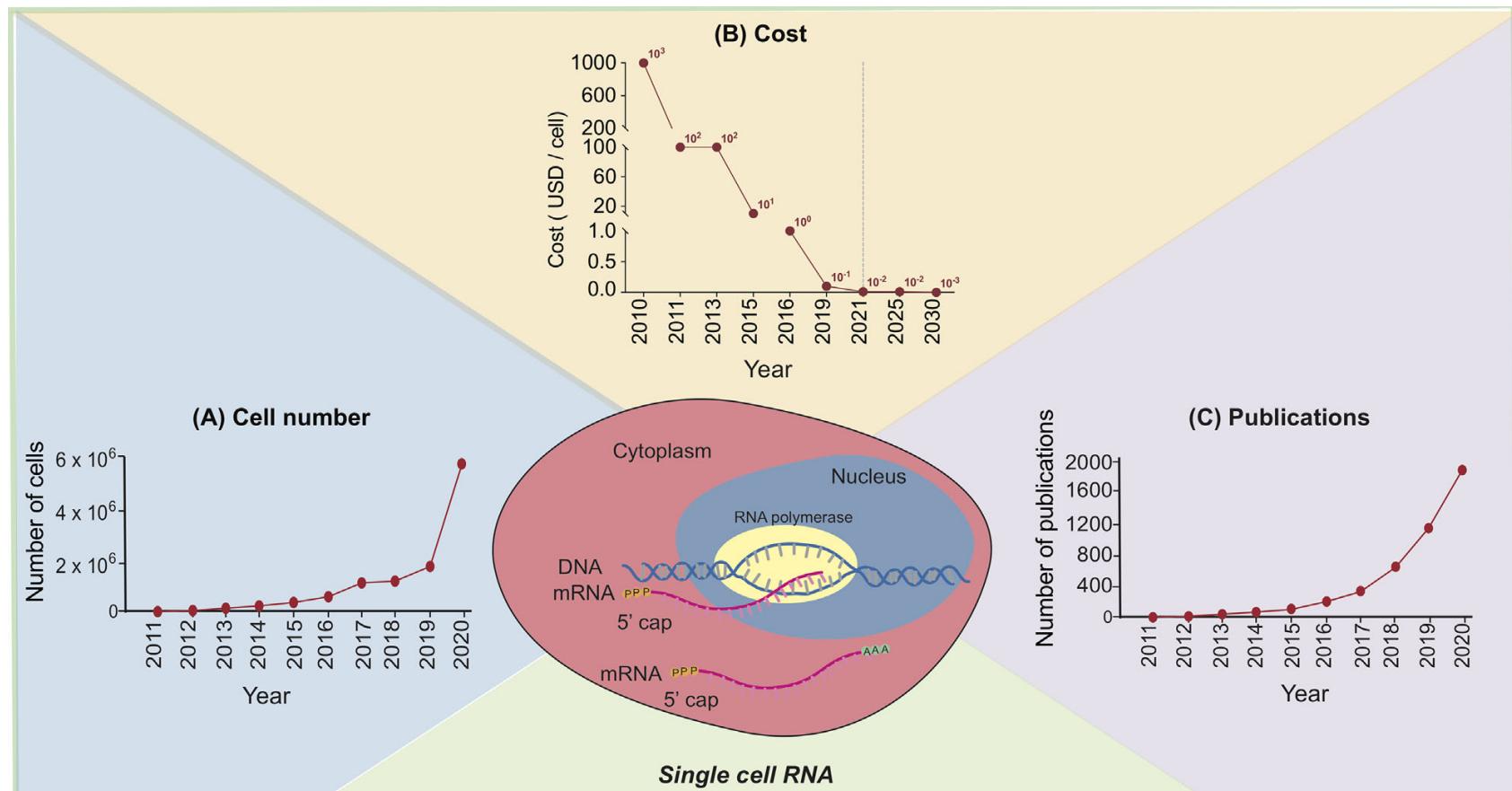


2005: 454 (Roche)

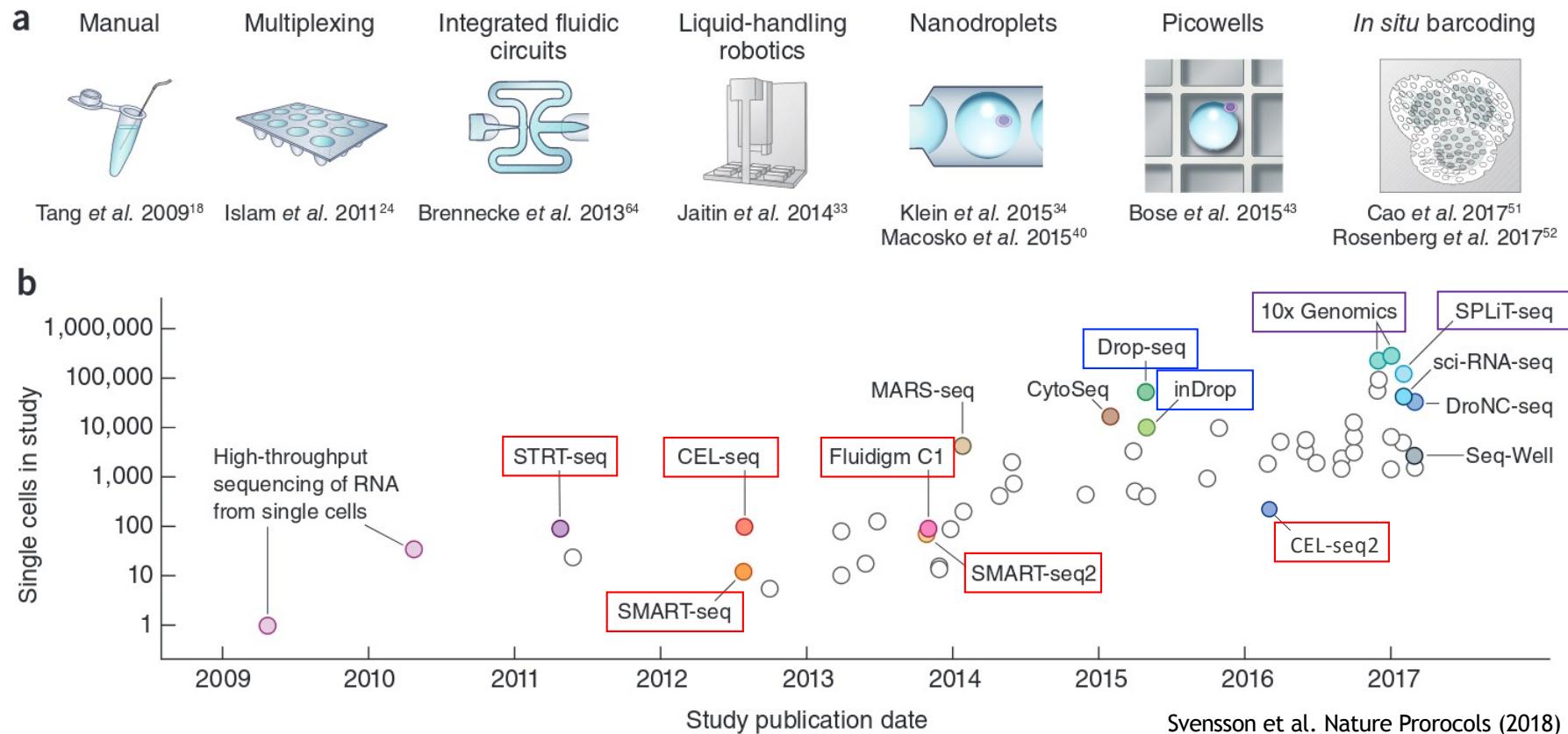
2007: Illumina

2008: SOLid (ABI)

Evolution of scRNAseq techniques



Evolution of scRNASeq techniques

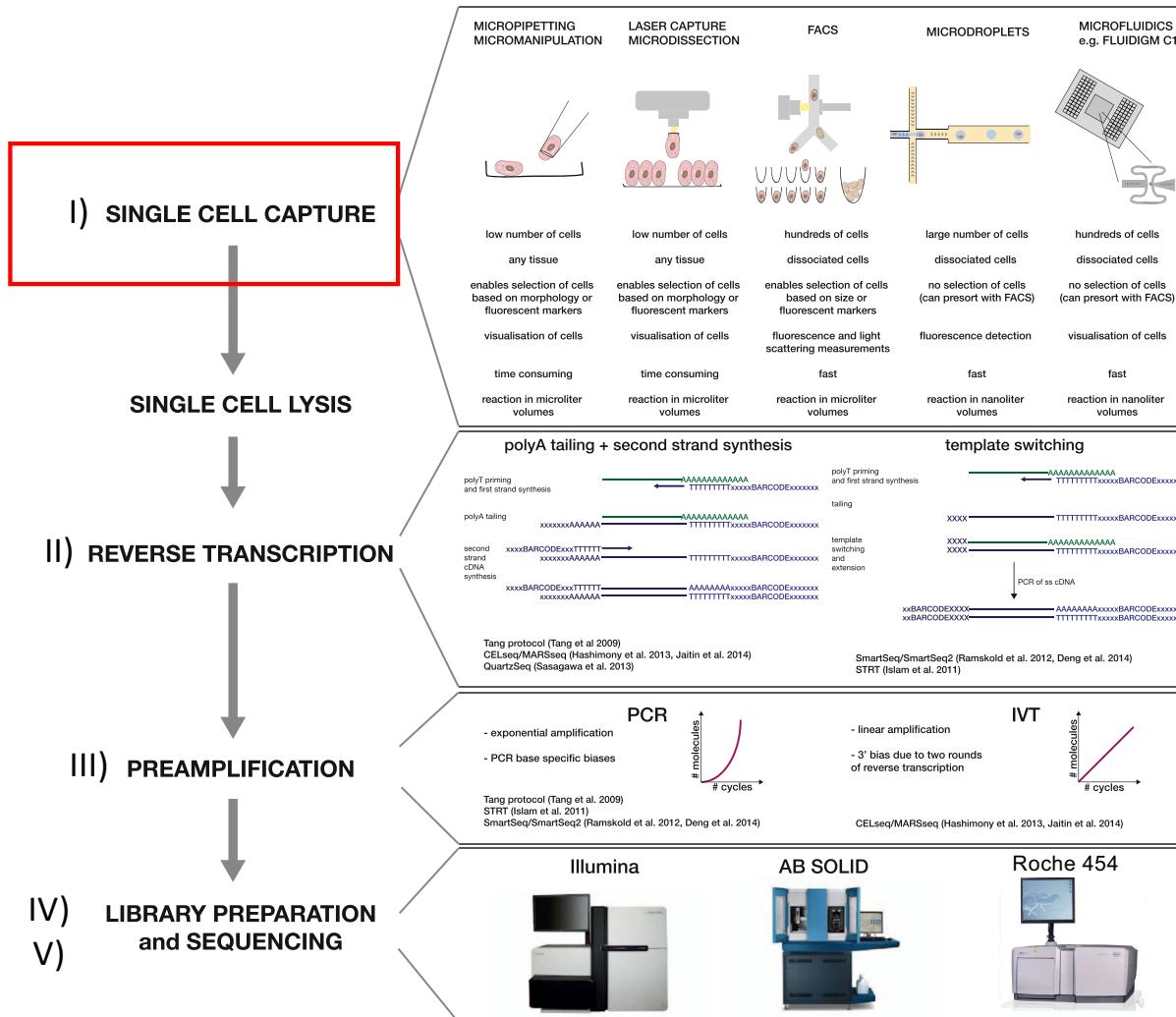


~100s cells thanks to **multiplexing (barcode)**

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

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Single-cell RNA sequencing experiment workflow



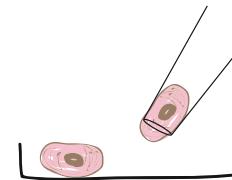
I) Single cell capture

10X Genomics,
Drop-seq, InDrop

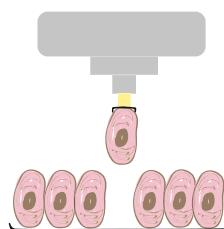
Fluidigm (C1),

Wafergen
(iCell8),
CellenOne

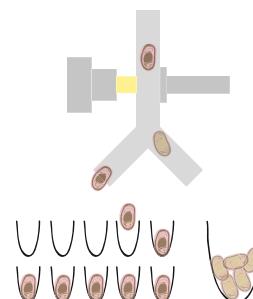
MICROPIPETTING MICROMANIPULATION



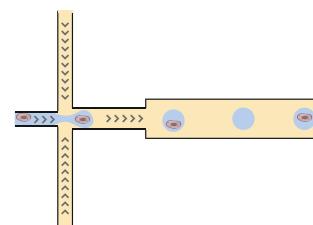
LASER CAPTURE MICRODISSECTION



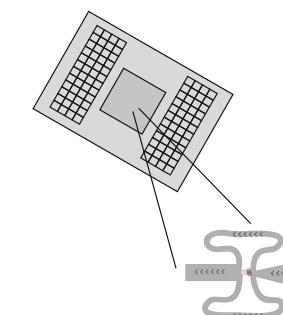
FACS



MICRODROPLETS



MICROFLUIDICS



Liquid-handling robotics

low number of cells

any tissue

enables selection of cells based on morphology or fluorescent markers

visualisation of cells

time consuming

reaction in microliter volumes

low number of cells

any tissue

enables selection of cells based on morphology or fluorescent markers

visualisation of cells

time consuming

reaction in microliter volumes

hundreds of cells

dissociated cells

enables selection of cells based on size or fluorescent markers

fluorescence and light scattering measurements

fast

reaction in microliter volumes

large number of cells

dissociated cells

no selection of cells (can presort with FACS)

fluorescence detection

fast

reaction in nanoliter volumes

hundreds of cells

dissociated cells

no selection of cells (can presort with FACS)

visualisation of cells

fast

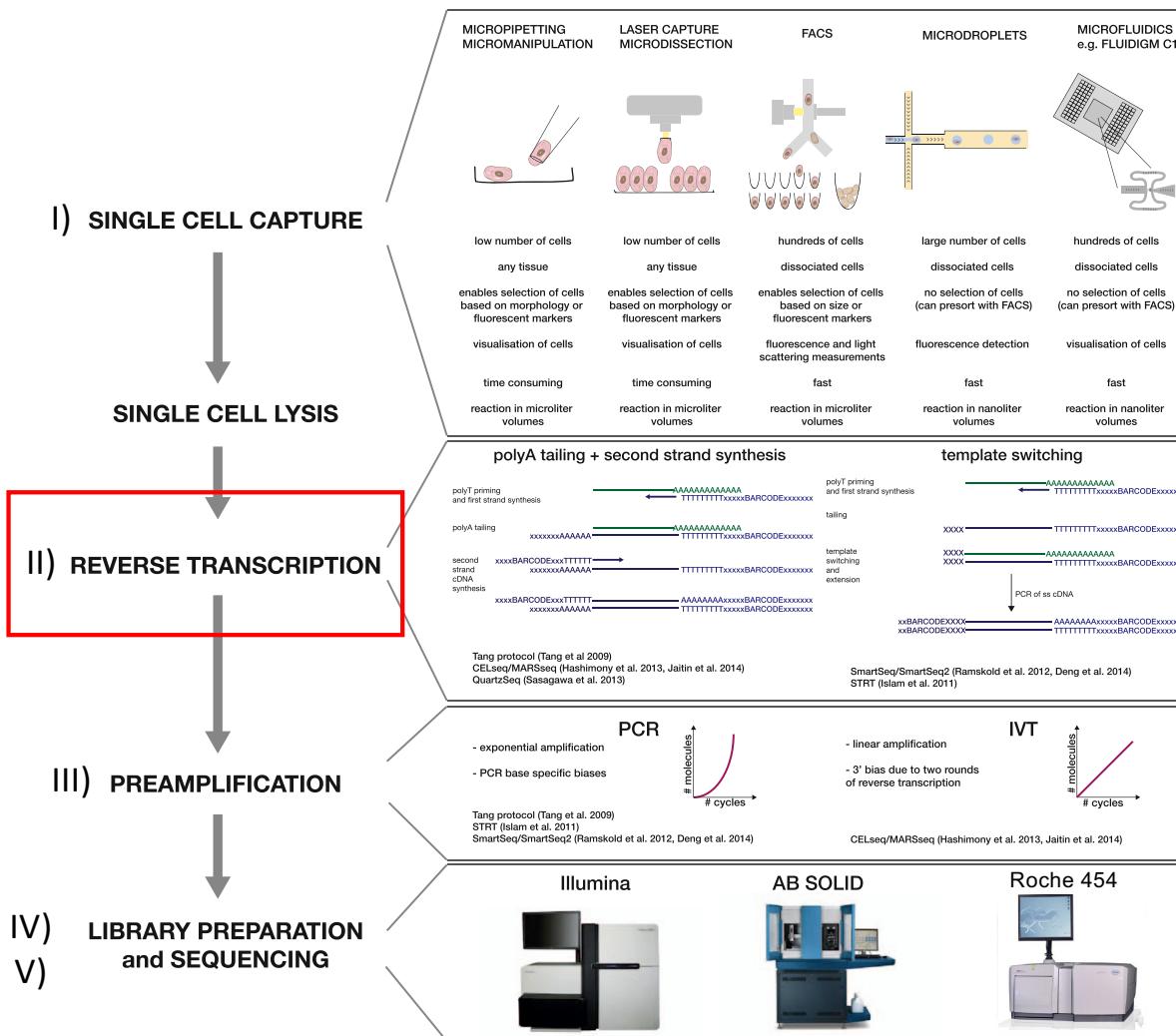
reaction in nanoliter volumes

medium-Large number of cells

some selection criteria

fast

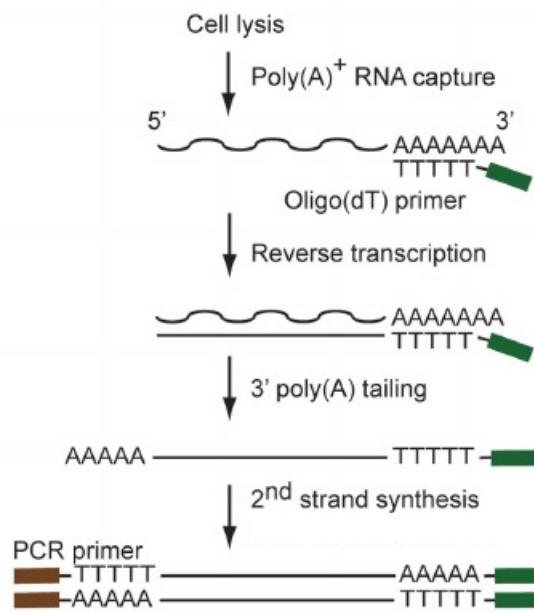
Single-cell RNA sequencing experiment workflow



