

# NCH\_Coder\_Upgrade

scRGOT 2025



Single Cell Research Group for Omics Technology

# Introduction to scRNA-seq

**Katie Miller, PhD**

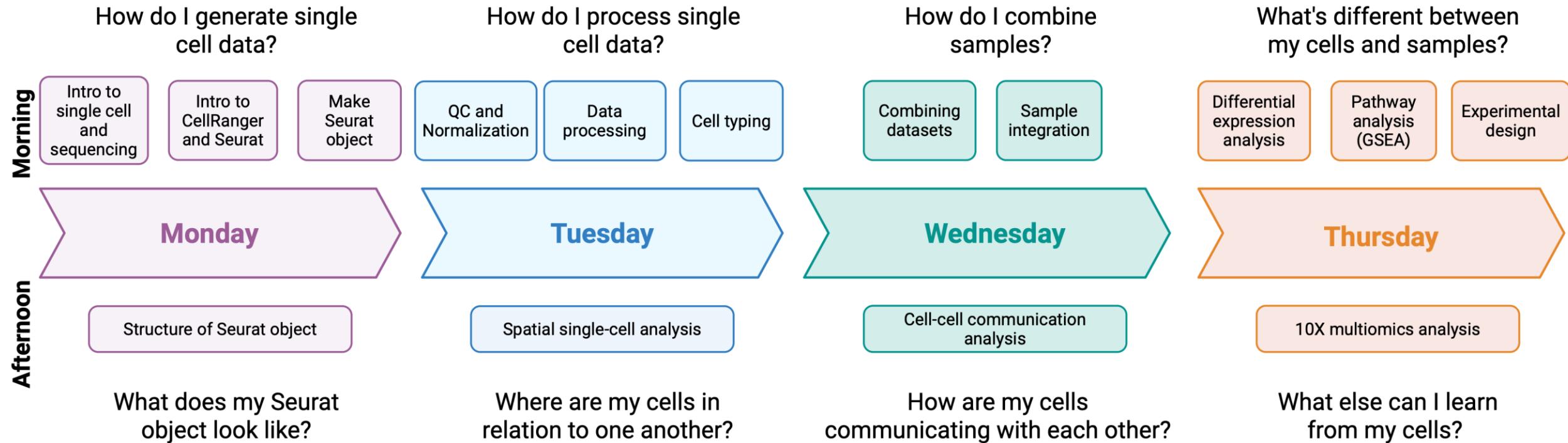
*Principal Investigator & Director of Genomic Services*

**Anthony Miller, PhD**

*Director of Technology Development*



# Welcome!

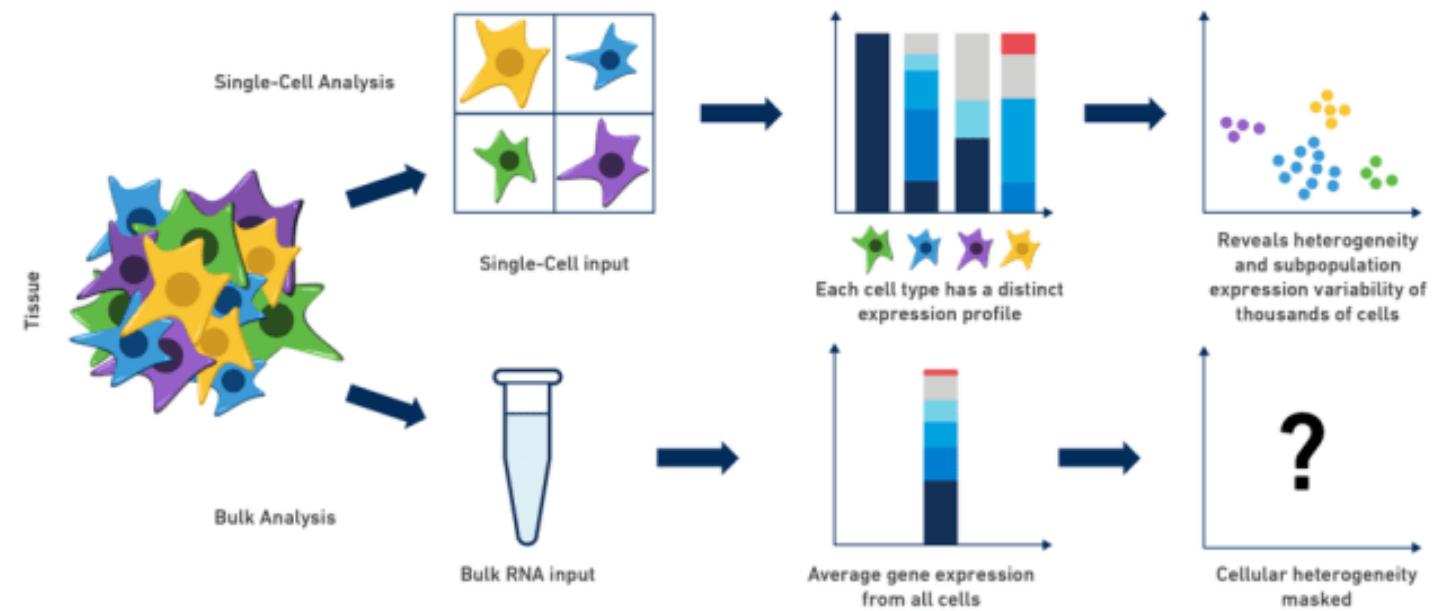


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*When your child needs a hospital, everything matters.<sup>SM</sup>*

# Single Cell Theory

## Advantages of Single Cell vs. Bulk RNA-seq

Image credit: 10x  
Genomics, LIT000027



# Single Cell Theory

## Advantages of Single Cell vs. Bulk RNA-seq

- Differential gene expression between cell types

- genes expressed highly in blue vs. green

- Rare cell population detection

- only three blue legos

- Lineage and development relationships

- large vs. small lego

- Cell type proportions

- how many green vs. yellow legos



Bulk RNA-seq



Single Cell RNA-seq

# The advent of single cell RNA-sequencing: First report of single cell RNA-seq

Article | Published: 06 April 2009

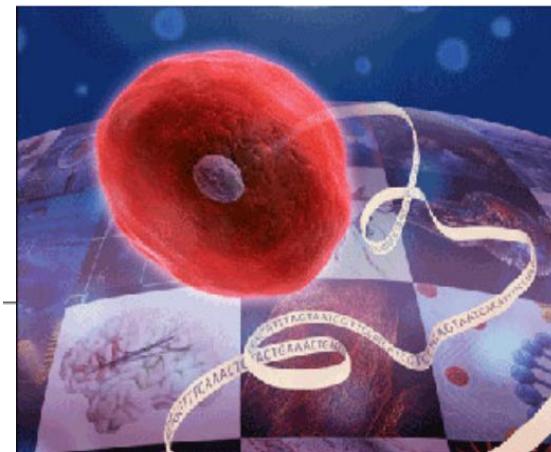
## mRNA-Seq whole-transcriptome analysis of a single cell

[Fuchou Tang](#), [Catalin Barbacioru](#), [Yangzhou Wang](#), [Ellen Nordman](#), [Clarence Lee](#), [Nanlan Xu](#), [Xiaohui Wang](#),

[John Bodeau](#), [Brian B Tuch](#), [Asim Siddiqui](#), [Kaiqin Lao](#)✉ & [M Azim Surani](#)✉

*Nature Methods* **6**, 377–382 (2009) | [Cite this article](#)

**57k** Accesses | **1961** Citations | **155** Altmetric | [Metrics](#)

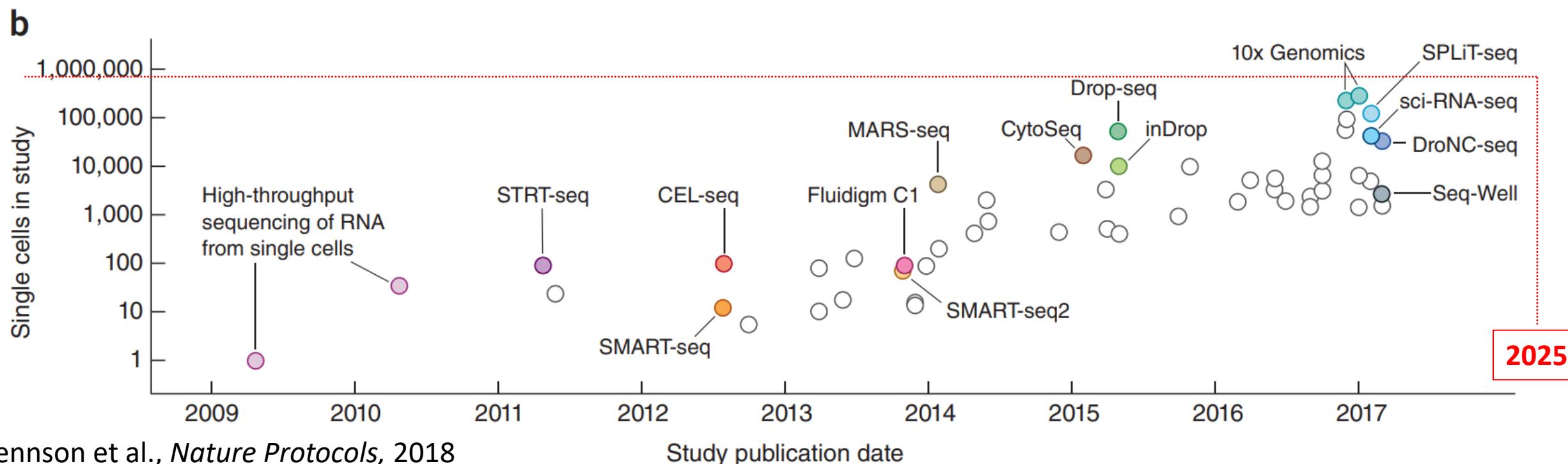
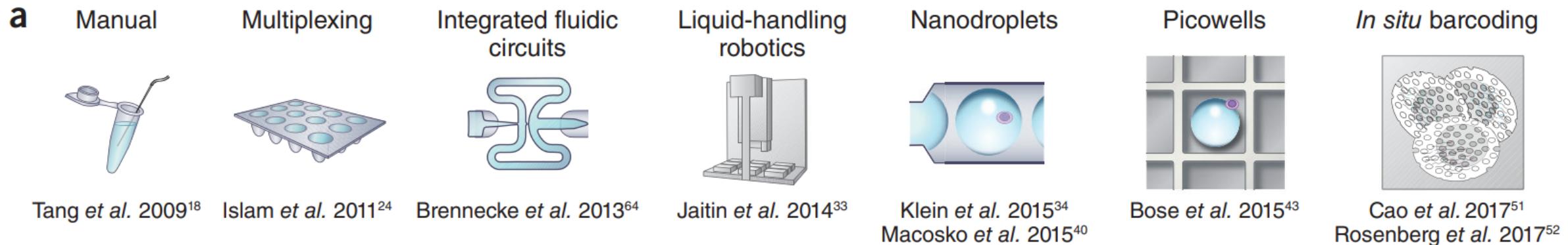