II) Reverse transcription

Cel-Seq(2), (InDrop)

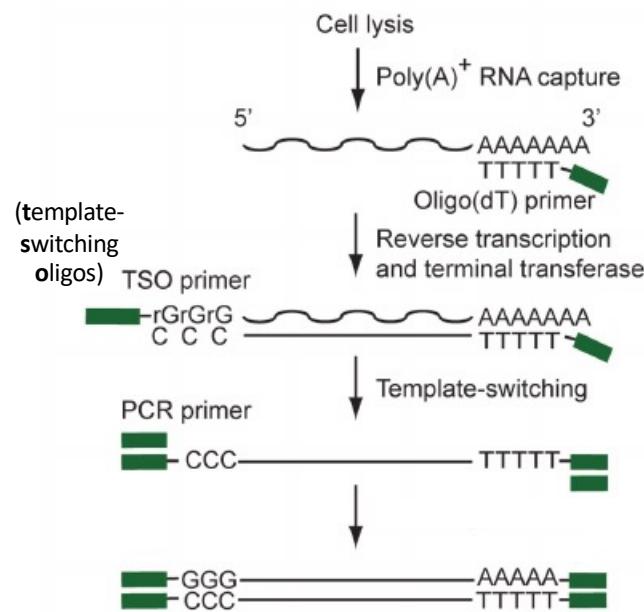
1) PolyA tailing + 2nd strand synthesis



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

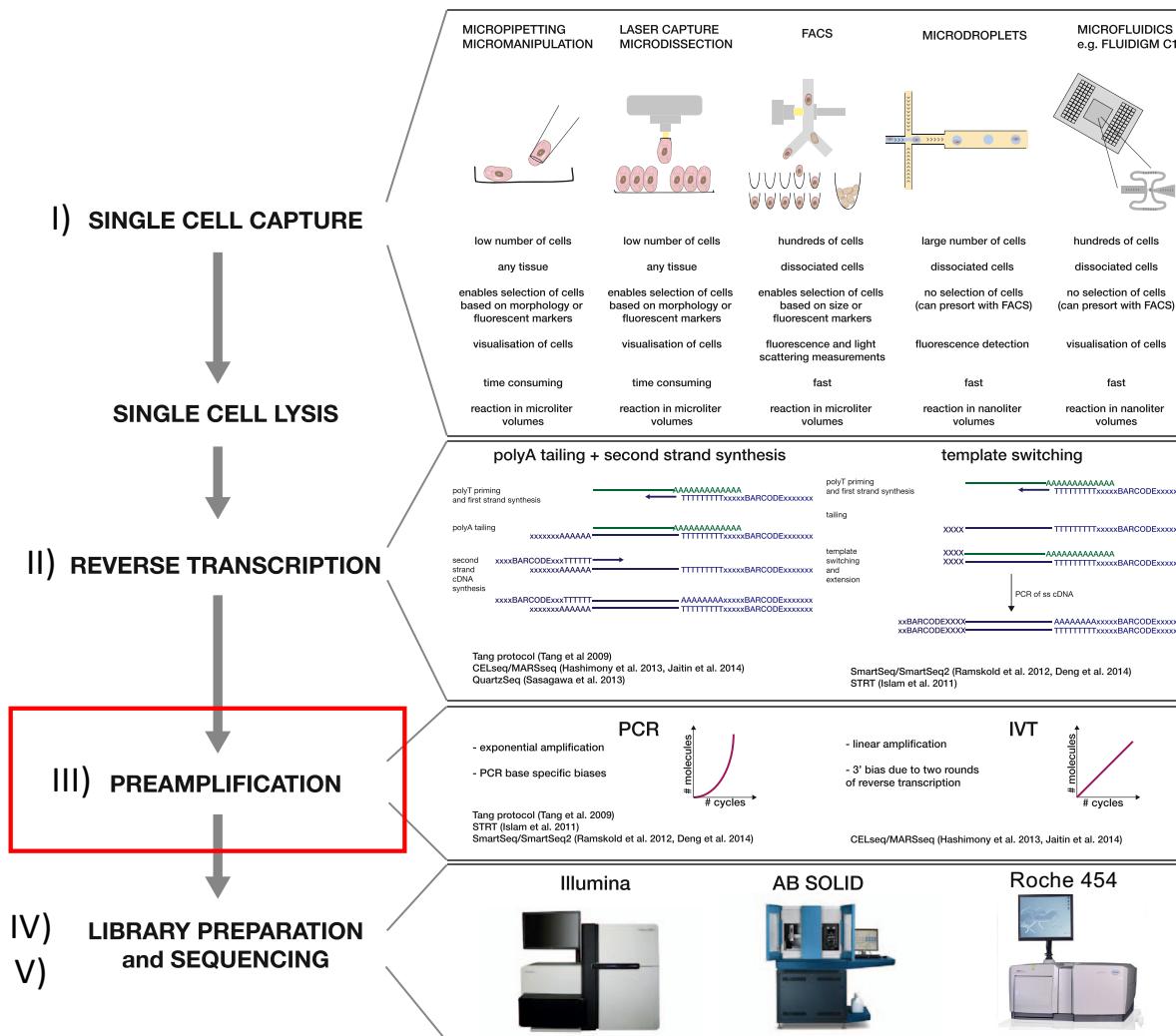
Smart-Seq(2/3), STRT-Seq, (Drop-seq, 10X)

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

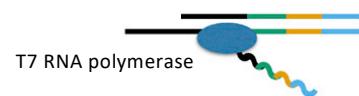


III) Preamplification

Cel-Seq(2), (InDrop)

(In vitro transcription)
IVT

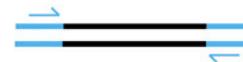
- linear amplification (slow)
- less error



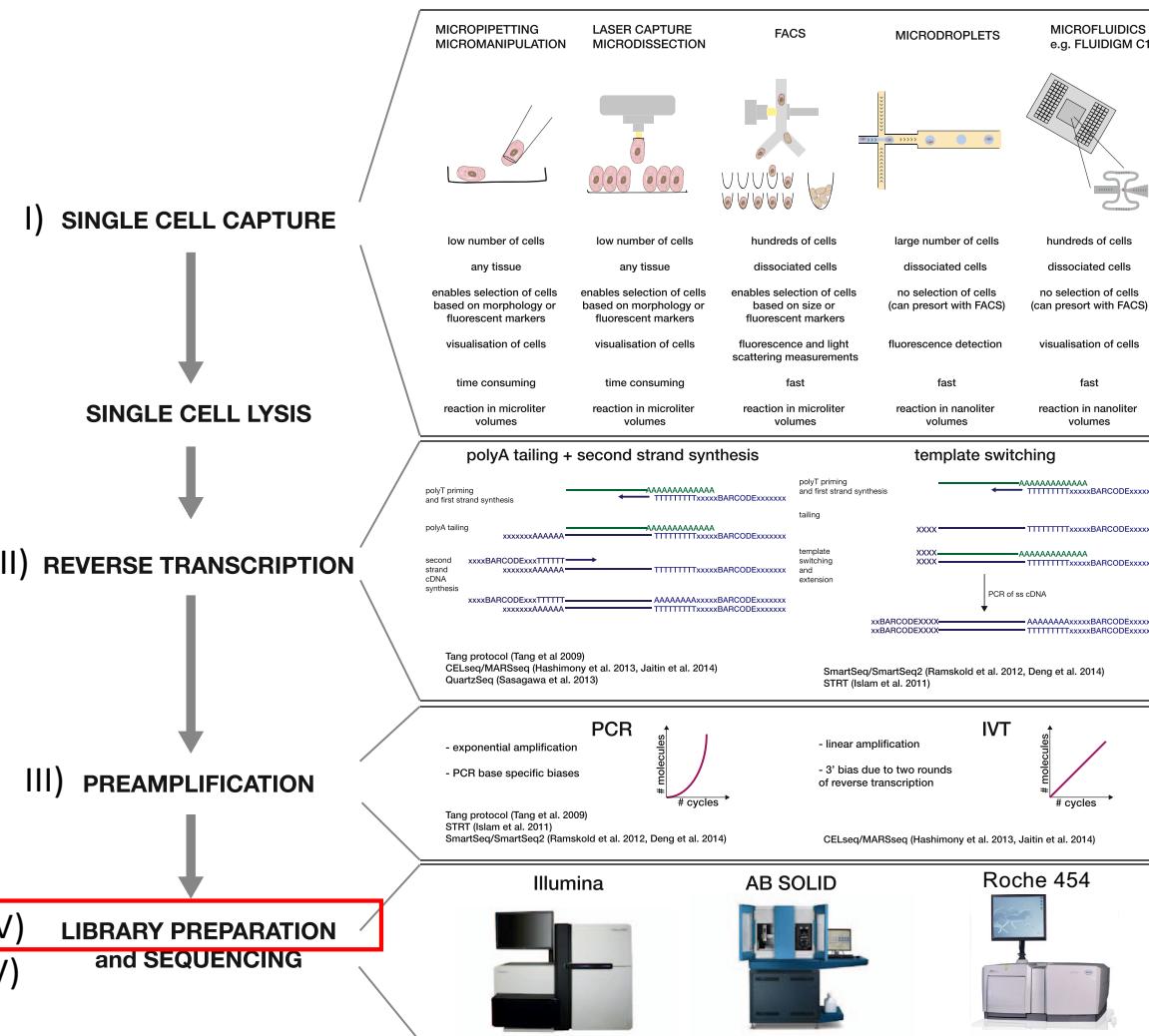
Smart-Seq(2), STRT-Seq, (Drop-seq, 10X)

PCR

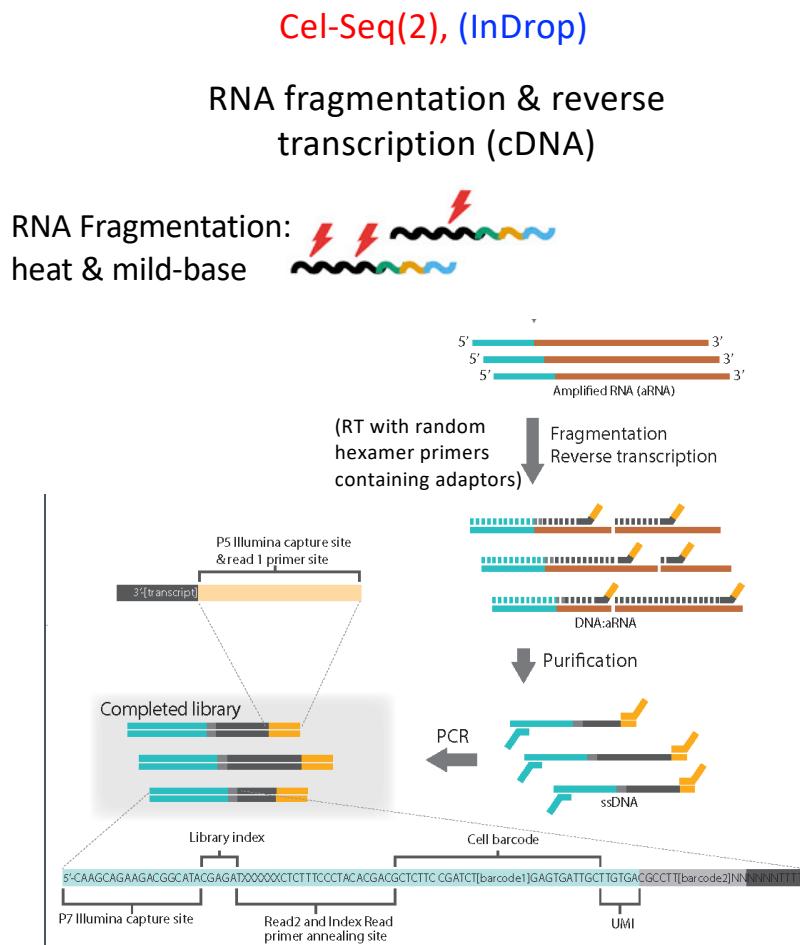
- exponential amplification (fast)
- error prone



Single-cell RNA sequencing experiment workflow

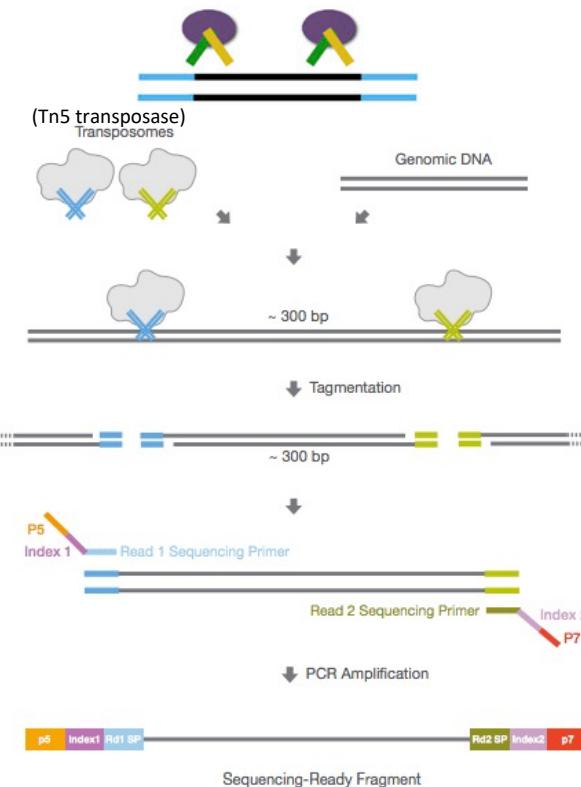


IV) Library preparation

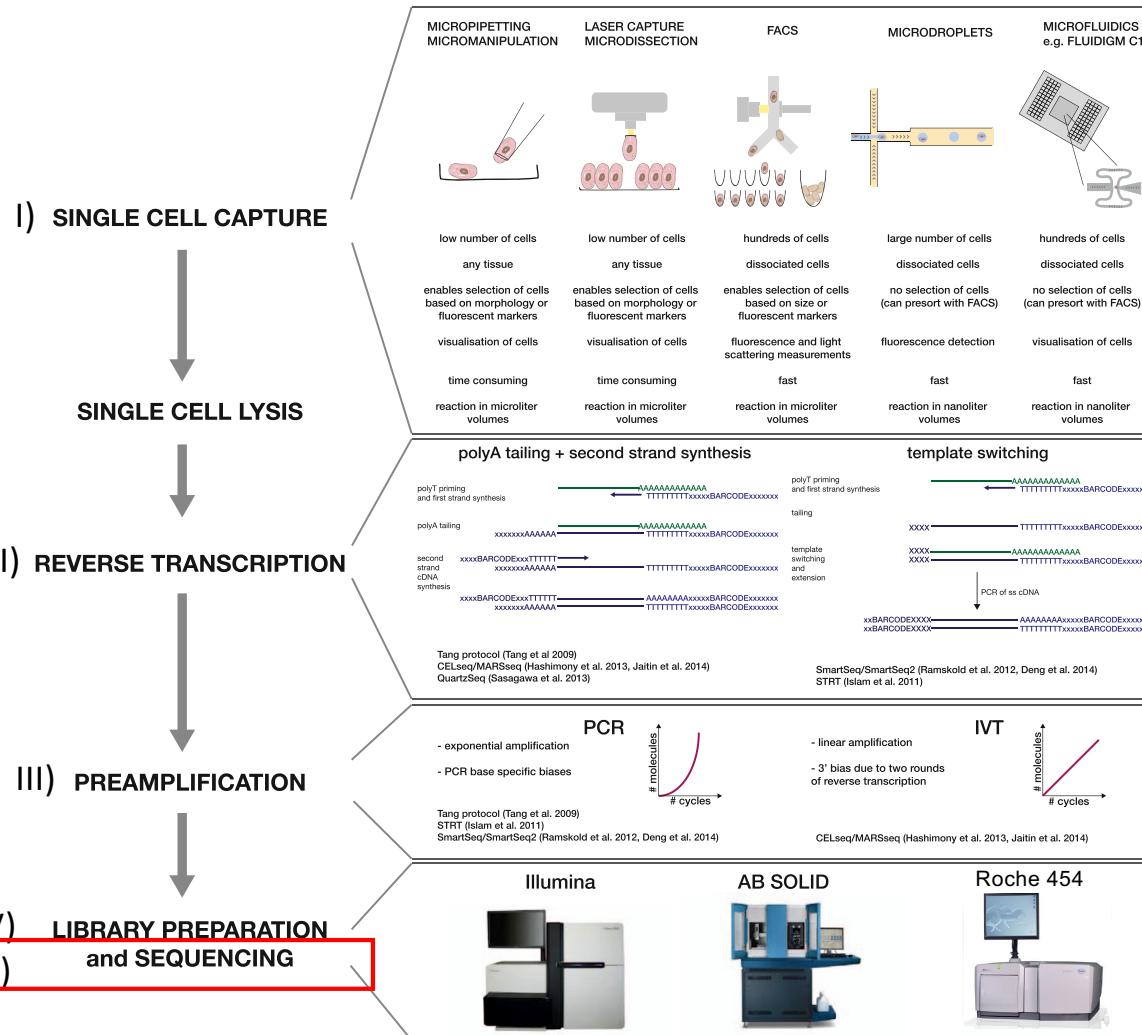


Smart-Seq(2), (Drop-seq, 10X)

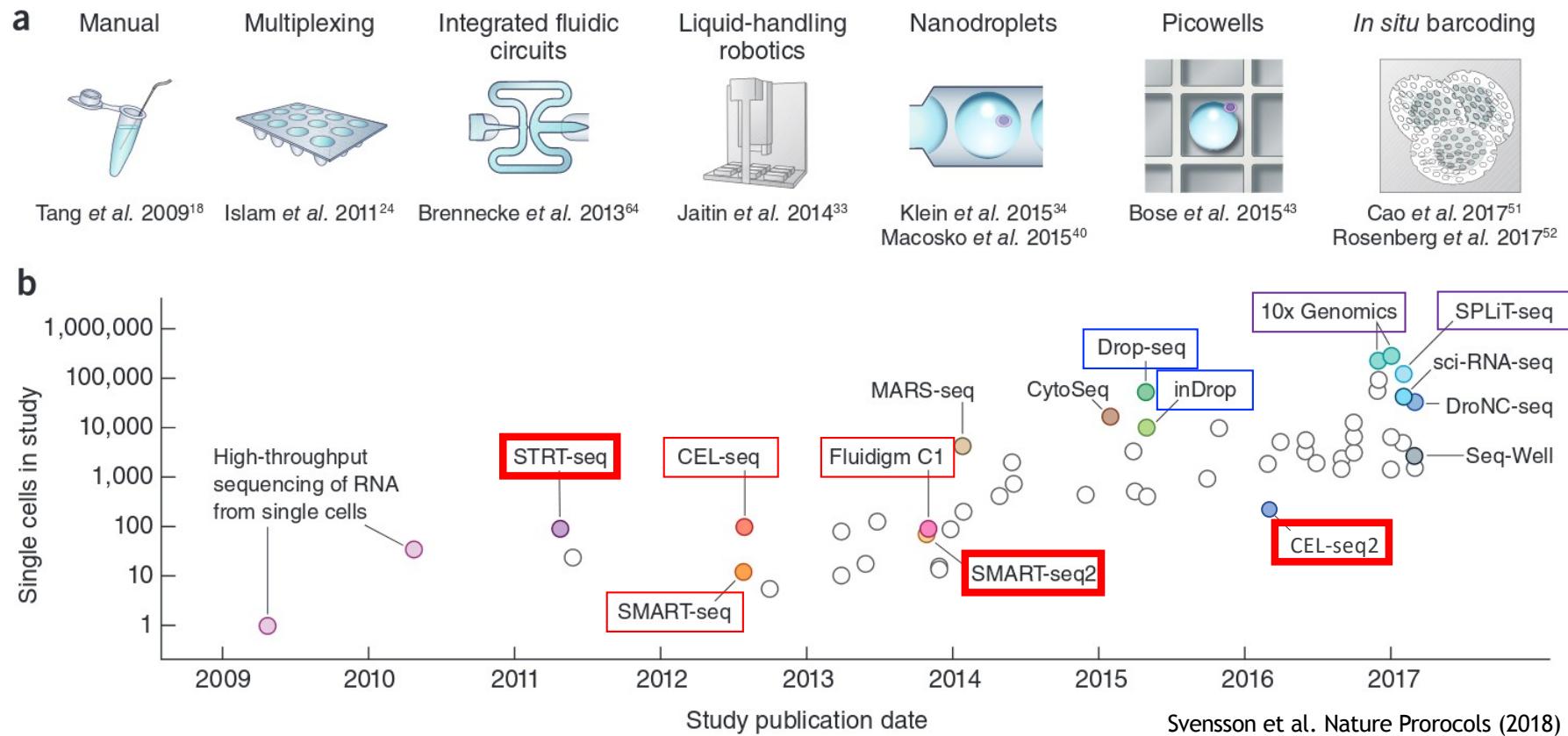
Tagmentation



Single-cell RNA sequencing experiment workflow



Evolution of scRNASeq techniques



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

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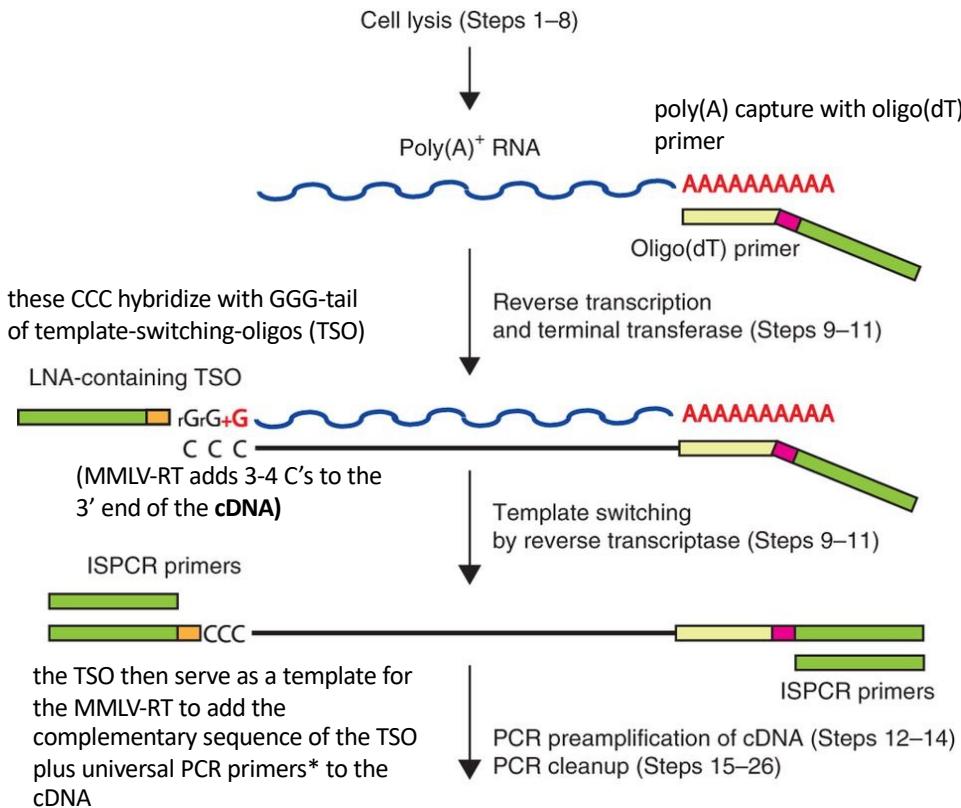
Three most popular plate-based scRNaseq

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

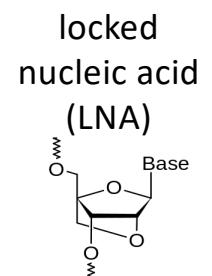
SMART-seq2

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

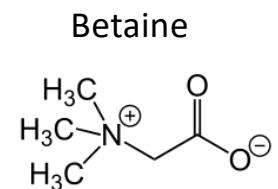
RNA capture and cDNA synthesis



- 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

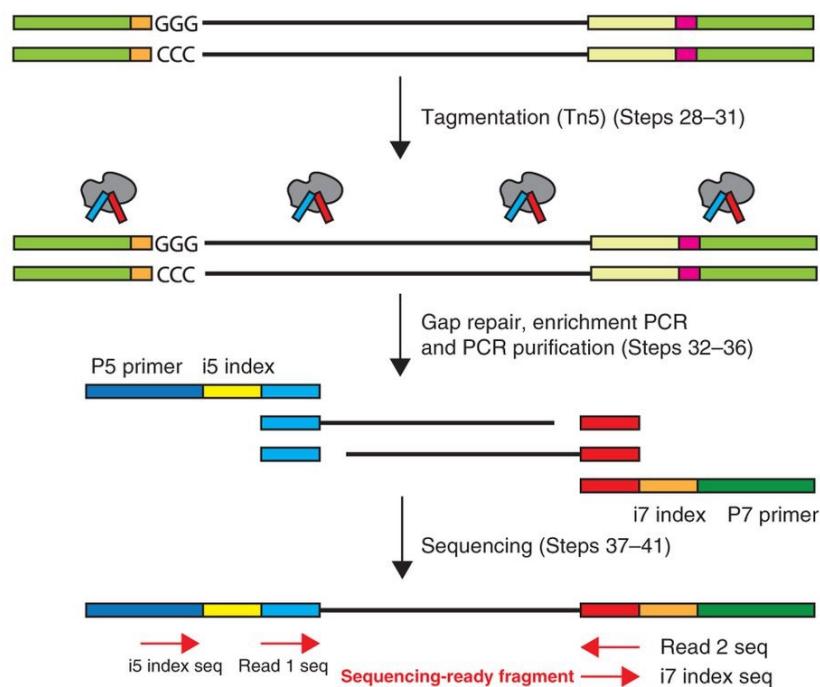


- Betaine**, a methyl group donor, can remove the steric hindrance of secondary structures of RNAs (such as hairpins or loops) so that early termination of chain elongation can be blocked.



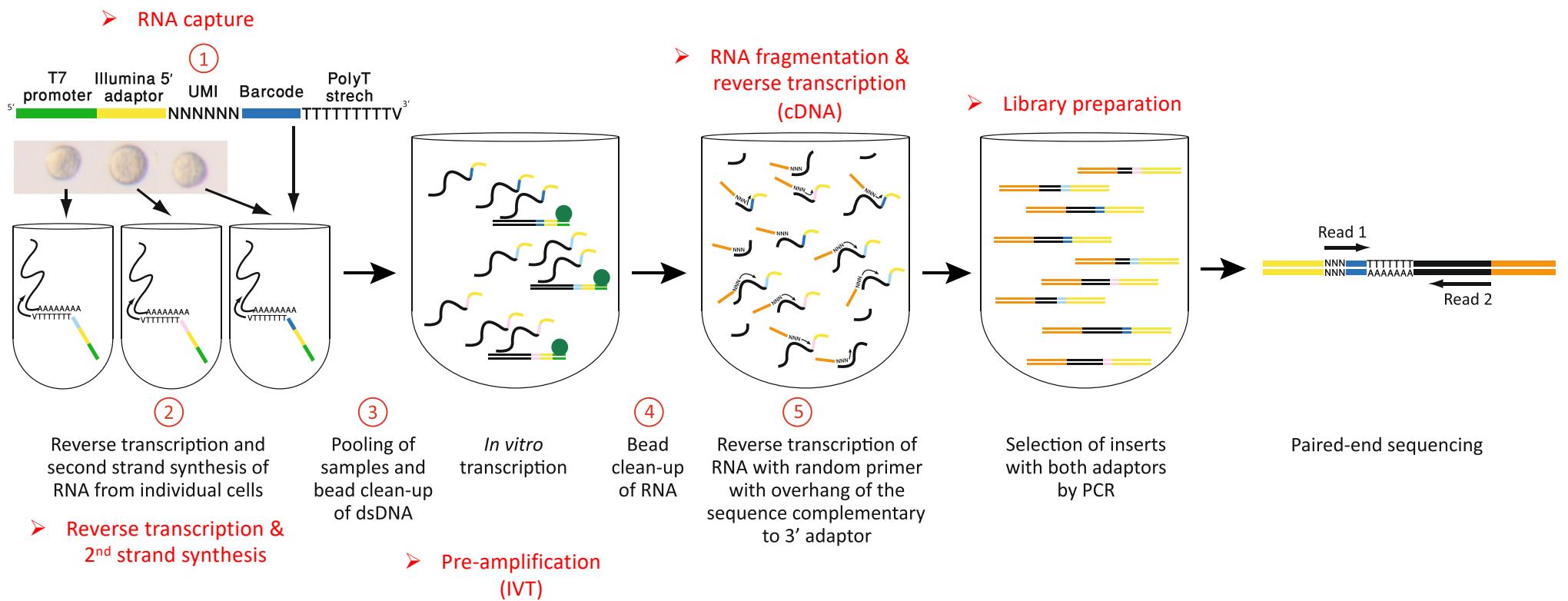
SMART-seq2

Library preparation



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

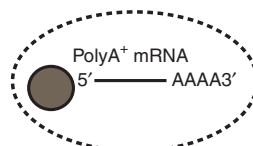
CEL-seq2



STRT-seq: single-cell tagged reverse transcription sequencing

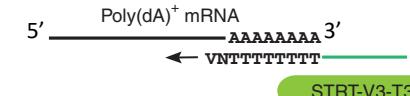


Prepare cell capture plate and collect 96 single cells in 96 different wells

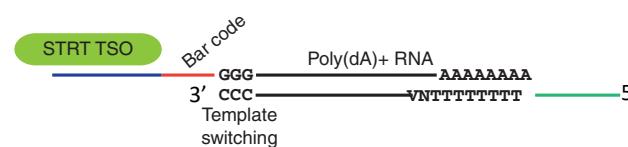


Cell lysis

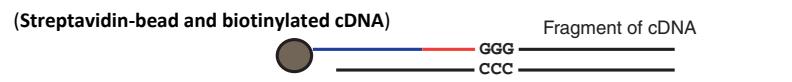
An upstream sequence must be introduced at the 3' end of the cDNA (5' end of the mRNA) to serve as template for the amplification using a universal primer (**biotinylated primer**)



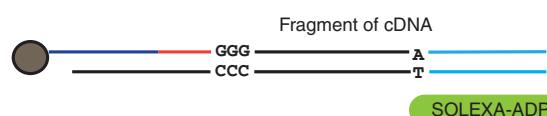
Oligo-dT(30) annealed to poly(dA)+ mRNA



First-strand synthesis by reverse transcriptase, template switching and introduction of bar code



Purification, PCR amplification, bead immobilization and fragmentation (dsDNA fragmentase)



End repair, dA-tailing and adapter ligation (SOLEXA-ADP2 adaptor ligation via T4 ligase)