EDITORIAL

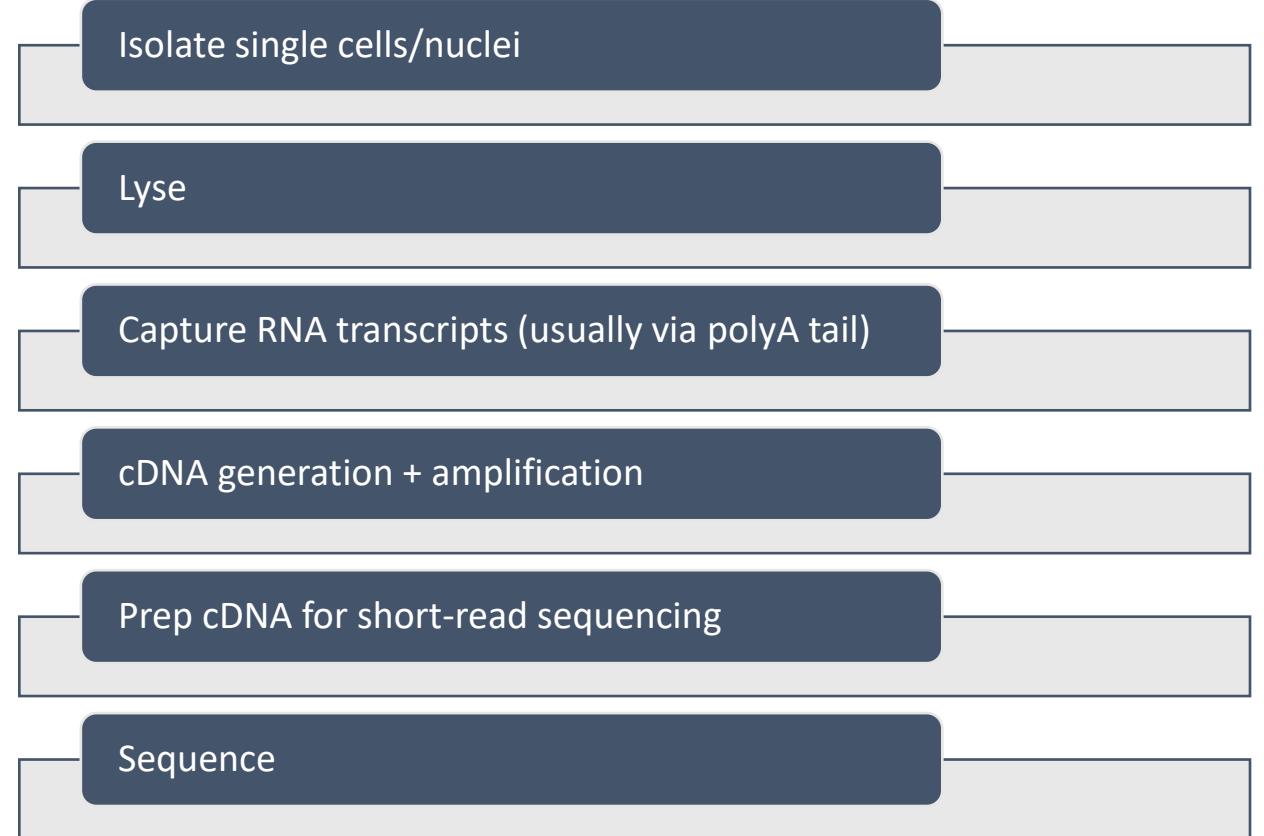
### Method of the Year 2013

Methods to sequence the DNA and RNA of single cells are poised to transform many areas of biology and medicine.

# SINGLE CELL PLATFORMS - Evolution: EXPONENTIAL INCREASE IN THROUGHPUT



# Basics of Single Cell



# SAMPLE PREP MATTERS!

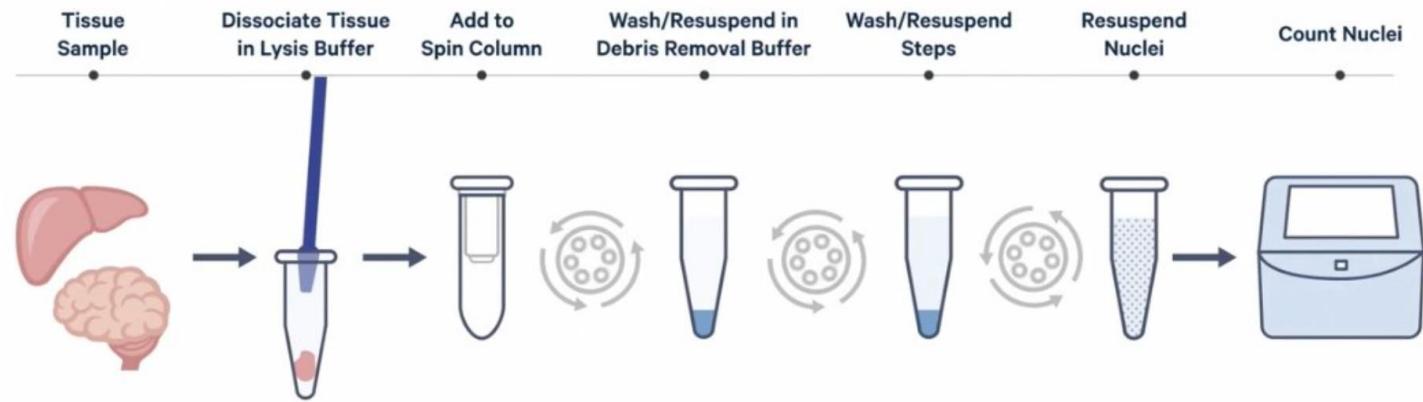
- Dead cells/RNA degradation
  - RNA degradation → RNA loss
  - Fragments of cells → extra RNA / droplet
  - Free-floating RNA → extra RNA / droplet
- Cell clumps → 2+ cells / droplet
- Aim for 80% viability; no clumps

# Sample Prep (fresh – cells vs. frozen – nuclei)

Fresh Tissue Dissociation – chemical vs. mechanical,  
automated vs. manual



Frozen Tissue Dissociation – isolate nuclei and  
remove debris



How many cells?

# Single Cell Theory

To what depth?

When sequencing costs or capacity are limiting, there is often a trade-off between sequencing more cells (**breadth**) and fewer cells with higher read depth (**depth**).

Cell Ranger (10x) provides a 'Sequencing Saturation' metric:

- E.g., sequencing RNA-rich cells (e.g., cell lines or tumors) at 50,000 read pairs per cell may yield only 30-50% saturation, but may sufficient for **clustering** and **cell-type identification**.
- lowly expressed transcripts** = deeper sequencing
- rare cell types** = maximize number of cells

#HELP!!!!

Do I need replicates?

# Single Cell Theory:

- Cell and sample #
- Sequencing depth

#HELP!!!!

"In order to guarantee biologically meaningful findings using transcriptomic experiments, it is important to consider various experimental factors in a systematic way through statistical power analysis."

Table 2

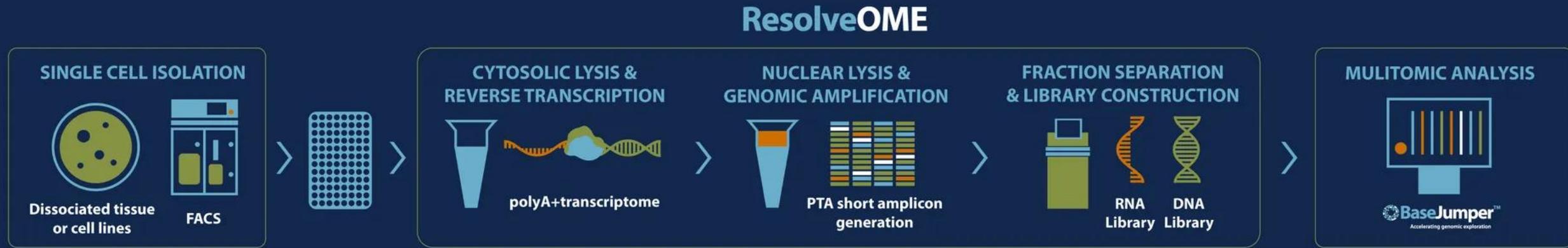
A table with information about different software tools for scRNA-seq power analysis with two distinct detection targets. Experimental Factors: cell number (1), individual number (2), Sequencing depth (3).

Detection Target	# of Samples	Tool Name	Experimental Factor	Software	Model	Power Assessment
Cell sub-population	Single sample	'SCOPIT' [37]	(1)	R package & Web application	Multinomial	Analytical
		'howmanycells'		Web application	Negative Binomial	
		'Sensei' [38]		Web application	Beta Binomial	
	Multi sample	'scPOST' [39]	(1), (2)	R package	Linear mixed model	Simulation-based
		'scPower' [40]	(1), (2), (3)	R package & Web server	Negative Binomial	Pseudobulk
		'hierarchicell' [41]				
DEG	Single sample	'powsimR' [42]	(1)			Simulation-based
		'POWSC' [43]		R package	A mixture of zero-inflated Poisson and log-normal Poisson distributions	
		'scDesign' [44]	(1), (3)		Gamma-Normal mixture model	

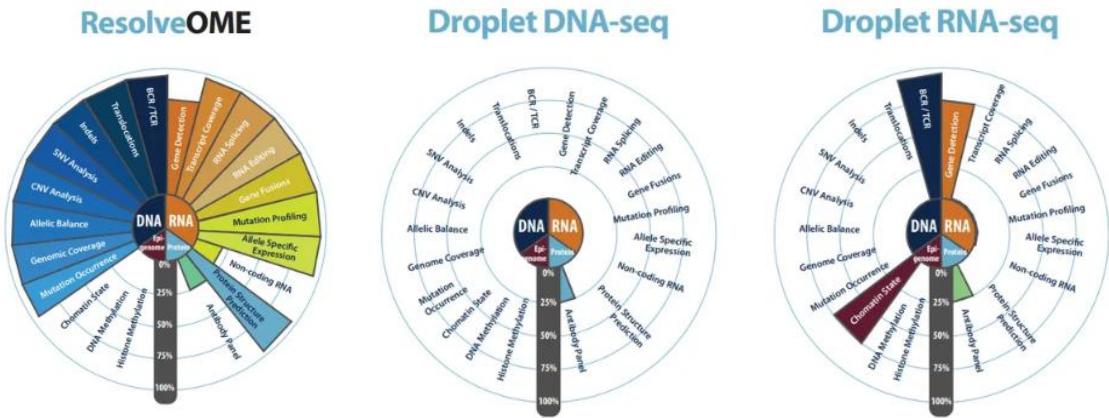


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# SINGLE CELL PLATFORMS – Plate-based



^physical separation of cells into 96-well plate



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# SINGLE CELL PLATFORMS – Plate-based

## Combinatorial indexing

### single cell RNA (no instrument)

> **Science** (IF: 47.73; Q1). 2018 Apr 13;360(6385):176-182. doi: 10.1126/science.aam8999.  
Epub 2018 Mar 15.

#### Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding

Alexander B Rosenberg <sup>1</sup>, Charles M Roco <sup>2</sup>, Richard A Muscat <sup>3</sup>, Anna Kuchina <sup>3</sup>, Paul Sample <sup>3</sup>, Zhen Yao <sup>4</sup>, Lucas T Graybuck <sup>4</sup>, David J Peeler <sup>2</sup>, Sumit Mukherjee <sup>3</sup>, Wei Chen <sup>5</sup>, Suzie H Pun <sup>2</sup>, Drew L Sellers <sup>2</sup> <sup>6</sup>, Bosiljka Tasic <sup>4</sup>, Georg Seelig <sup>1</sup> <sup>5</sup> <sup>7</sup>

Affiliations + expand

PMID: 29545511 PMCID: PMC7643870 DOI: 10.1126/science.aam8999

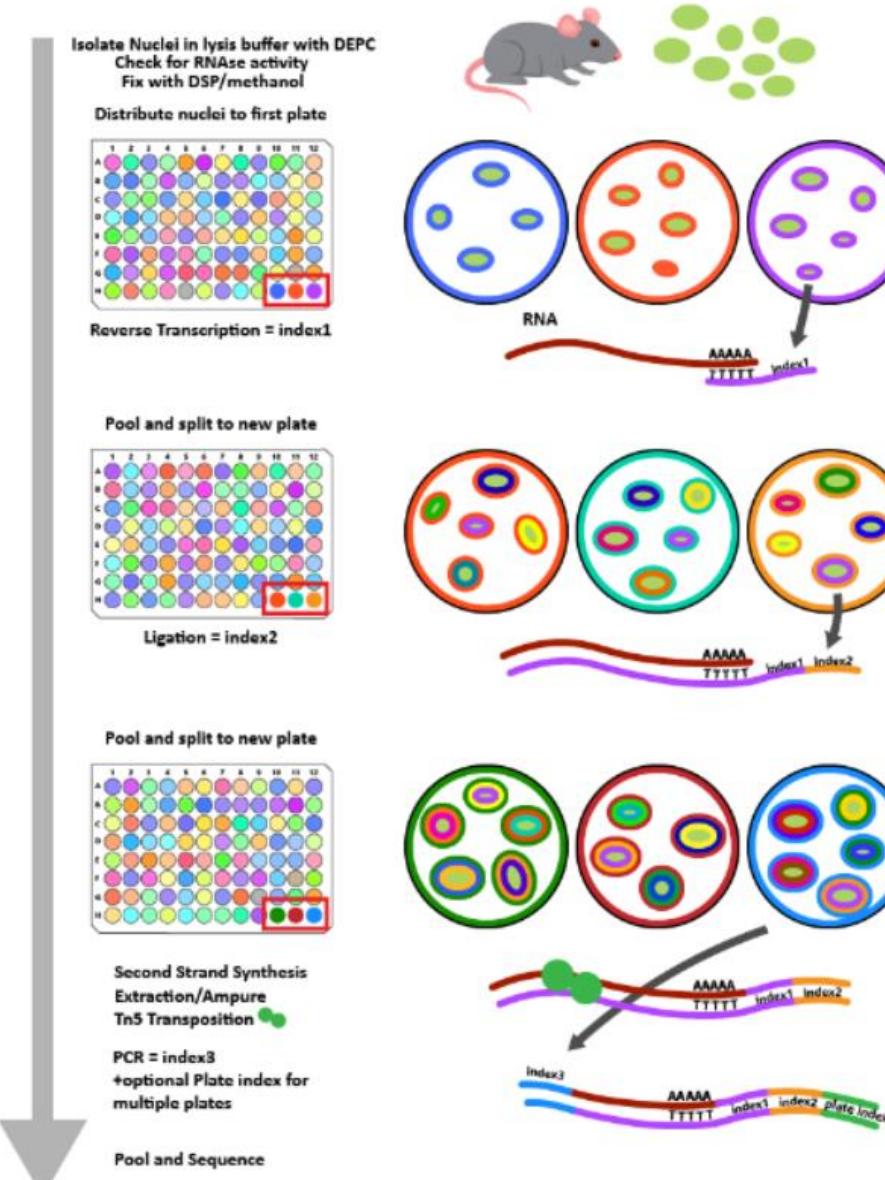
> **Science** (IF: 47.73; Q1). 2017 Aug 18;357(6352):661-667. doi: 10.1126/science.aam8940.

#### Comprehensive single-cell transcriptional profiling of a multicellular organism

Junyue Cao <sup>1</sup> <sup>2</sup>, Jonathan S Packer <sup>1</sup>, Vijay Ramani <sup>1</sup>, Darren A Cusanovich <sup>1</sup>, Chau Huynh <sup>1</sup>, Riza Daza <sup>1</sup>, Xiaojie Qiu <sup>1</sup> <sup>2</sup>, Choli Lee <sup>1</sup>, Scott N Furlan <sup>3</sup> <sup>4</sup> <sup>5</sup>, Frank J Steemers <sup>6</sup>, Andrew Adey <sup>7</sup> <sup>8</sup>, Robert H Waterston <sup>9</sup>, Cole Trapnell <sup>9</sup>, Jay Shendure <sup>9</sup> <sup>10</sup>

Affiliations + expand

PMID: 28818938 PMCID: PMC5894354 DOI: 10.1126/science.aam8940



Combinatorial indexing now commercialized



MORE CELLS, MORE SAMPLES, MORE CLARITY

# Smash the limits of single cell sequencing



## Discover biology at single cell resolution at scale

Our easy-to-use combinatorial indexing kits deliver robust, reliable, and flexible single-cell omics on millions of cells and hundreds of samples

# SINGLE CELL PLATFORMS – Droplet-based



# SINGLE CELL PLATFORMS – Droplet-based How it works (microfluidics)



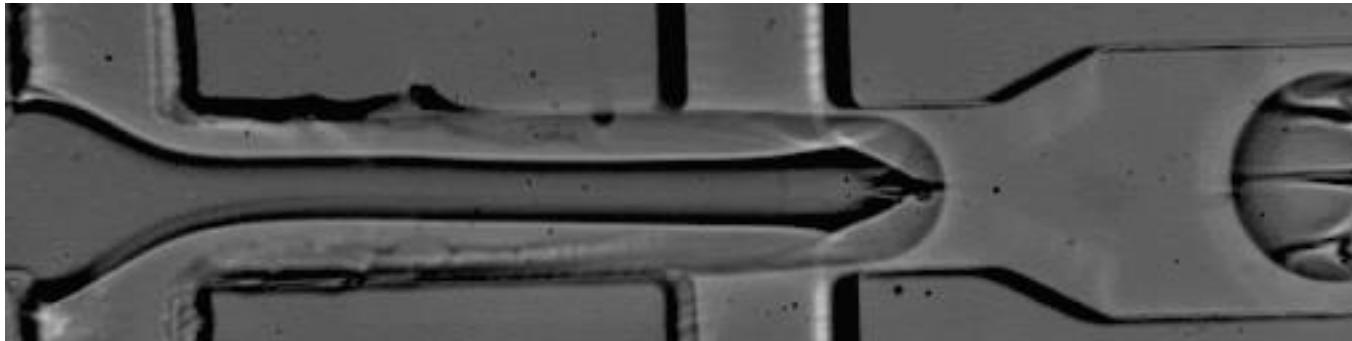
Cell

Volume 161, Issue 5, 21 May 2015, Pages 1202-1214

Resource

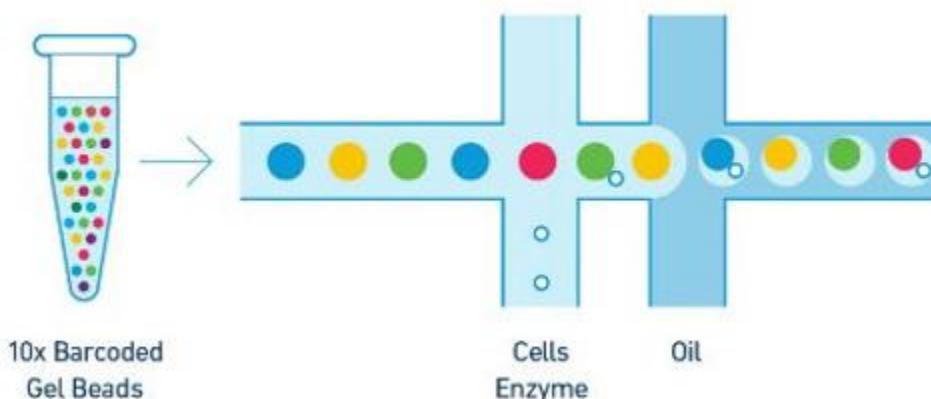
Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets

Evan Z. Macosko<sup>1 2 3</sup>, Anindita Basu<sup>4 5</sup>, Rahul Satija<sup>4 6 7</sup>, James Nemesh<sup>1 2 3</sup>, Karthik Shekhar<sup>4</sup>, Melissa Goldman<sup>1 2</sup>, Itay Tirosh<sup>4</sup>, Allison R. Bialas<sup>8</sup>, Nolan Kamitaki<sup>1 2 3</sup>, Emily M. Martersteck<sup>9</sup>, John J. Trombetta<sup>4</sup>, David A. Weitz<sup>5 10</sup>, Joshua R. Sanes<sup>9</sup>, Alex K. Shalek<sup>4 11 12</sup>, Aviv Regev<sup>4 13 14</sup>, Steven A. McCarroll<sup>1 2 3</sup>