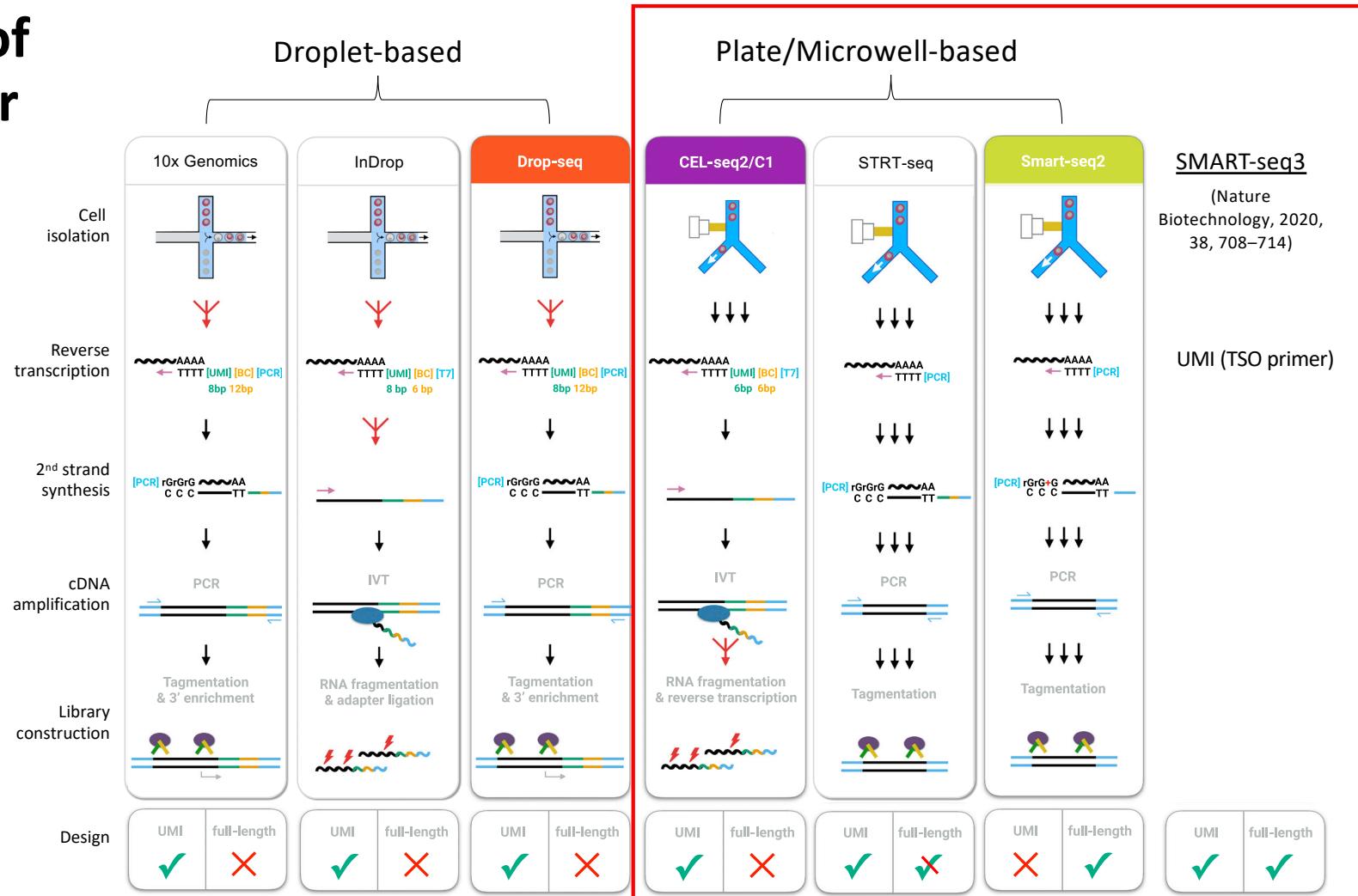
Library amplification and introduction of P1 motif



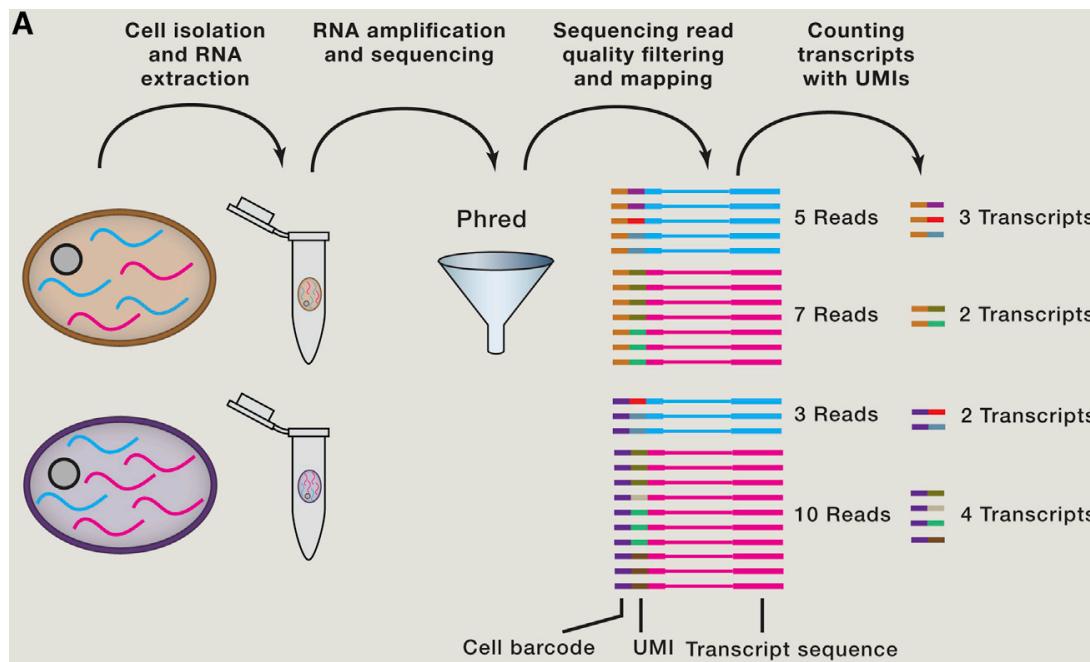
Illumina sequencing

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)



Conclusion for Part I (plate-based scRNASeq)

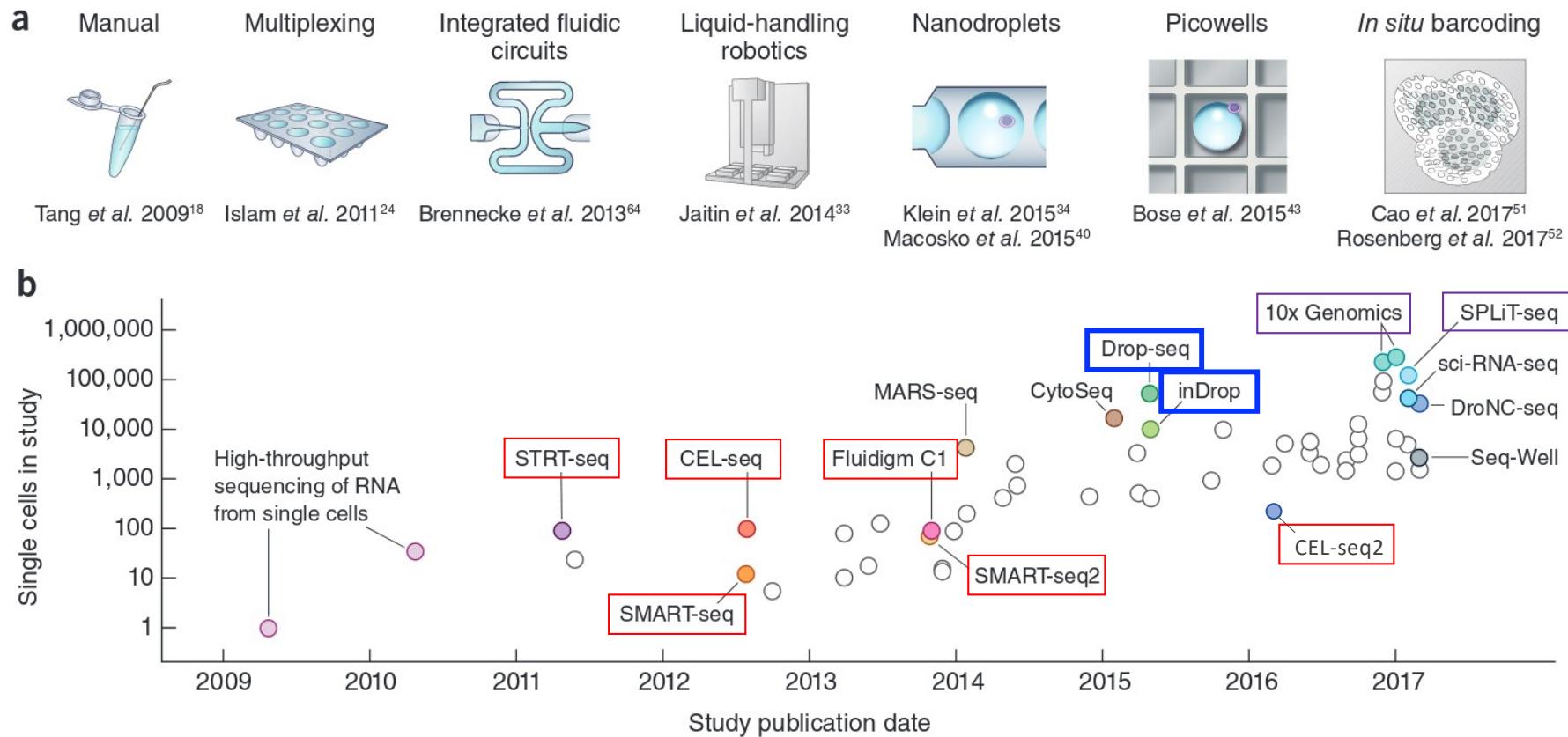
- **General introduction about scRNASeq**
 - scSeq vs NGS
- **Different types of plate-based scRNASeq**
 - SMART-seq2/3, CEL-seq2, STRT-seq
- **Workflow of different plate-based scRNASeq**
 - Single cell capture, cell lysis, reverse transcription, pre-amplification, library preparation, sequencing

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

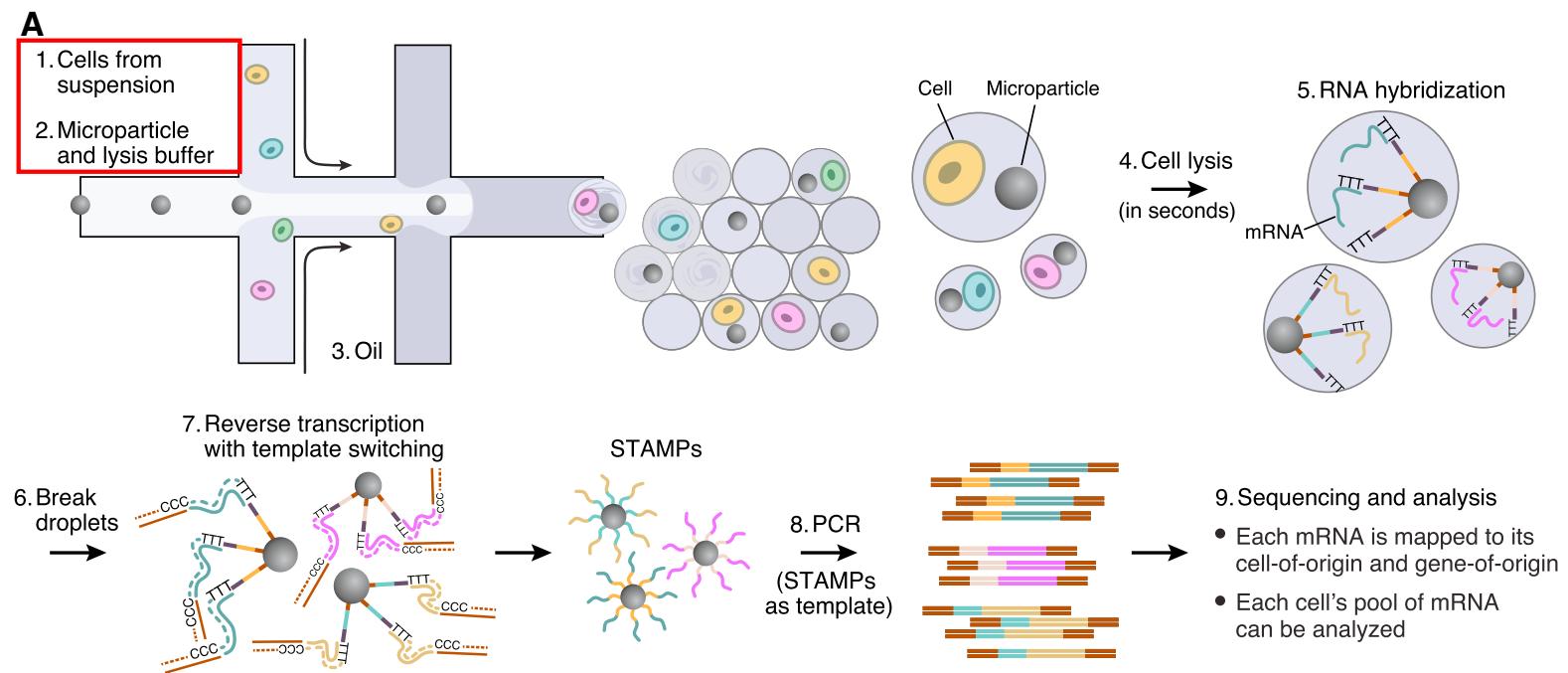


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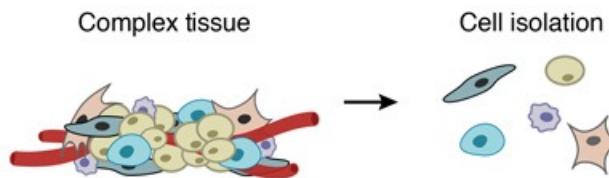
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DropSeq overview

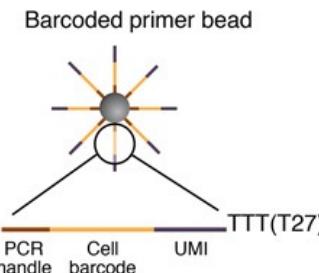


DropSeq overview

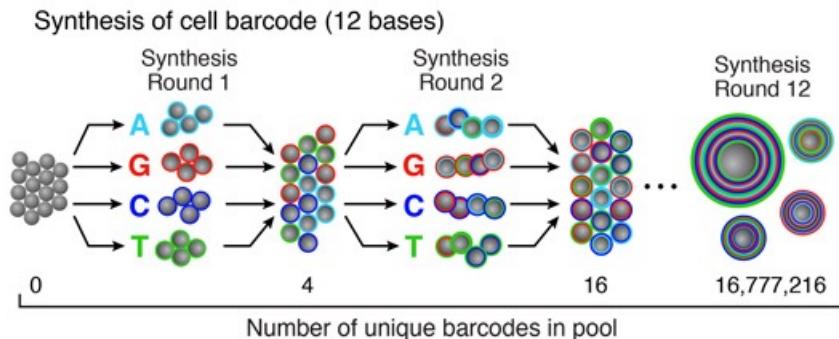
1. PREPARE A SINGLE-CELL SUSPENSION FROM A COMPLEX TISSUE



2. MICROPARTICLE PREPARATION



Split-and-pool synthesis of the cell barcode



Synthesis of a unique molecular identifier (UMI)

Synthesis of UMI (8 bases)

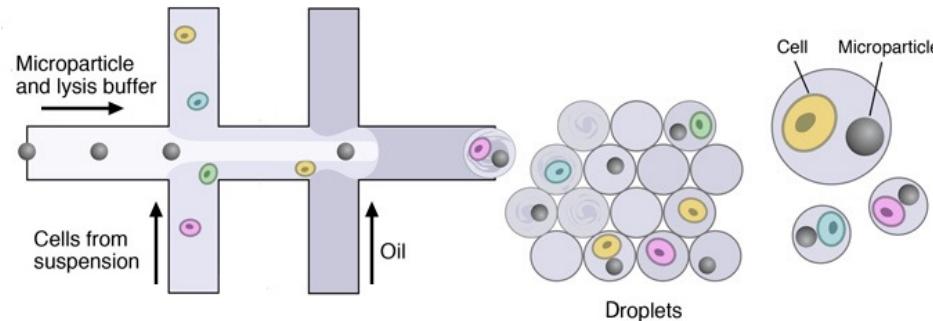


- Millions of the same cell barcode per bead
- 4^8 different molecular barcodes (UMIs) per bead

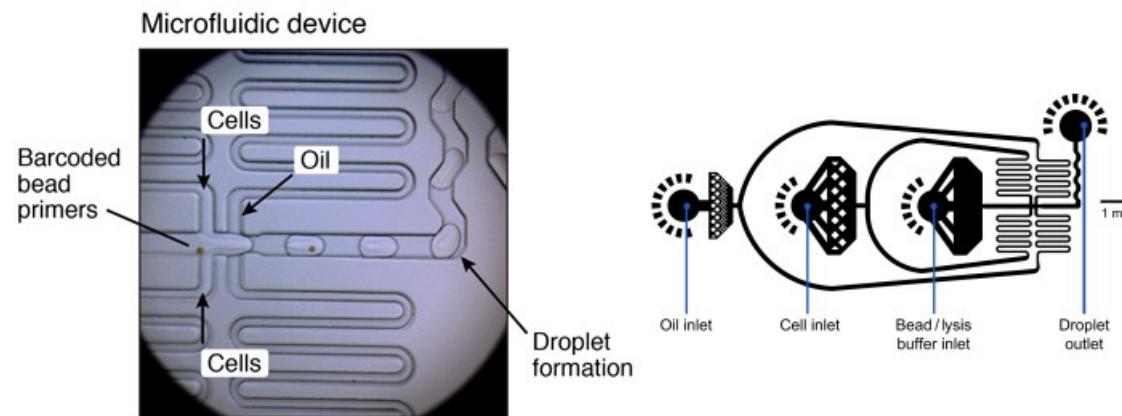
Cell, 2015, 161, 1202–1214

DropSeq workflow

3. MICROFLUIDIC DEVICE



Microfluidic setup

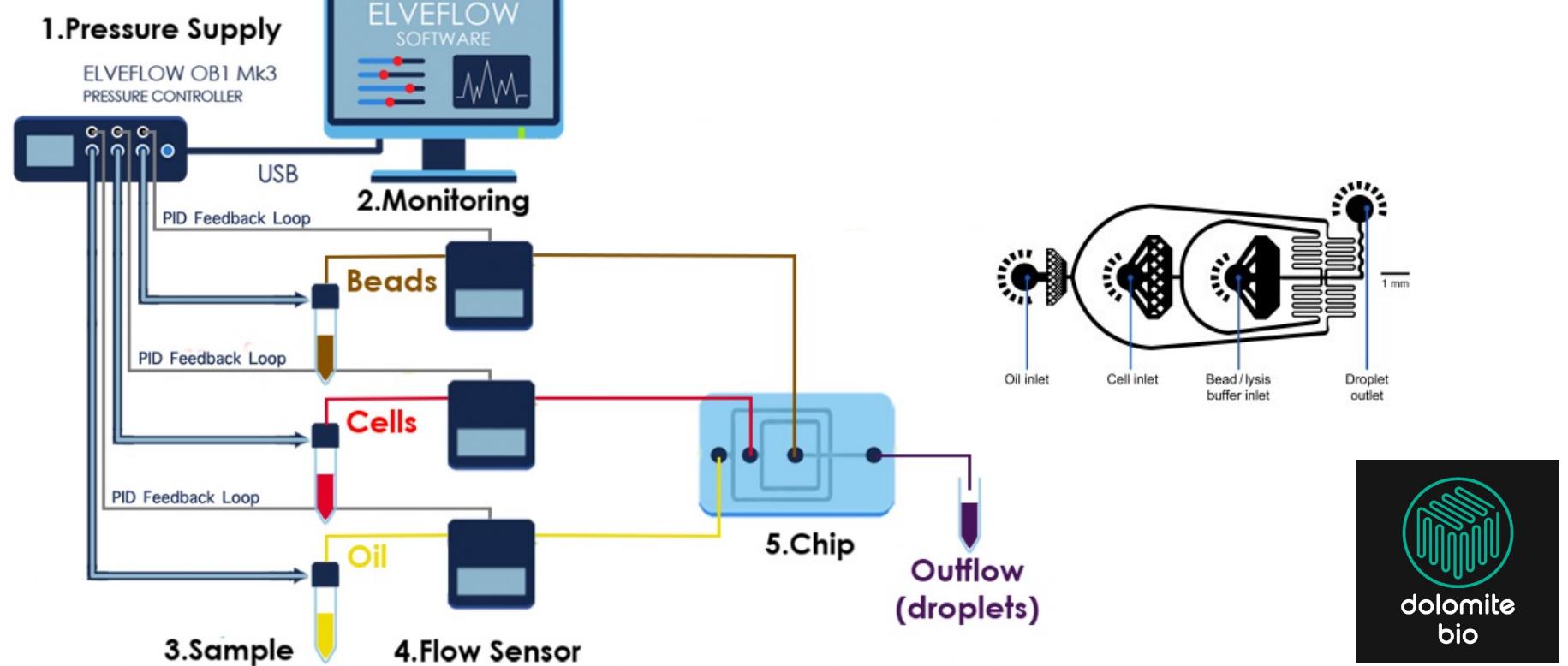


Cell, 2015, 161, 1202–1214

DropSeq workflow

3. MICROFLUIDIC DEVICE

Set-up Diagram

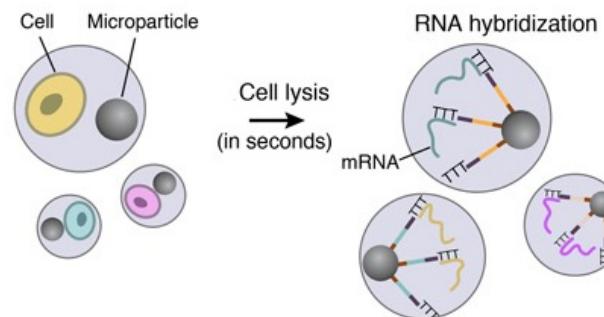


Cell, 2015, 161, 1202–1214



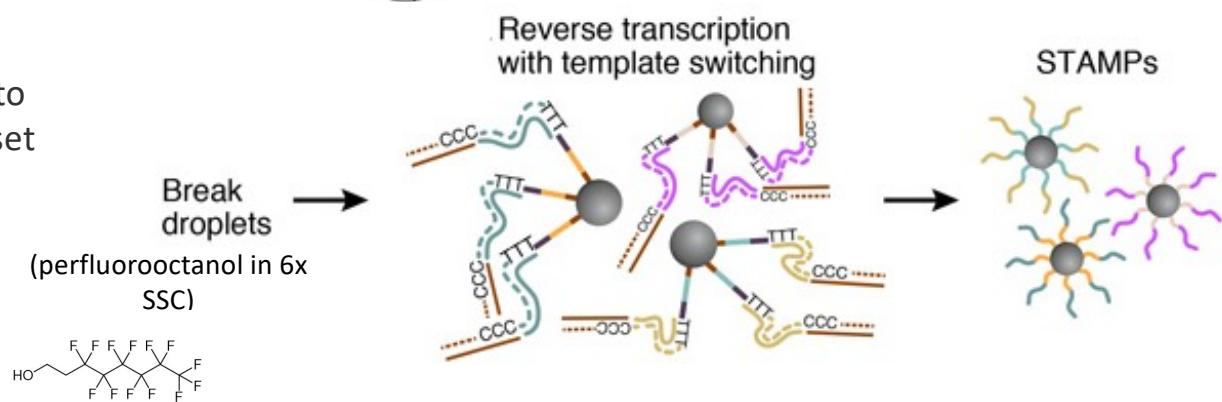
DropSeq workflow

4. CELL LYSIS AND RNA HYBRIDIZATION



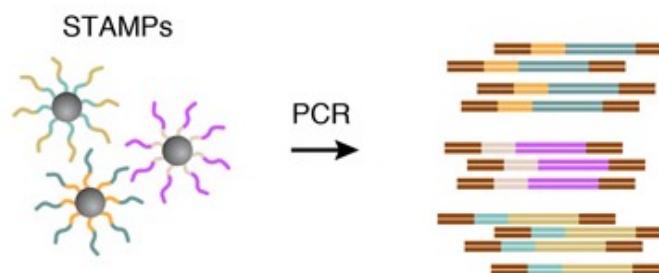
5. STAMPS GENERATION

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).

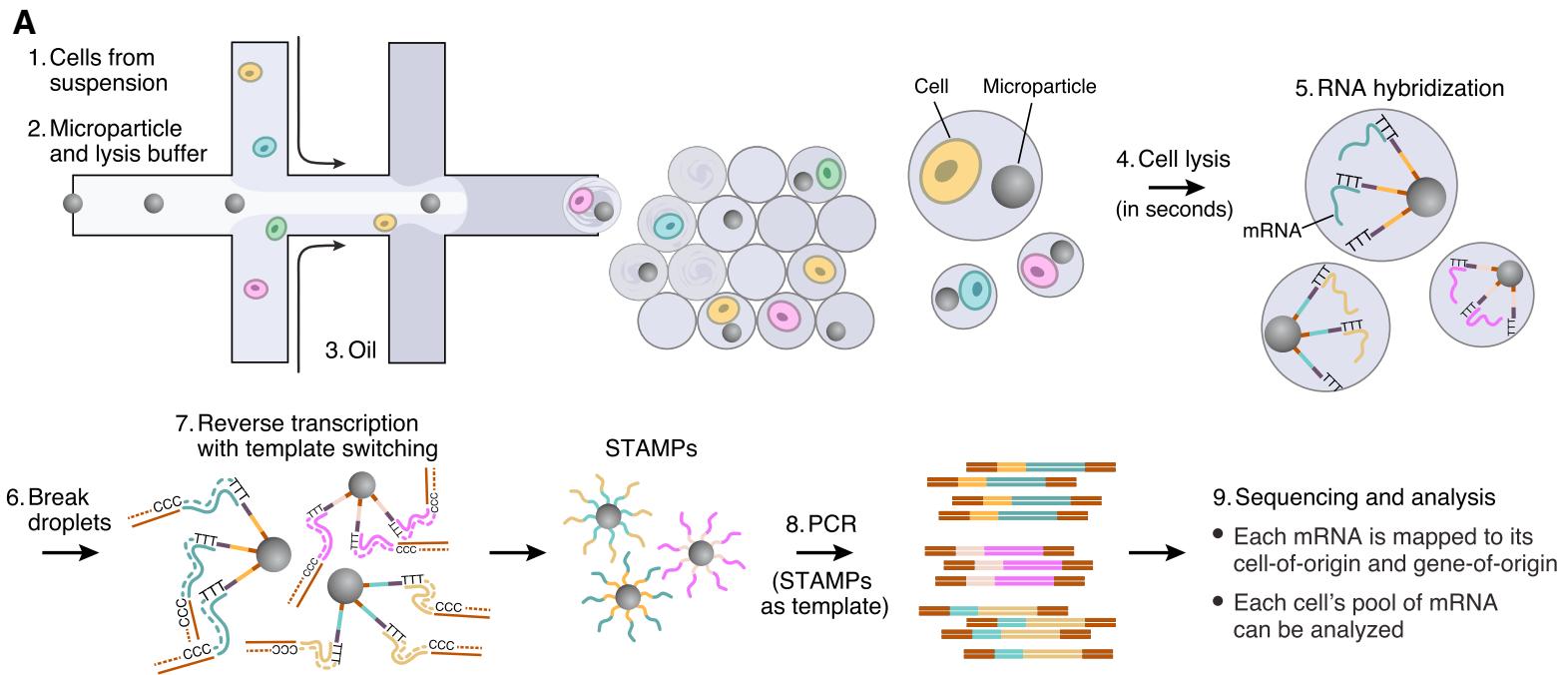


6. AMPLIFICATION OF STAMPS

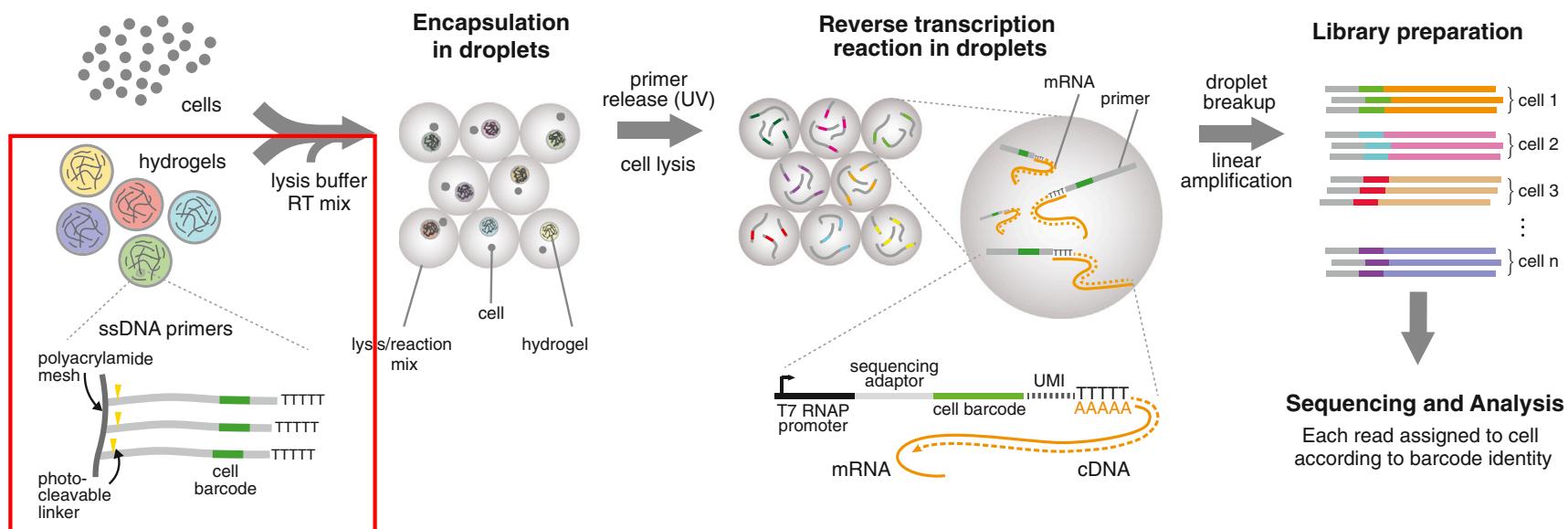
The barcoded STAMPs can then be amplified in pools by PCR reaction for high-throughput mRNA-sequencing, to analyze any desired number of individual cells.