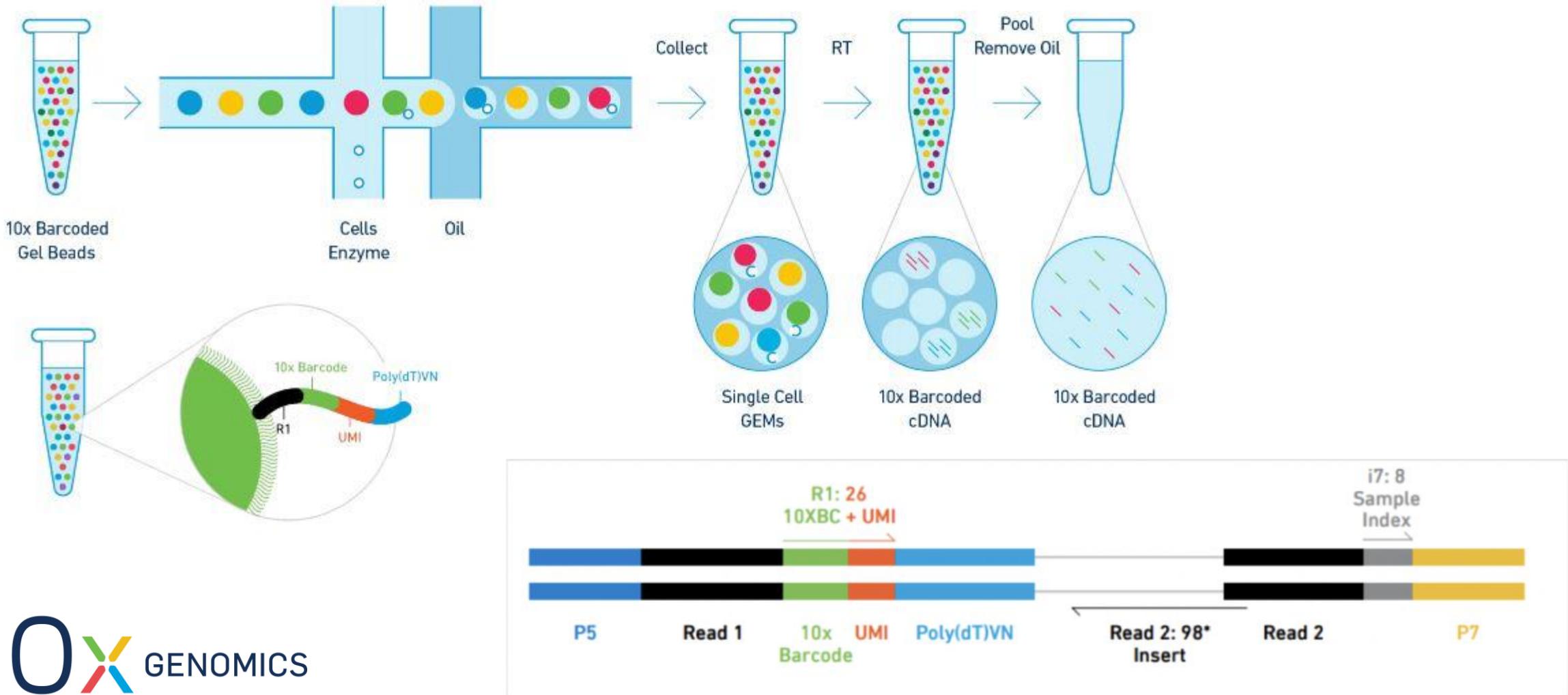


<http://mccarrolllab.org/dropseq/>

Commercial implementations of droplet-based single-cell RNA-seq now exist (e.g., 10x Genomics):



# SINGLE CELL PLATFORMS – Droplet-based



# Numbers game

- Each droplet:
  - 1 in [?]: get a cell
  - 1 in [?]: get a bead
  - 1 in [large number]: get both
  - 1 in [very large number]: get multiple

Cell Stock Concentration (Cells/ $\mu$ l)	Targeted Cell Recovery										
	500	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
100	8.3 35.0	16.5 26.7	33.0 10.2	n/a							
200	4.1 39.1	8.3 35.0	16.5 26.7	24.8 18.5	33.0 10.2	41.3 2.0	n/a	n/a	n/a	n/a	n/a
300	2.8 40.5	5.5 37.7	11.0 32.2	16.5 26.7	22.0 21.2	27.5 15.7	33.0 10.2	38.5 4.7	n/a	n/a	n/a
400	2.1 41.1	4.1 39.1	8.3 35.0	12.4 30.8	16.5 26.7	20.6 22.6	24.8 18.5	28.9 14.3	33.0 10.2	37.1 6.1	41.3 2.0
500	1.7 41.6	3.3 39.9	6.6 36.6	9.9 33.3	13.2 30.0	16.5 26.7	19.8 23.4	23.1 20.1	26.4 16.8	29.7 13.5	33.0 10.2
600	1.4 41.8	2.8 40.5	5.5 37.7	8.3 35.0	11.0 32.2	13.8 29.5	16.5 26.7	19.3 24.0	22.0 21.2	24.8 18.5	27.5 15.7
700	1.2 42.0	2.4 40.8	4.7 38.5	7.1 36.1	9.4 33.8	11.8 31.4	14.1 29.1	16.5 26.7	18.9 24.3	21.2 22.0	23.6 19.6
800	1.0 42.2	2.1 41.1	4.1 39.1	6.2 37.0	8.3 35.0	10.3 32.9	12.4 30.8	14.4 28.8	16.5 26.7	18.6 24.6	20.6 22.6

Volume of Cell Suspension Stock per reaction ( $\mu$ l) | Volume of PBS per reaction ( $\mu$ l)



# Numbers game

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4%	~825	~500
~0.8%	~1,650	~1,000
~1.6%	~3,300	~2,000
~2.4%	~4,950	~3,000
~3.2%	~6,600	~4,000
~4.0%	~8,250	~5,000
~4.8%	~9,900	~6,000
~5.6%	~11,550	~7,000
~6.4%	~13,200	~8,000
~7.2%	~14,850	~9,000
~8.0%	~16,500	~10,000

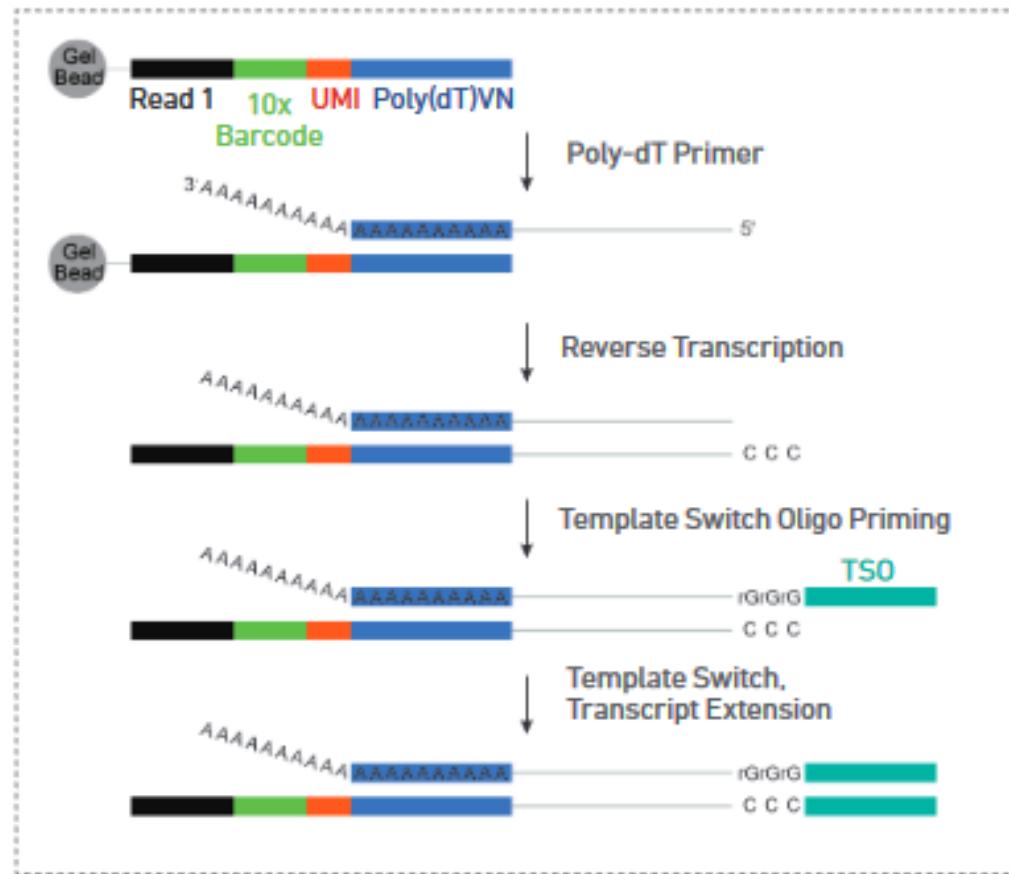
<https://kb.10xgenomics.com/hc/en-us/articles/360001378811-What-is-the-maximum-number-of-cells-that-can-be-profiled->