DropSeq overview



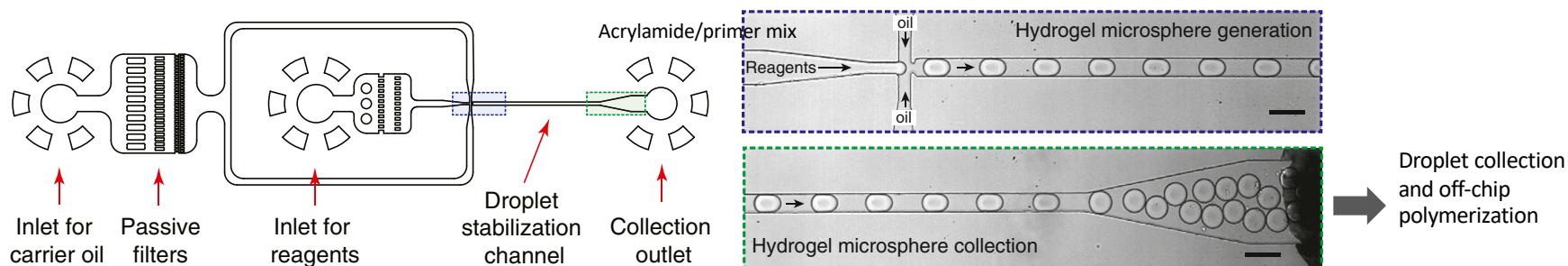
InDrop overview



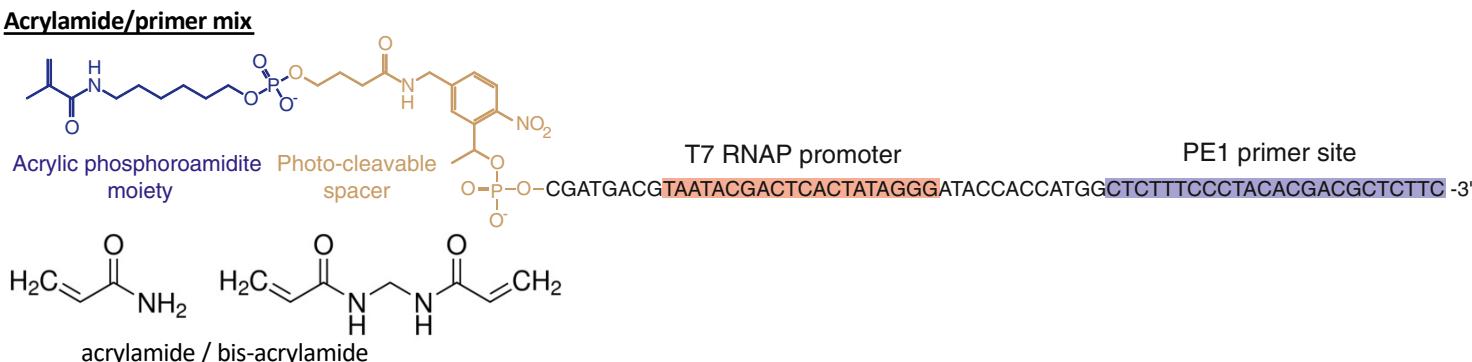
InDrop workflow

I) Synthesis of barcoded hydrogel beads

A



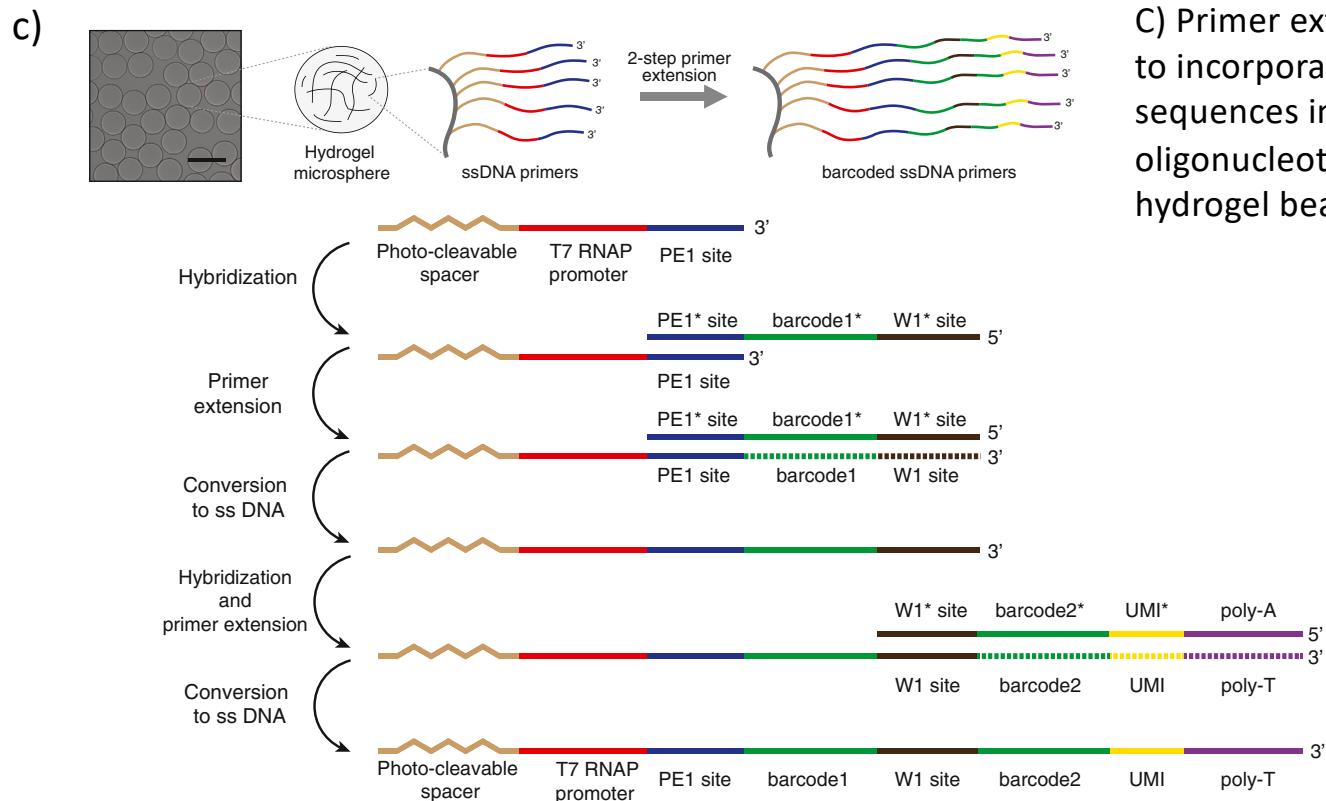
B



(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.

InDrop workflow

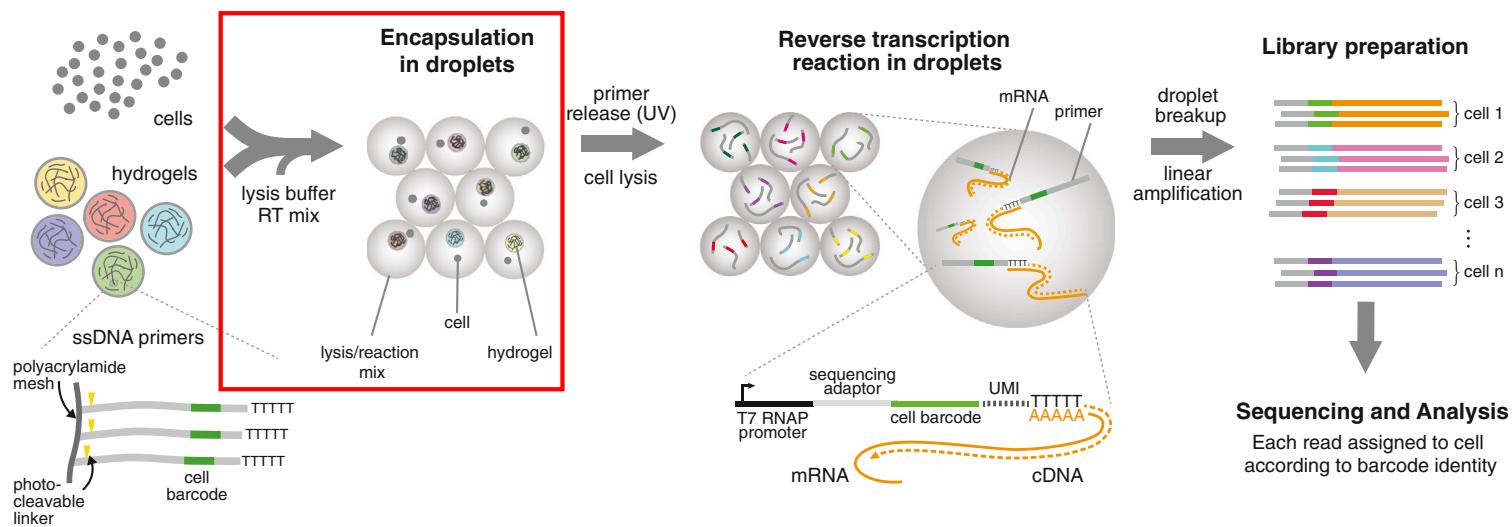
I) Synthesis of barcoded hydrogel beads



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

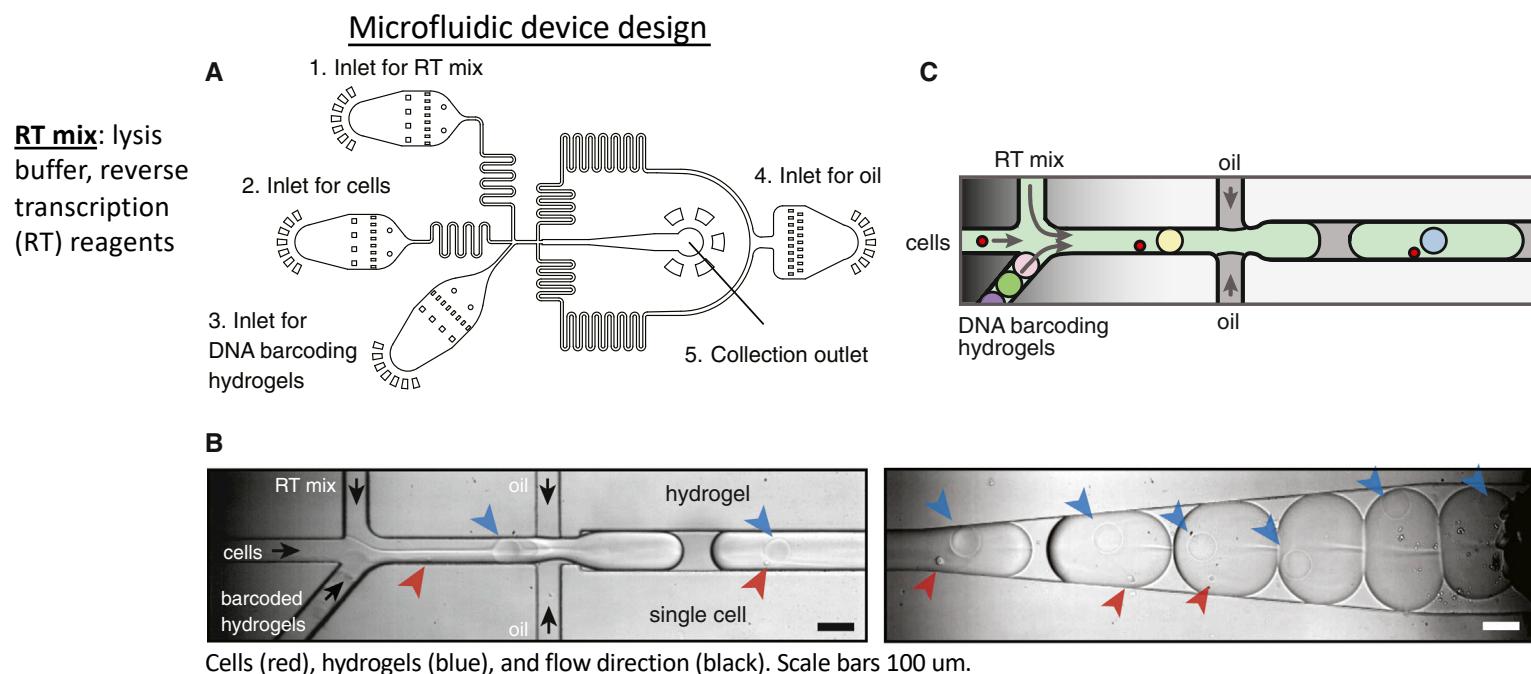
5'- /5Acryd//iSpPC/ CGATGACG TAATACGACTCACTATAGGG ATACCACCATGG CTCTTTCCCTACACGACGCTCTC
CGATCT [barcode1] AAGGCGTCACAAGCAATCACTC [barcode2] NNNNNN TTTTTTTTTTTTTTTTV-3'

InDrop overview

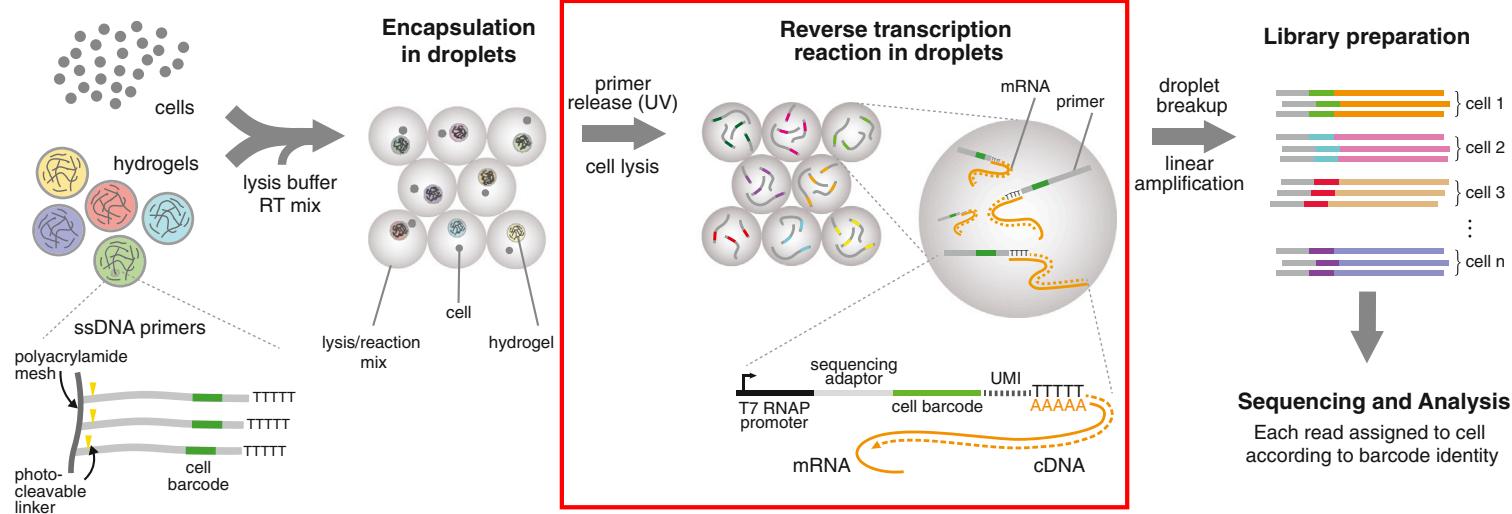


InDrop workflow

II) Encapsulation in droplets



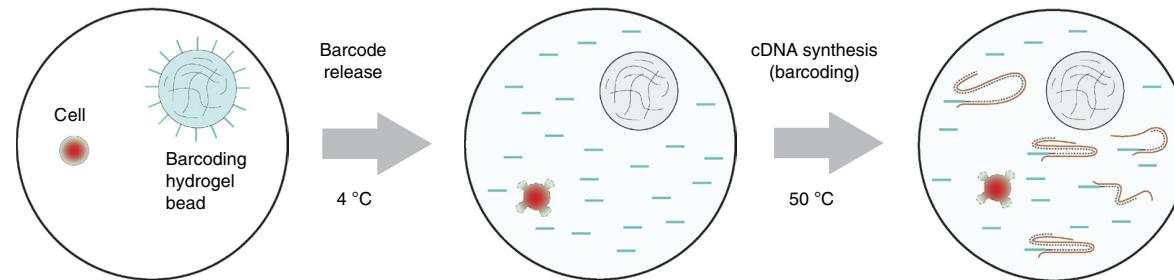
InDrop overview



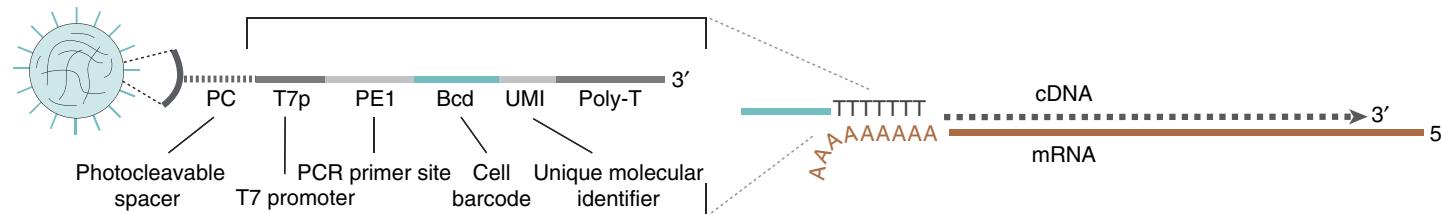
InDrop workflow

III) Reverse transcription in droplets

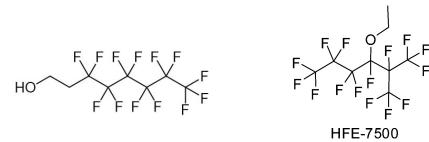
- After cell and hydrogel bead encapsulation, the barcoded cDNA primers are released from the beads using **365 nm UV light** ($\sim 10 \text{ mW/cm}^2$; which is not damaging to DNA/RNA), followed by mRNA capture and reverse transcription



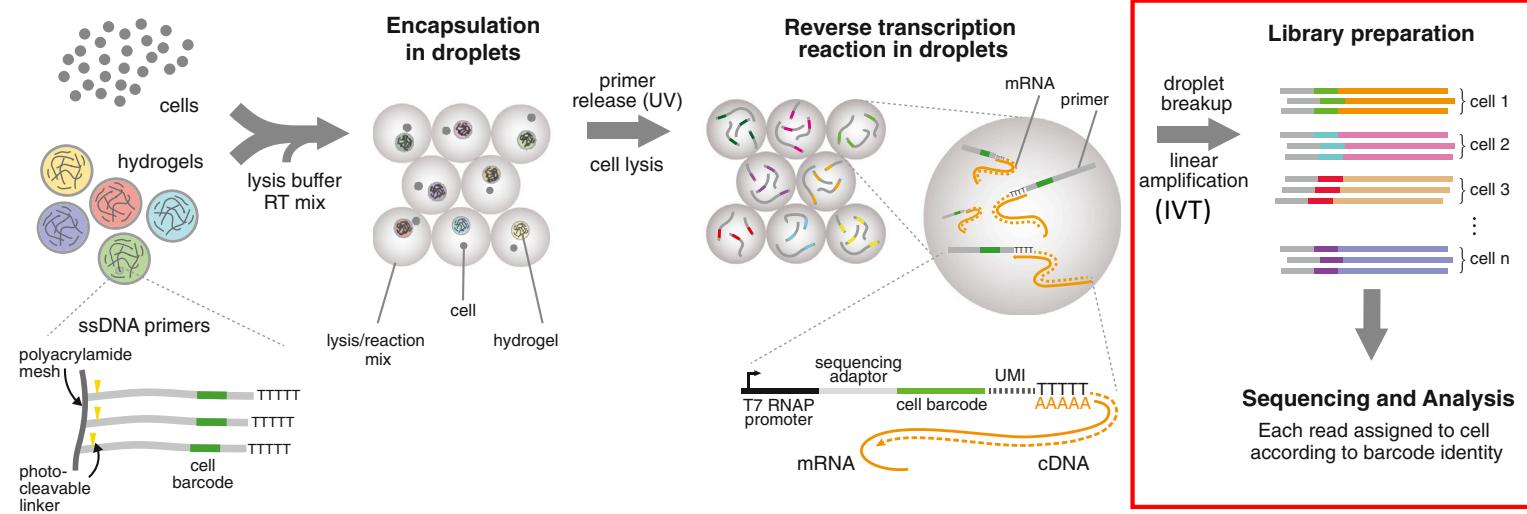
- mRNA capture and reverse transcription



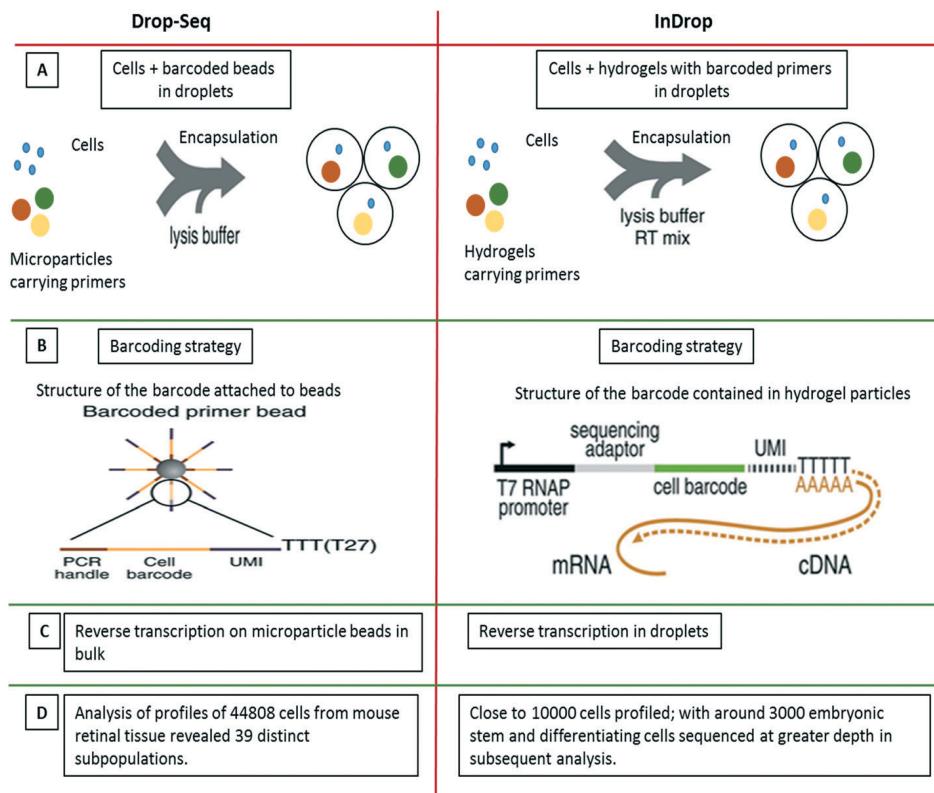
InDrop overview



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



DropSeq vs InDrop



Common

- Move throughput from hundreds (plate-based) to thousands of cells
- Droplet-based processing using microfluidics
- 3'-end
- Use UMI (unique molecular identifier)

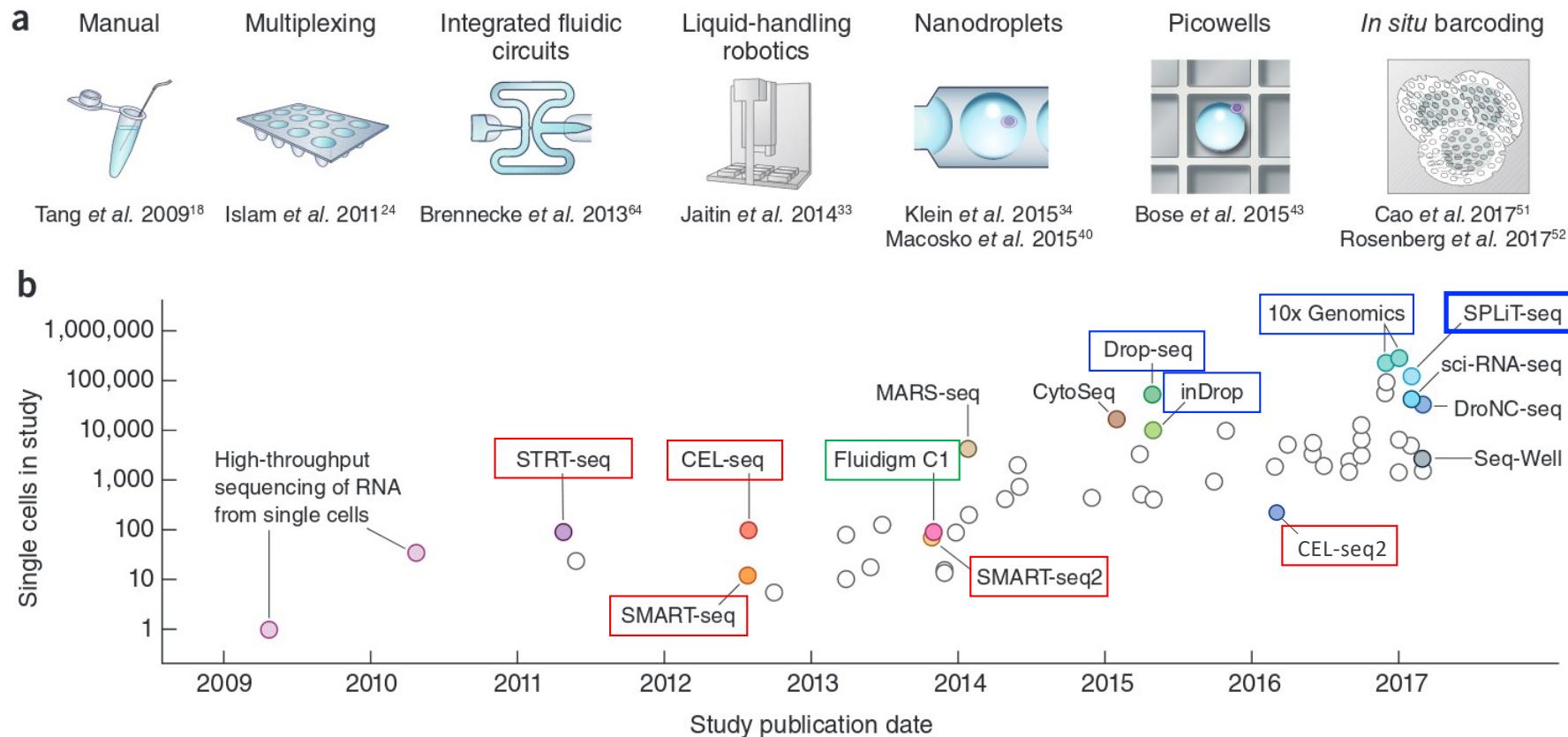
DropSeq

- 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



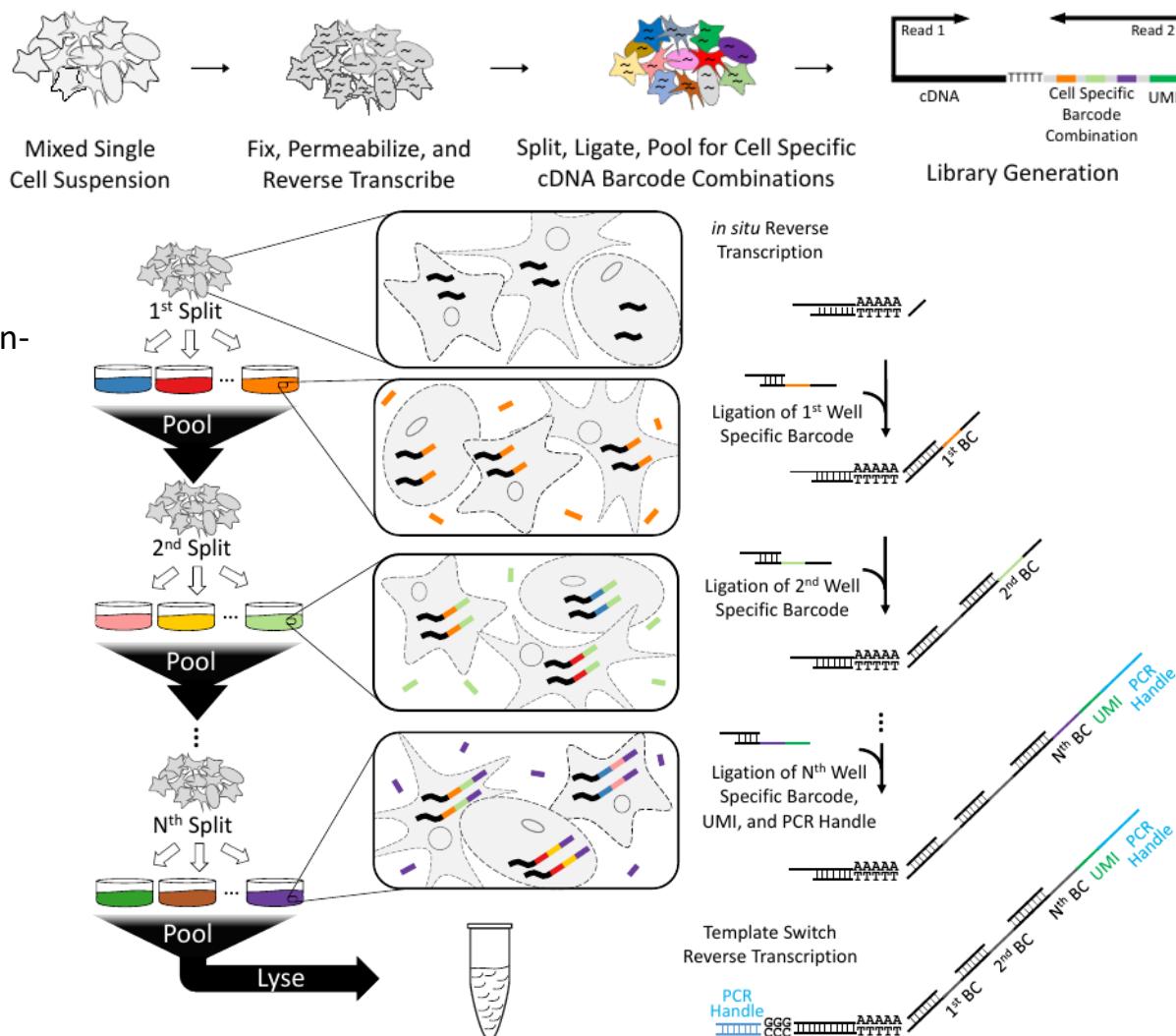
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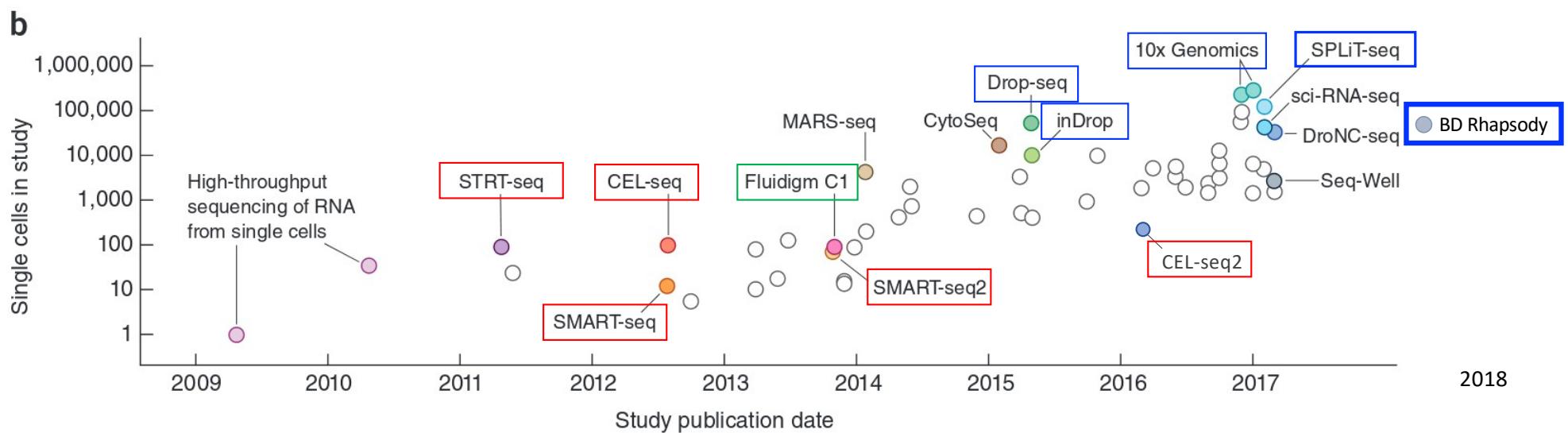
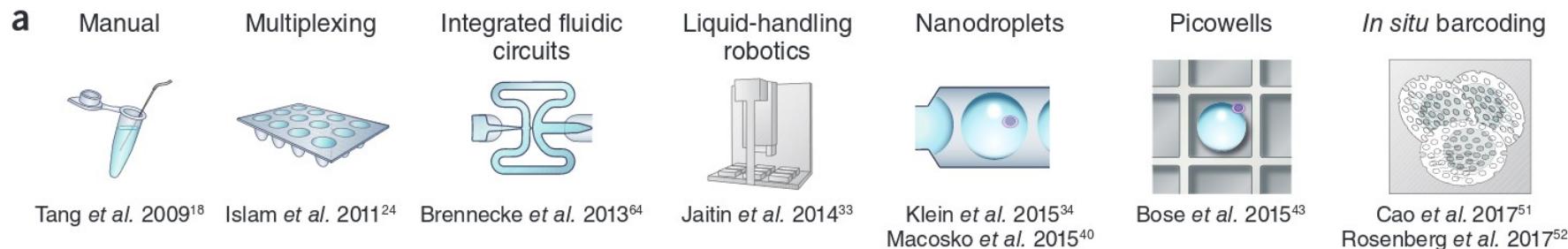
In situ barcoding

SPLIT-seq:
Split Pool Ligation-based
Transcriptome sequencing



Rosenburg et al, 2018, Science

Evolution of scRNASeq techniques



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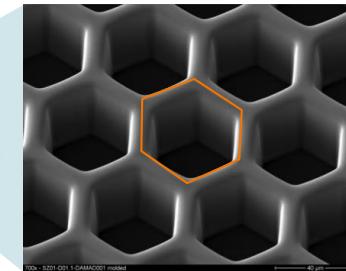
Svensson *et al. Nature Protocols* (2018)

BD Rhapsody™ Instrument

Rhapsody imaging
Scanner for QC
Measurements

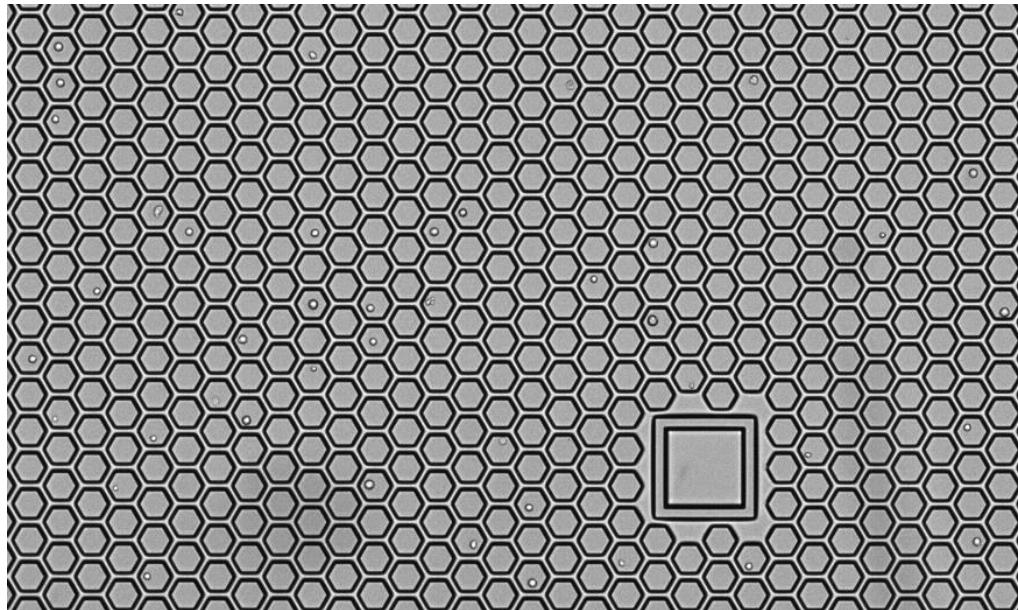
One system for analyzing
both protein and RNA at a
single cell level