Slide courtesy of Matt Cannon

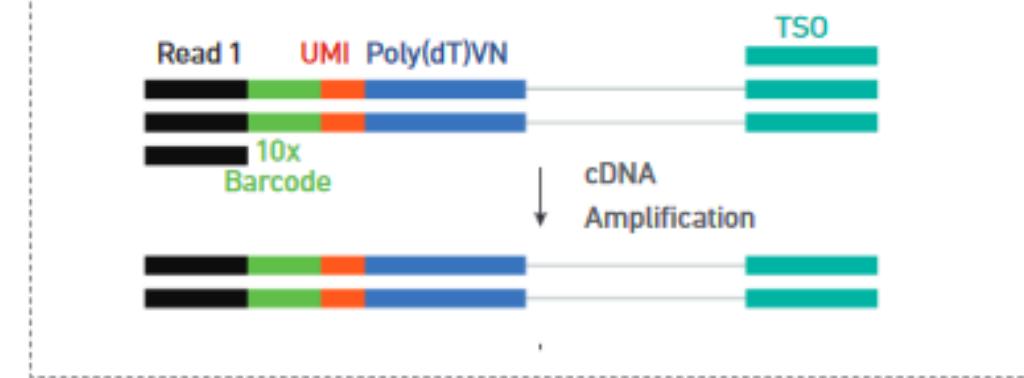


# 3' Library prep – 10xG

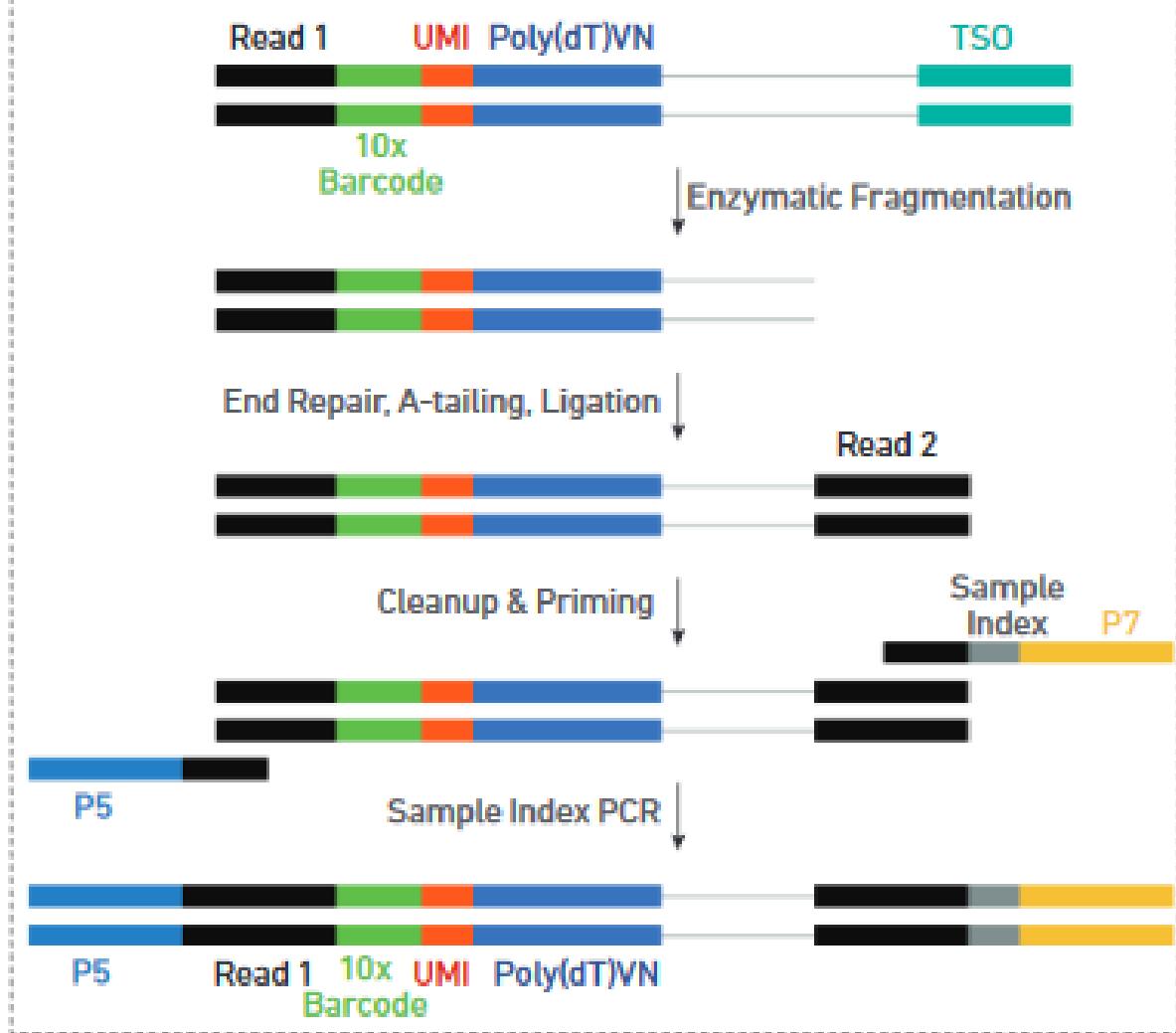
Inside individual GEMs



## Pooled cDNA amplification



## Pooled amplified cDNA processed in bulk

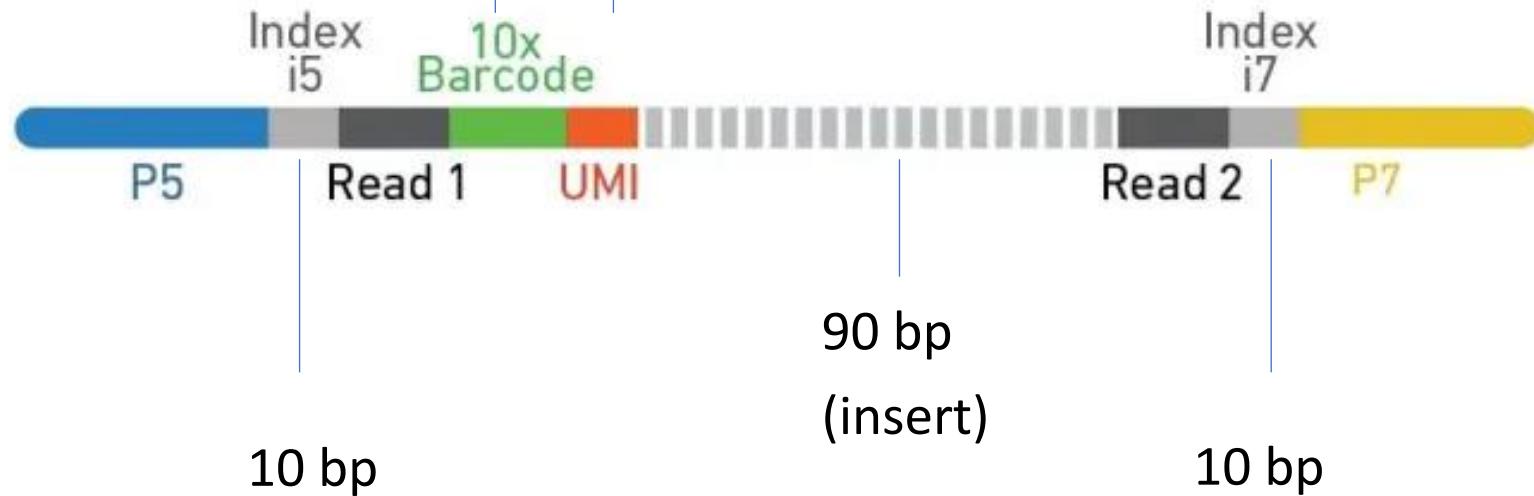


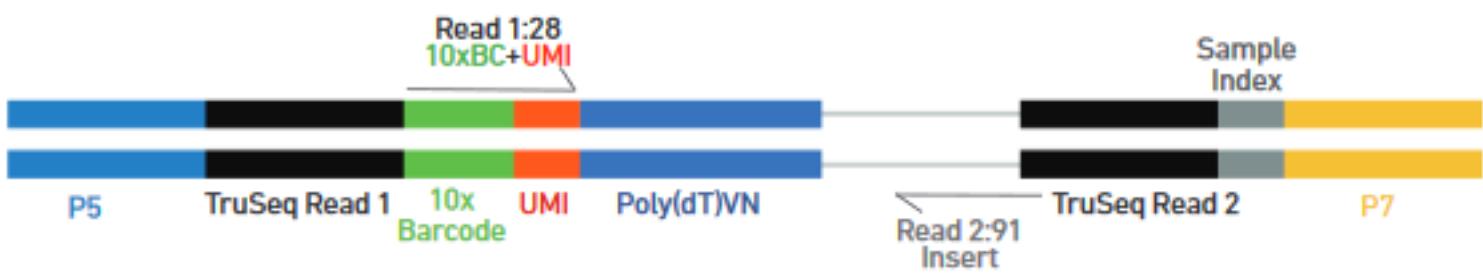
# Sequence data

---

“Run recipe” =  $28 \times 10 \times 10 \times 90$

$$28 = 16 + 12 \text{ bp}$$





## Read 1

```
@A00498:356:H53CMDRXY:1:2101:4390:1266 1:N:0:CATGCGAT
AATAGAGAGTCTGTACTTTGACAACCGT
```

+

```
FFFFFFFFFFFFFFFFFFFFFFFFFFFFFF
```

```
@A00498:356:H53CMDRXY:1:2101:15130:1141 1:N:0:CATGCGAT
```

```
ATCTCTATCCAACTGACAGTTAACTGGT
```

+

```
:FFFFFF, FFFFFFFFFFFFFFFFFFFF
```

```
@A00498:356:H53CMDRXY:1:2101:15167:1141 1:N:0:CATGCGAT
```

```
GATGGAGTCGTGGACCACTCGGGCAGCC
```

+

```
FFFFFFFFFFFF:FFFF:FFFFFFFFF
```

```
@A00498:356:H53CMDRXY:1:2101:15528:1141 1:N:0:CATGCGAT
```

**10X barcode (cell)**

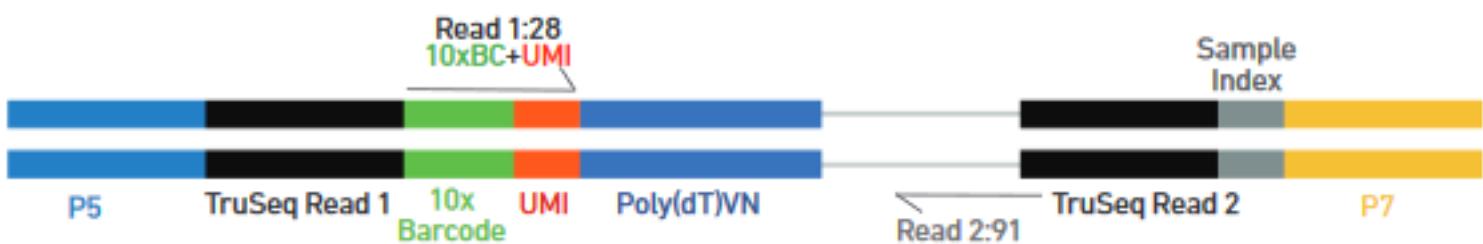
**FCTACATAGTCTACCA**TCCAAACGAAC **Unique molecular identifier (UMI)**

+

```
FFFFFFFFFFFFFFFFFFFFFFFF
```



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Read 2 = insert = transcript of interest

@A00498:356:H53CMDRXY:1:2101:28221:1204 2:N:0:CATGCGAT  
 AGATGATCTGTCAGTGCTAACCGTAGGATGTTGAAGTCCCCACTGTATTGTGGAGTCTATTGTCTCTTAGGTTAATAATATT  
 +  
 FFF:FFFFFFF:FFFF:F,F,FFFF,F,:FFFFFF:FFF:FFF,FFFF:FFF:,FFF,FF:FFFFFF:FFF,FFFF  
 @A00498:356:H53CMDRXY:1:2101:28583:1204 2:N:0:CATGCGAT  
 ATGCCCTAGCCCACCTTACCACAAAGGCACACCTACACCCATTATCCCCATACTACTTATAATCGAAACCACAGACTACACATTCAACC  
 +  
 F::FFF,F,FFFFFF,FF:FFFF,FFF:FFF,FF:FF:,F:FF,:F,,F,,,F:FFFF:FFF:FFF,,FFF,FF:FF,  
 @A00498:356:H53CMDRXY:1:2101:28673:1204 2:N:0:CATGCGAT  
 GTGAAGAGGATCTGAATTCTTAATGCATCAAAGCCTCTGGCAAACCATTGCCGAAATCTAAAAAAACTTGTAGCAAAGGCAGTAAA  
 +  
 FFFFFFF:FFFFFF:FFFFFF:FFFFFF:FFFFFF:FFFFFF:FFFFFF:FFFFFF:FFFFFF:FFFFFF:FFFFFF:FFFFFF:FFFFFF  
 @A00498:356:H53CMDRXY:1:2101:28709:1204 2:N:0:CATGCGAT  
 CGCGAGGTGGGGCGTCGTGTAAGCAGCGGAGGATGGGGGGCGGTGCACGTGGGTGGCGTGGCTGAGATCTAAGTGTCTGCAGCTGTG  
 +  
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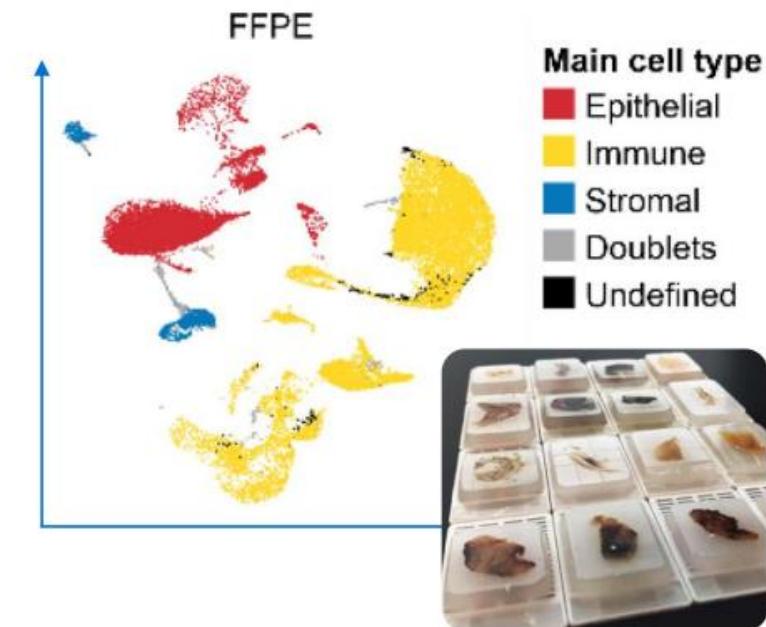
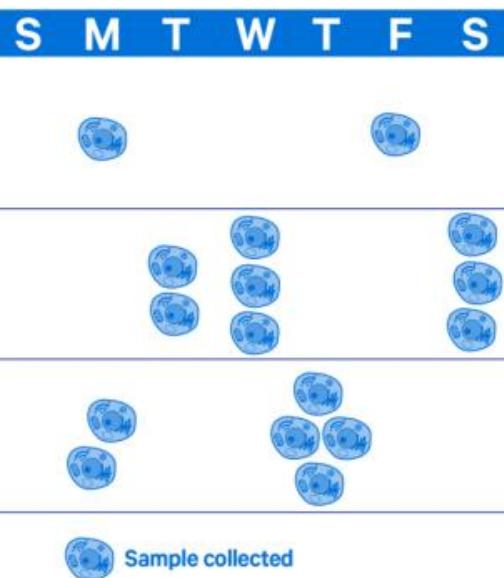
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# 10x Genomics – FLEX/'Fixed RNA Profiling'