Rhapsody Express
For manipulating fluidics
from cell capture and processing



Microwell Cartridge

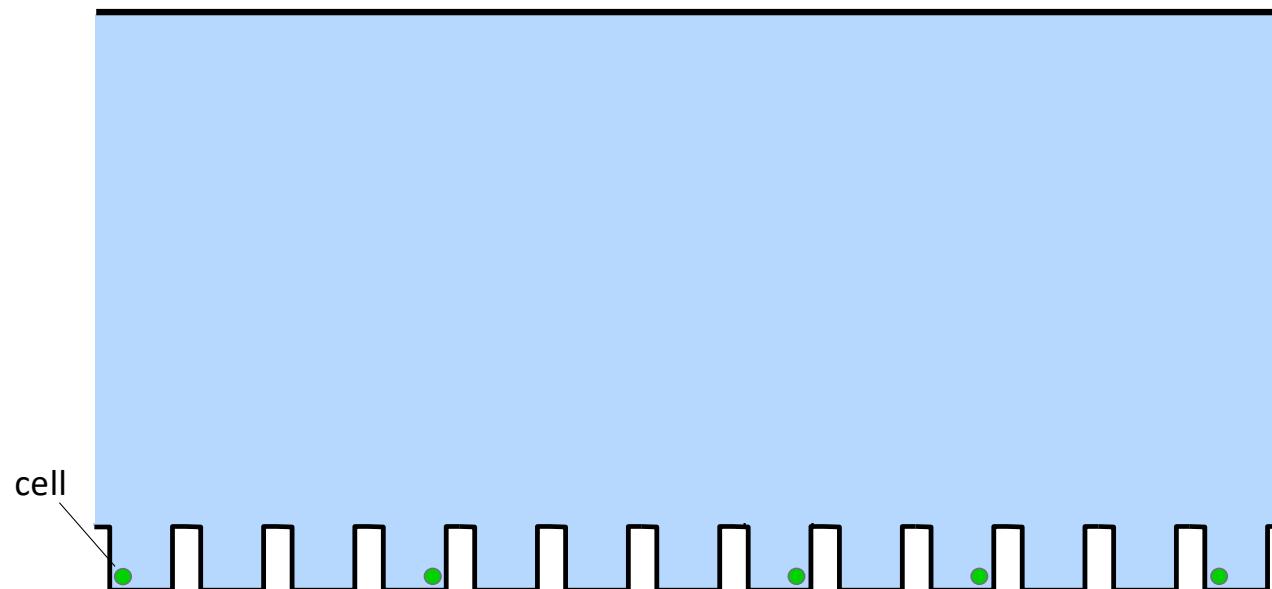
BD Rhapsody – micowell cartridge



- 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

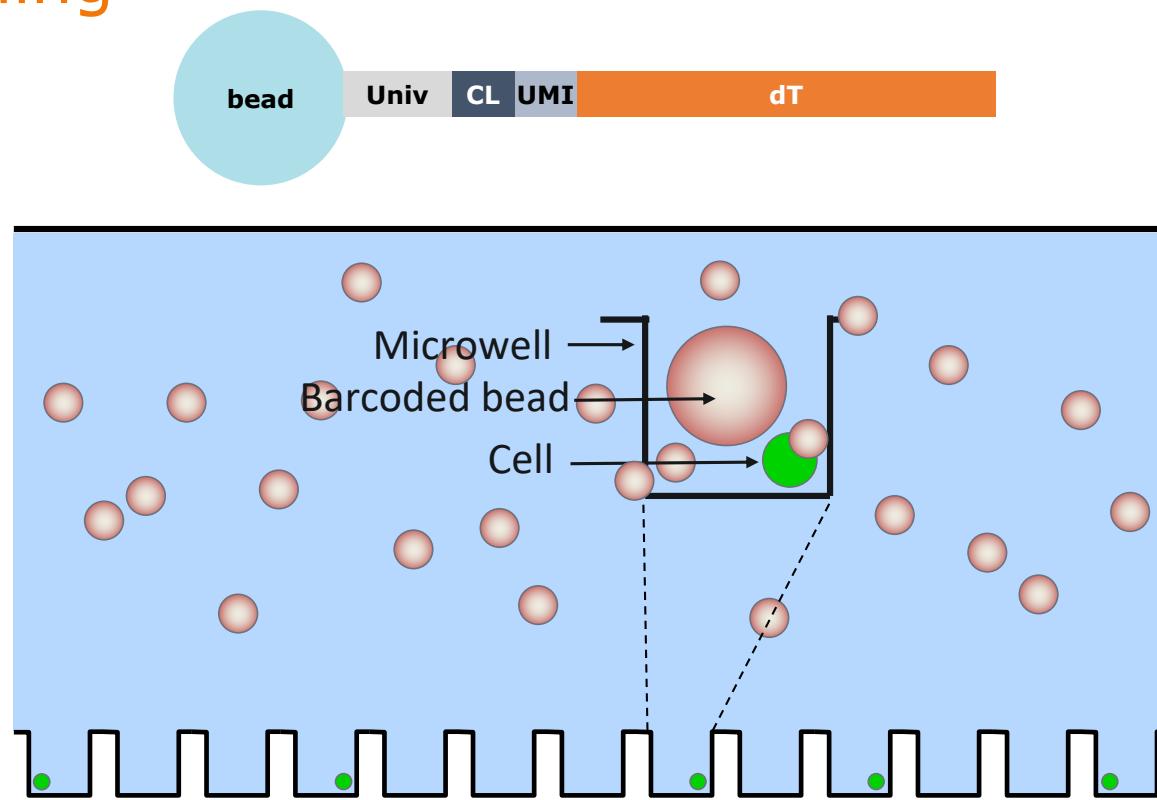
BD Rhapsody – microwell cartridge workflow

Cell loading



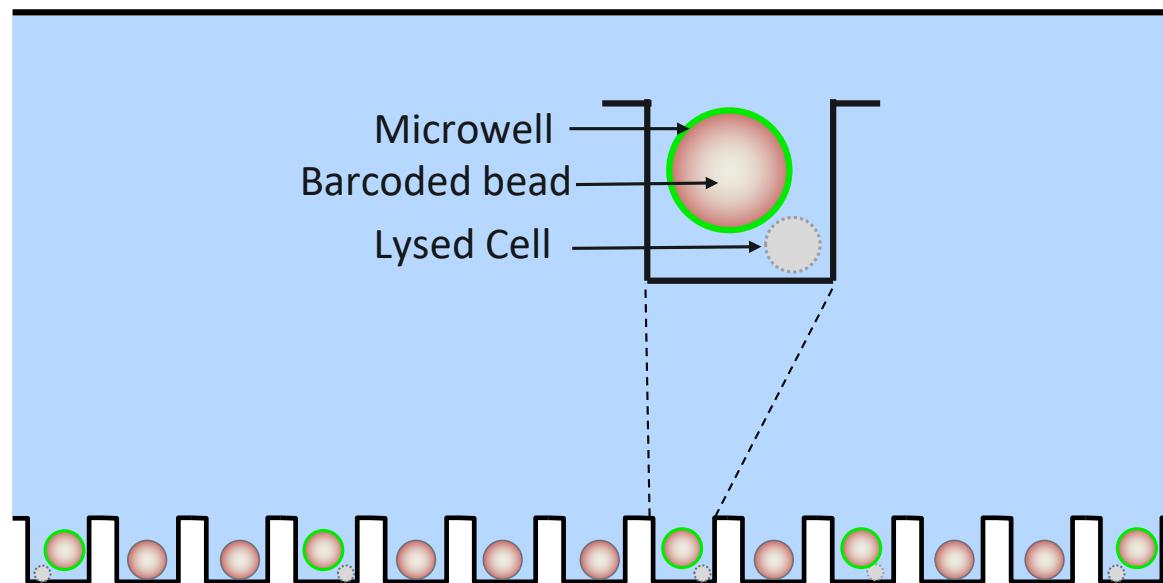
BD Rhapsody – microwell cartridge workflow

Bead loading



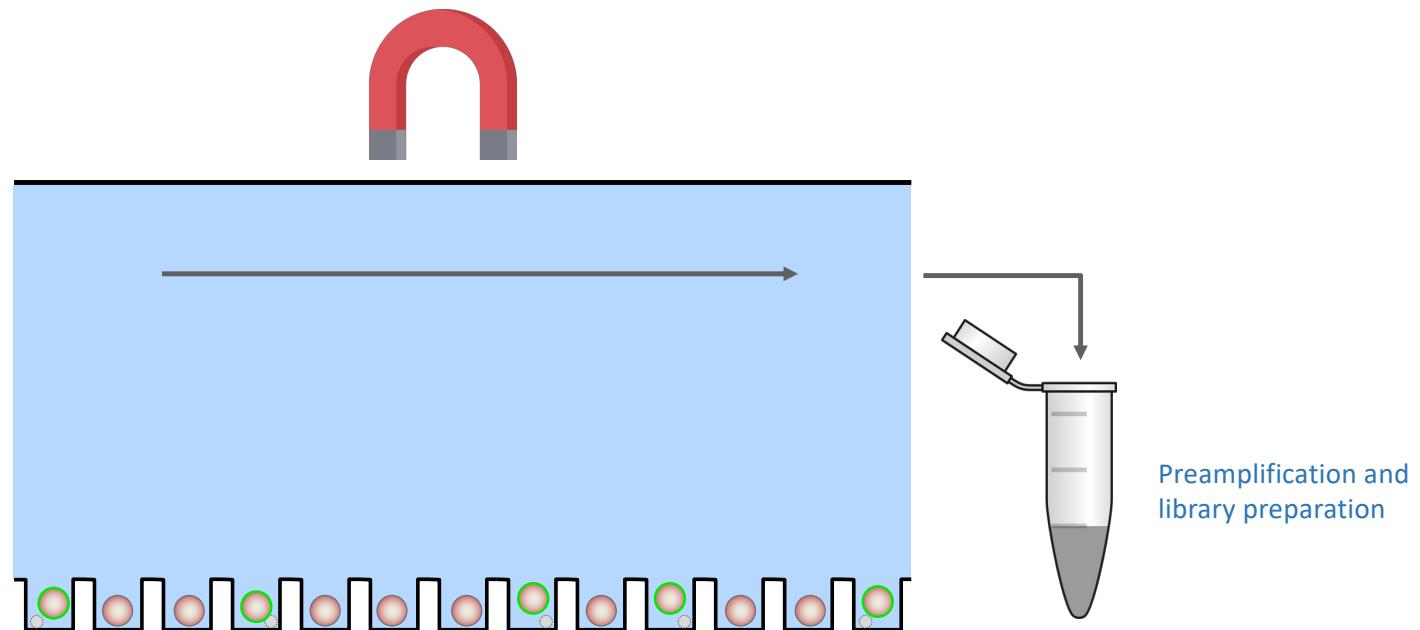
BD Rhapsody – microwell cartridge workflow

Lysis and mRNA hybridization

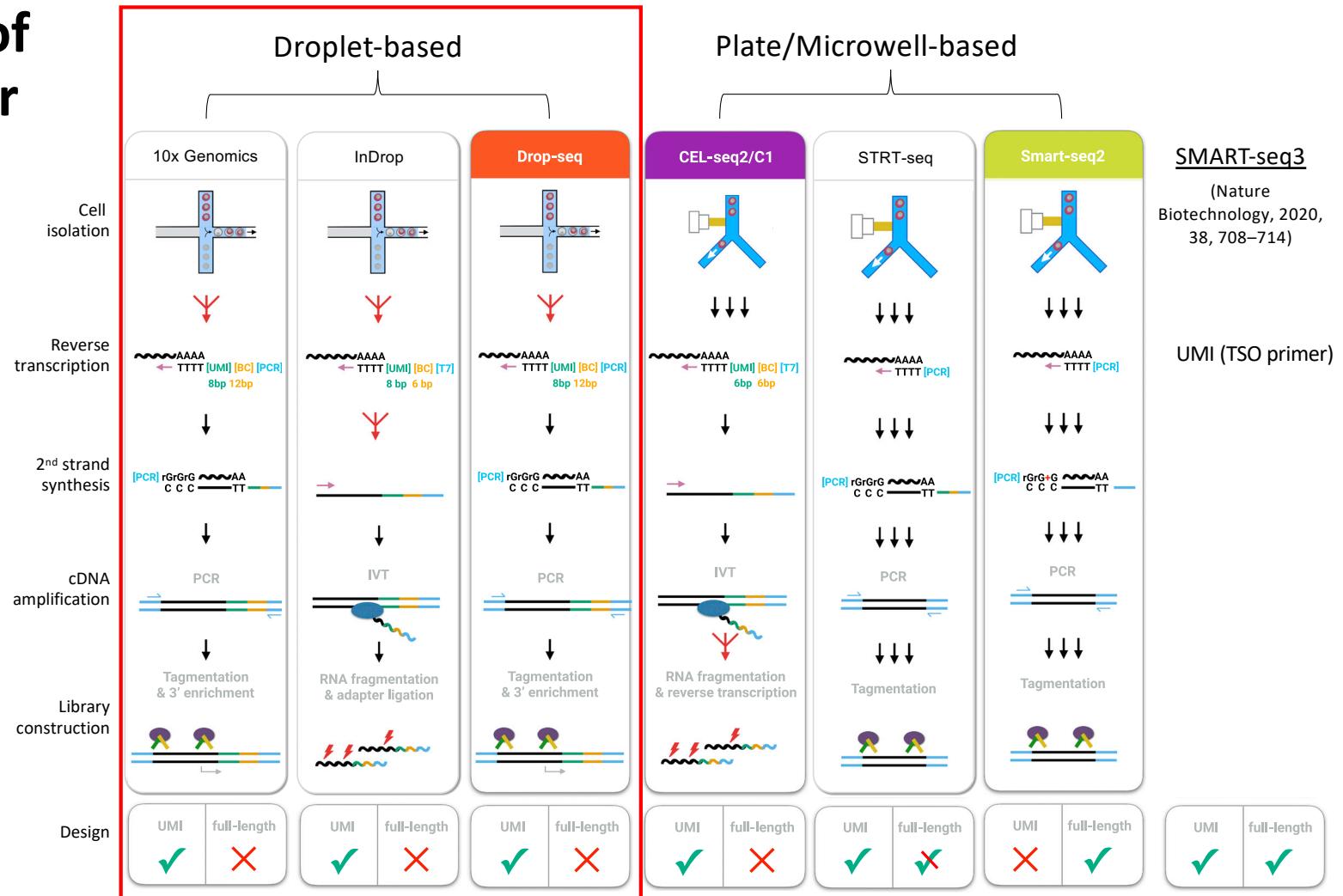


BD Rhapsody – micowell cartridge workflow

Retrieval of beads



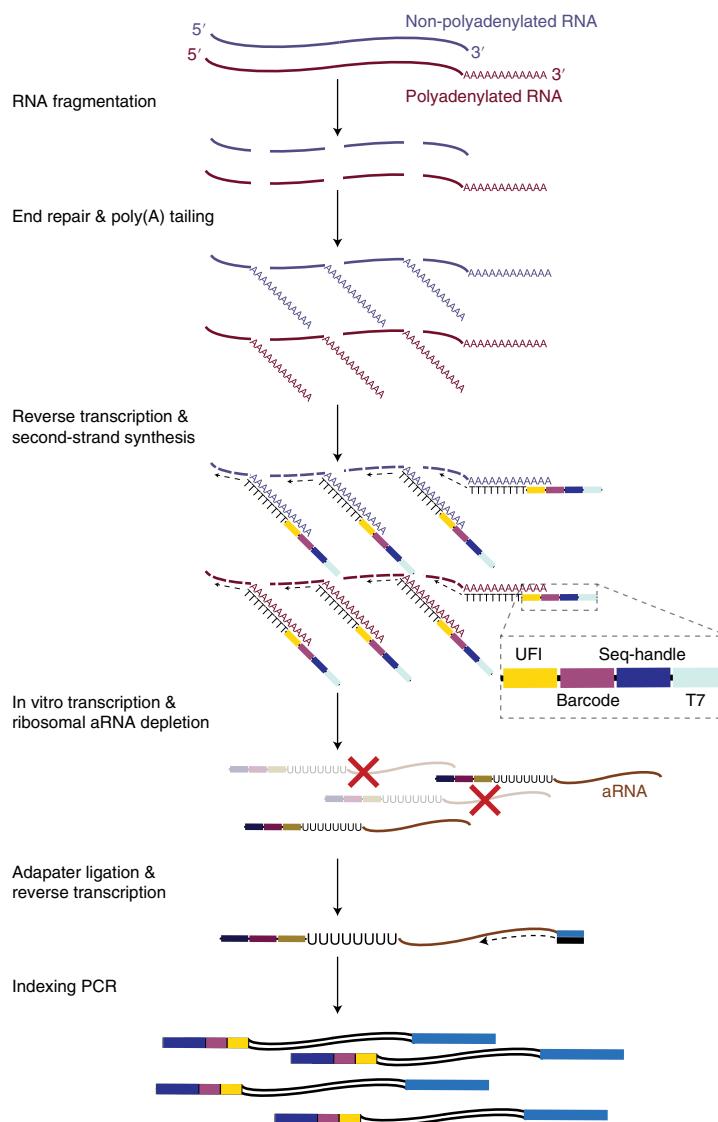
Summary of the popular scRNASeq methods



Molecular Cell, 2017 65, 631–643

VASA-seq: vast transcriptome analysis of single cells by dA-tailing

→ Profile full-length RNAs and both polyadenylated & non-polyadenylated RNAs



Nature Biotechnology, 2022

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 (of a small amount of cells)? 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, VASA-seq, (STRT-seq?)
- To have fewer errors? IVT-based amplification method (CEL-seq2, InDrop, VASA-seq)
- To study transcription start sites? STRT-seq, SMART-seq2/3, 10X Genomics 5' method, (VASA-seq)
- To capture poly(A)- and non-poly(A)-RNAs & study non-coding RNAs? VASA-seq