Sample fixation

Massive scale at low cost

Unlock FFPE tissue archives



10X GENOMICS

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Slide courtesy of Egon Ranghini (10X)

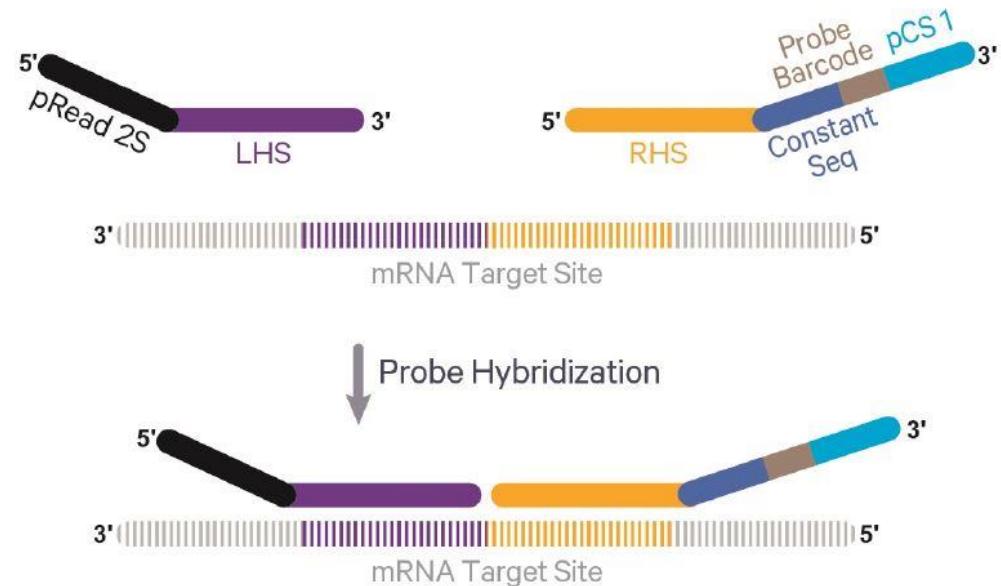


# 10x Genomics – 'Fixed RNA Profiling'

- Measures RNA levels in fixed samples using probes targeting the whole transcriptome (mouse or human only).
- After probe panels are added to the sample, each probe pair hybridizes to its target gene and is then ligated, followed by library generation + sequencing.
- Measures gene expression indirectly by counting barcoded probes rather than sequencing the native RNA molecules themselves.

## ADVANTAGES:

- Process FFPE or OCT samples
- Increased throughput (multiplexing)
- Higher-sensitivity (vs. 3'/5' kit)
- Lower cost for sequencing

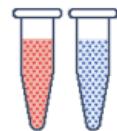


# GEM-X Flex Gene Expression – any sample at any scale

## Broad sample compatibility



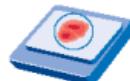
Fresh tissue  
Frozen tissue



Cell suspensions  
Nuclei suspensions



Whole blood



FFPE tissue



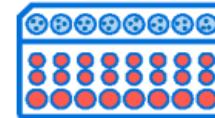
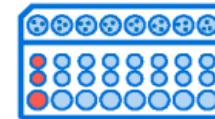
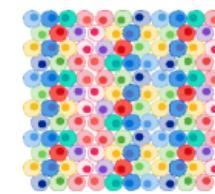
Human or mouse

## In-line multiplexing



- Up to 16 samples per channel
- Up to 128 samples per chip

## Flexible project input



- Up to 20,000 cells per sample
- Up to 320,000 cells per lane
- Up to 2.56M cells per chip
- Low cell input recommendations (25K cells)

10X GENOMICS

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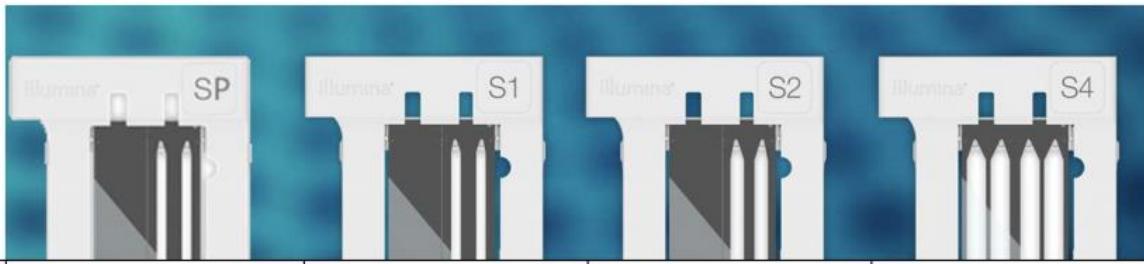
Slide courtesy of Egon Ranghini (10X)

# Sequencing depth specifications

3 samples \*  
10,000 cells \*  
25,000 reads/cell =  
**750M reads**

**NovaSeq6000  
SP flow cell**

**Illumina  
NovaSeq6000**



Flow cell	SP	S1	S2	S4
Lane number	2	2	2	4
M reads per lane	400	800	2'000	2'500
Total M reads	800	1'600	4'000	10'000

# The Pros



Industry standard; familiar; flexible; **Multiomics** – RNA + Protein



Seems similar to 10x, although lower initial investment for instrument



Microwells allow direct monitoring (lower multiplets, assesses viability, etc.)



1 million cells



**Multiomics**; DNA (panel) and protein from same cell



Up to 500K cells; can multiplex numerous samples; methylation



Lower cost; recently acquired by Illumina



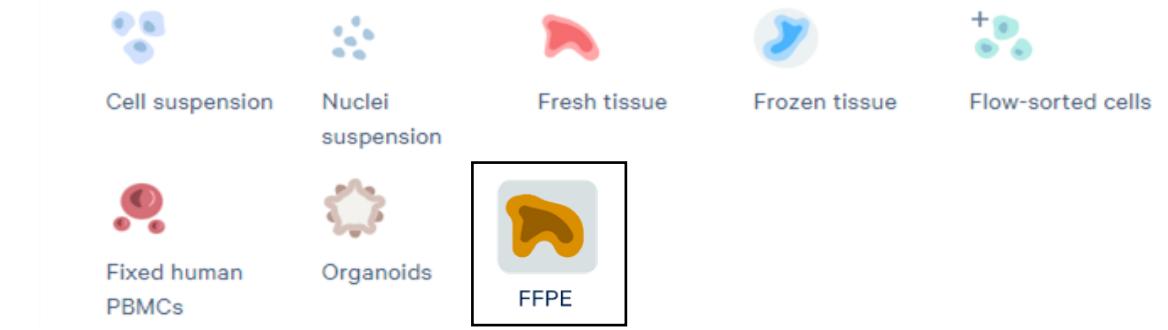
**Multiomics**; DNA (whole genome) and RNA from same cell

\*Instrument-free

# Genomics Services Laboratory @ NCH: 10xG Single Cell Workflows

- 3' & 5' tagging for **RNA**
- Probe-based (Hu/Mus) for **RNA**
- **TCR/BCR** sequencing
- **Protein** (surface markers)
- **ATAC-seq**
- **Long-read transcriptomics** (PacBio sequencing)

## Compatible samples



\*Director: [katherine.miller@nationwidechildrens.org](mailto:katherine.miller@nationwidechildrens.org)

\*Operations Manager: [amy.wetzel@nationwidechildrens.org](mailto:amy.wetzel@nationwidechildrens.org)

# The Future of Single Cell ‘Omics

---

\$ Cost  
efficiency

DNA Scalability

✓ Efficiency

Accessibility

Puzzle Multi-omics

Syringe Clinical  
Applications

# Illumina short-read sequencing

**Anthony Miller, PhD**

Director of Technology Development

Institute for Genomic Medicine



# Current and Emerging Short-Read Technologies

Ultima  
“UG 100”



Singular Genomics  
“G4”



Element Biosciences  
“AVITI”



Pacific Biosciences  
“Onso”



# Illumina Sequencers



## Benchtop

iSeq 100

MiniSeq

MiSeq Series +

NextSeq 550 Series +

NextSeq 1000 & 2000

Run Time	9.5–19 hrs	4–24 hours	4–55 hours	12–30 hours	11–48 hours
Maximum Output	1.2 Gb	7.5 Gb	15 Gb	120 Gb	360 Gb*
Maximum Reads Per Run	4 million	25 million	25 million †	400 million	1.2 billion*
Maximum Read Length	2 × 150 bp	2 × 150 bp	2 × 300 bp	2 × 150 bp	2 × 150 bp

# Illumina Sequencers



## Benchtop

	iSeq 100	MiniSeq	MiSeq Series +	NextSeq 550 Series +	NextSeq 1000 & 2000
Run Time	9.5–19 hrs	4–24 hours	4–55 hours	12–30 hours	11–48 hours
Maximum Output	1.2 Gb	7.5 Gb	15 Gb	120 Gb	360 Gb*
Maximum Reads Per Run	4 million	25 million	25 million †	400 million	1.2 billion*
Maximum Read Length	2 × 150 bp	2 × 150 bp	2 × 300 bp	2 × 150 bp	2 × 150 bp

## Production Scale



	NextSeq 550 Series +	NextSeq 1000 & 2000	NovaSeq 6000
Run Time	12–30 hours	11–48 hours	~13 - 38 hours (dual SP flow cells) ~13–25 hours (dual S1 flow cells) ~16–36 hours (dual S2 flow cells) ~44 hours (dual S4 flow cells)
Maximum Output	120 Gb	360 Gb*	6000 Gb
Maximum Reads Per Run	400 million	1.2 billion*	20 billion
Maximum Read Length	2 × 150 bp	2 × 150 bp	2 x 250**

# Illumina Sequencer – NovaSeq X



Table 1: NovaSeq X Series performance parameters<sup>a</sup>

Flow cell type	1.5B	10B	25B
Output per single flow cell run <sup>a</sup>			
2 × 50 bp	~165 Gb	1 Tb	–
2 × 100 bp	330 Gb	2 Tb	–
2 × 150 bp	500 Gb	3 Tb	8 Tb
Reads passing filter per flow cell <sup>a</sup>			
Single reads	1.6 billion	10 billion	26 billion
Paired-end reads	3.2 billion	20 billion	52 billion

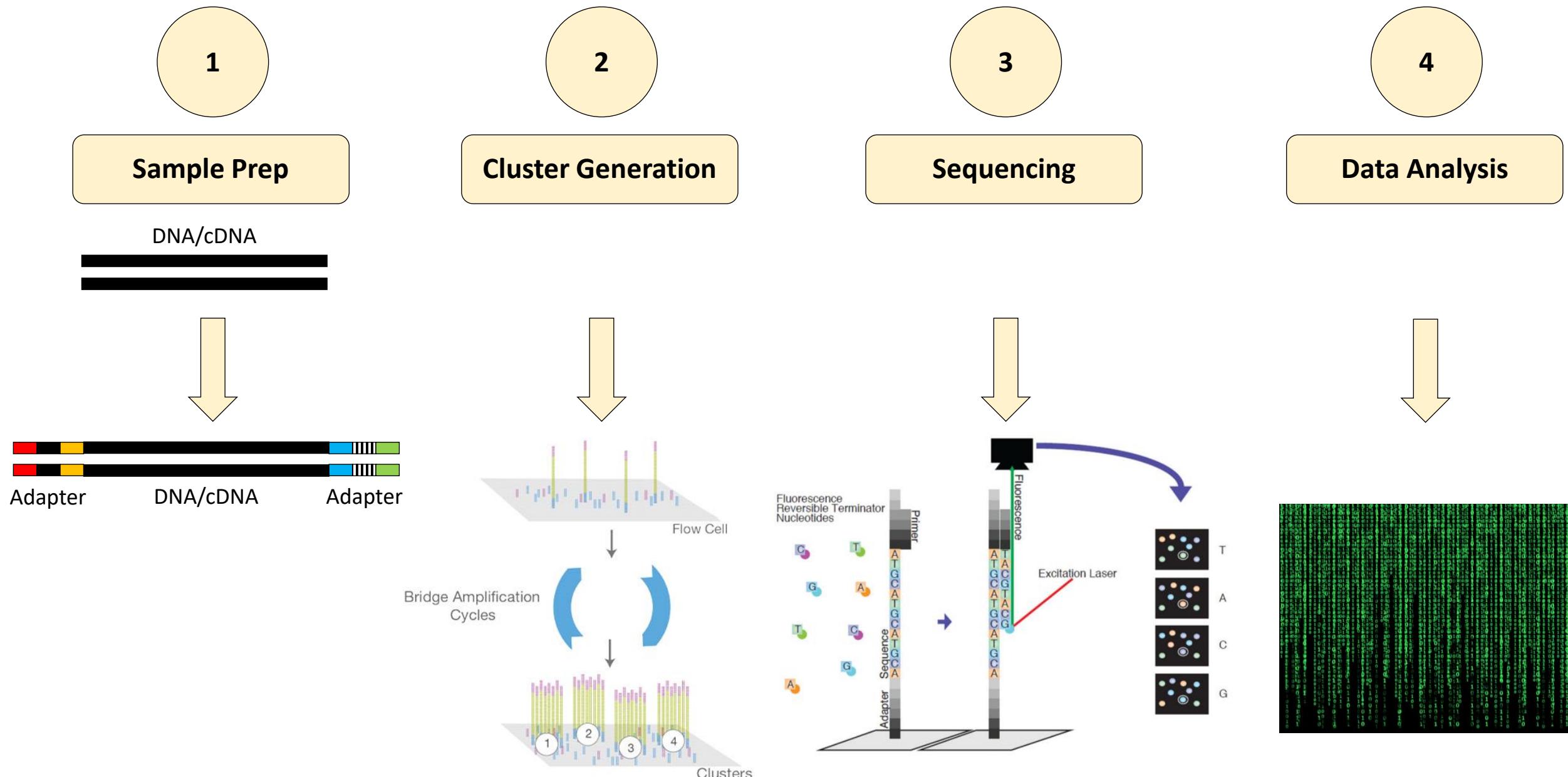
Table 2: Estimated sample throughput for key applications<sup>a</sup>

Flow cell type	Per single flow cell run			Per dual flow cell run <sup>b</sup>		
	1.5B	10B	25B	1.5B	10B	25B
Human genomes	~4	~24	~64	~8	~48	~128
Exomes	~41	~250	~750	~82	~500	~1500
Transcriptomes	~30	~200	~520	~60	~400	~1040

a. All sample throughputs are estimates. Human genomes estimates assume > 120 Gb of data per sample to achieve 30× coverage. Exomes estimates assume ~8 Gb per sample to achieve 100× coverage. Transcriptomes estimates assume ≥ 50M reads. Throughput may vary based on library preparation kit used. Performance metrics subject to change.

b. Dual flow cell runs only apply to the NovaSeq X Plus System.

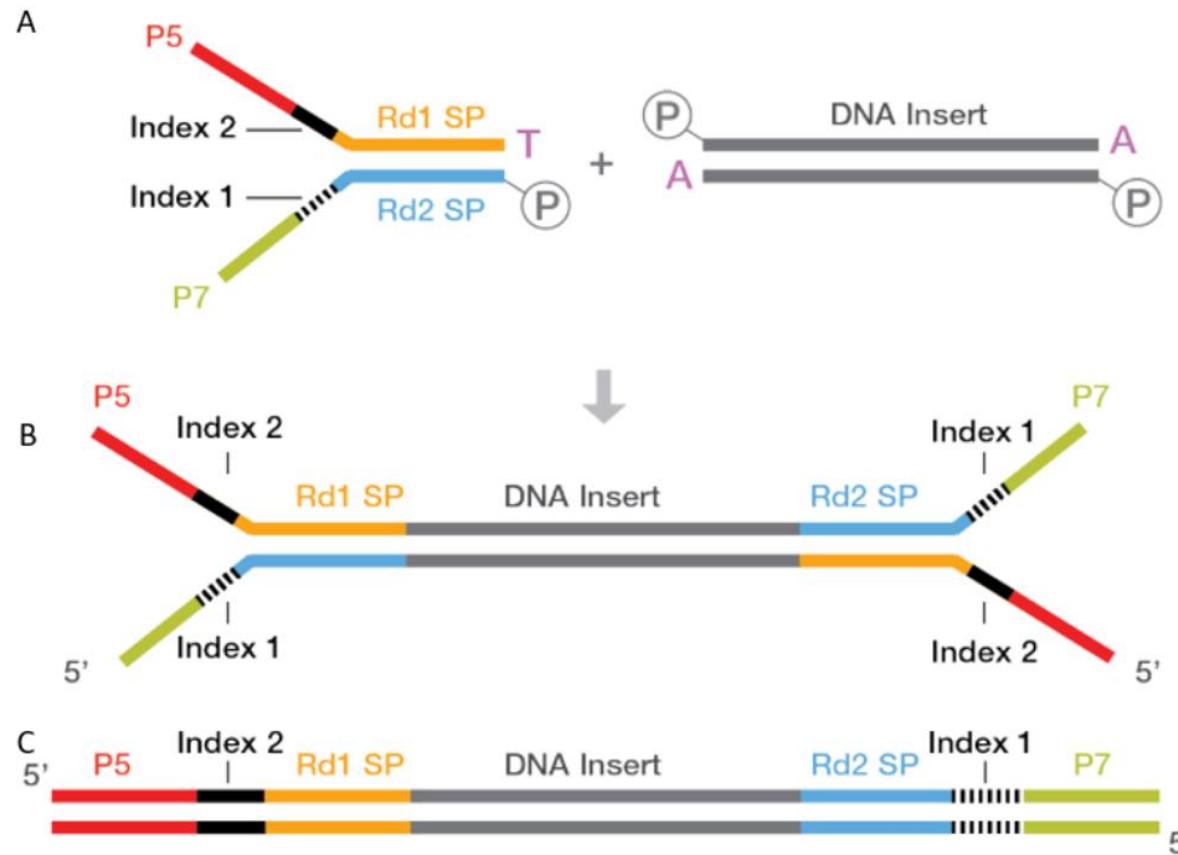
# Overview of Illumina Workflow



# Illumina Adapter – Foundation for Cluster Generation

Sample Prep

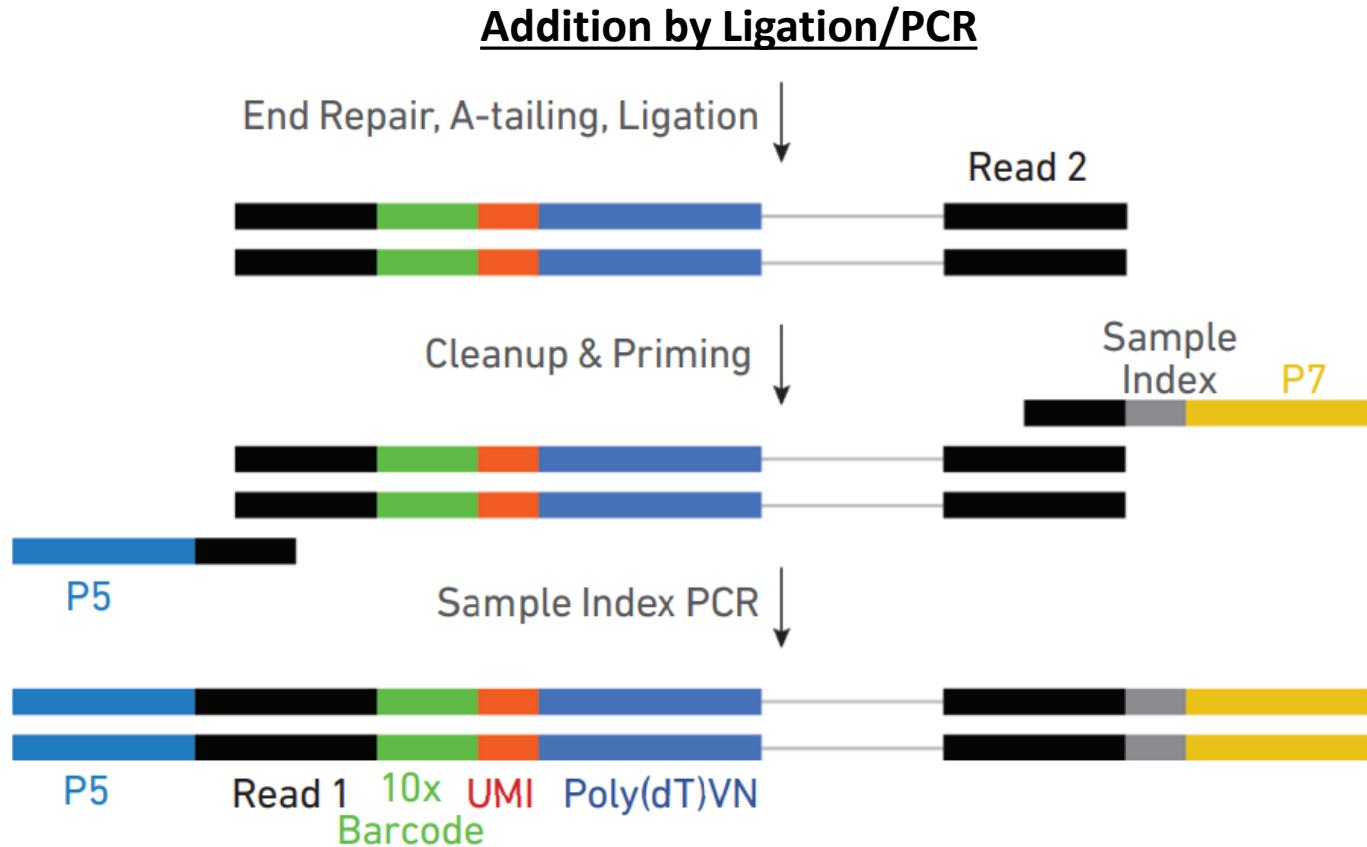
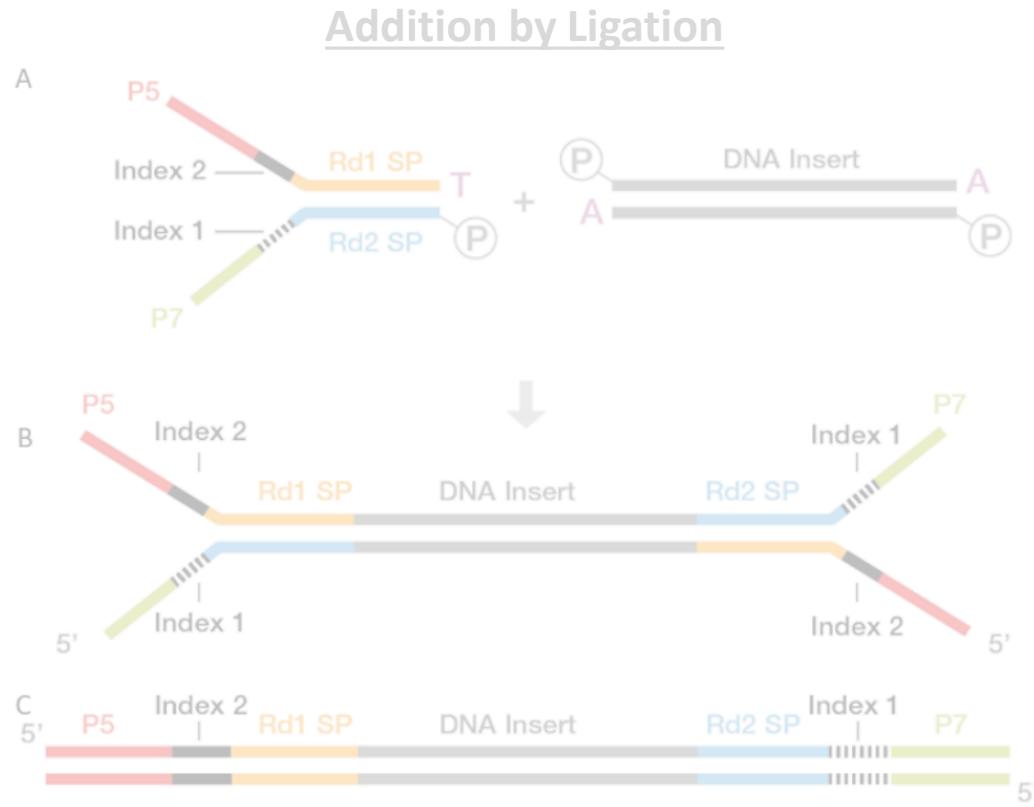
## Addition by Ligation



NATIONWIDE CHILDREN'S  
When your child needs a hospital, everything matters.<sup>SM</sup>

# Illumina Adapter – Foundation for Cluster Generation

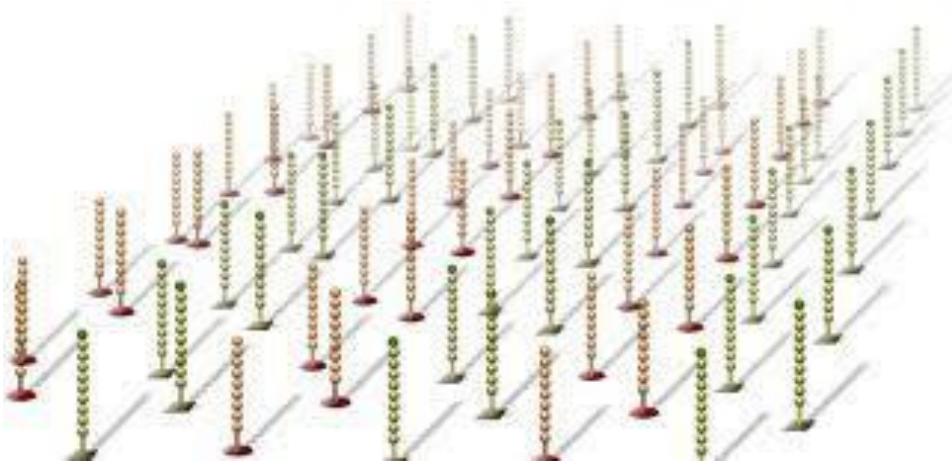
Sample Prep



NATIONWIDE CHILDREN'S  
*When your child needs a hospital, everything matters.*™

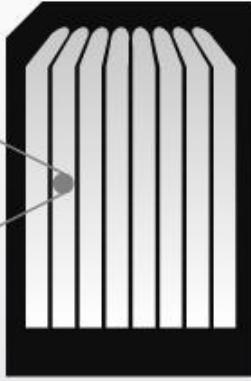
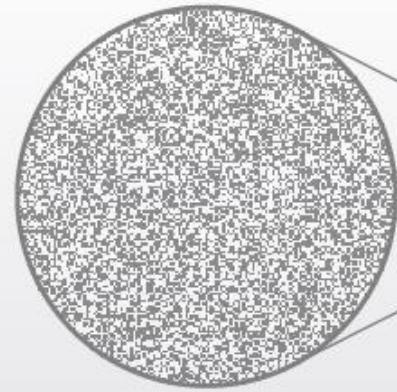
# Illumina Flowcell – Cluster Generation

## Cluster Generation

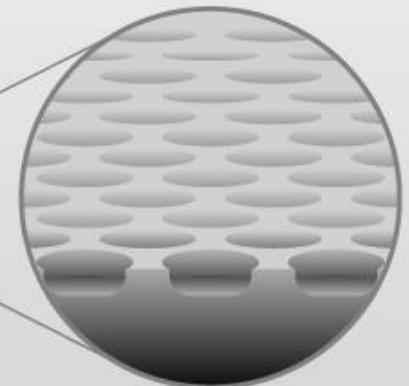
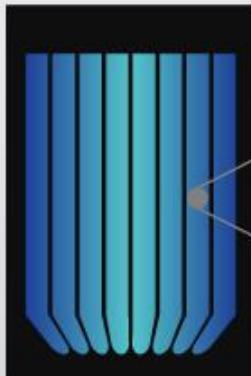


Flow cell contains lawn of oligos  
complementary to the P5/P7  
sequences

Random Flow Cell



Patterned flow cells



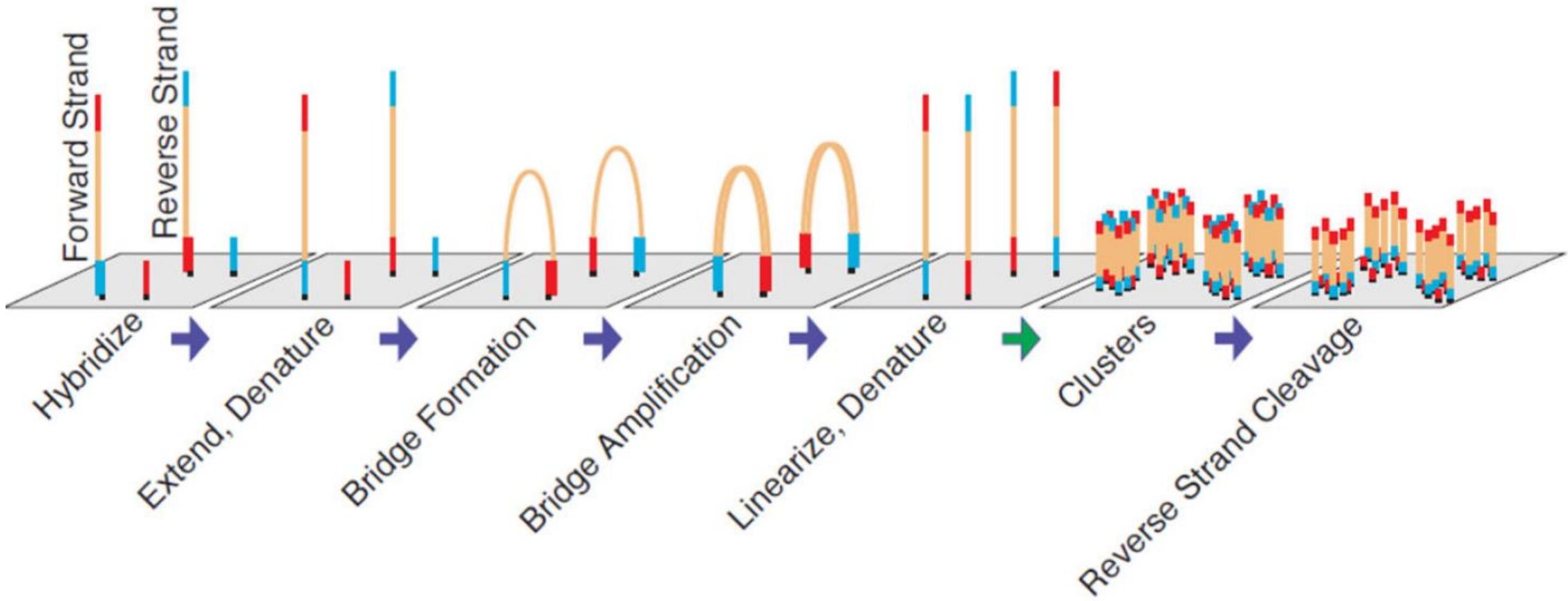
2

3

# Illumina Flowcell – Cluster Generation

Cluster Generation

Sequencing



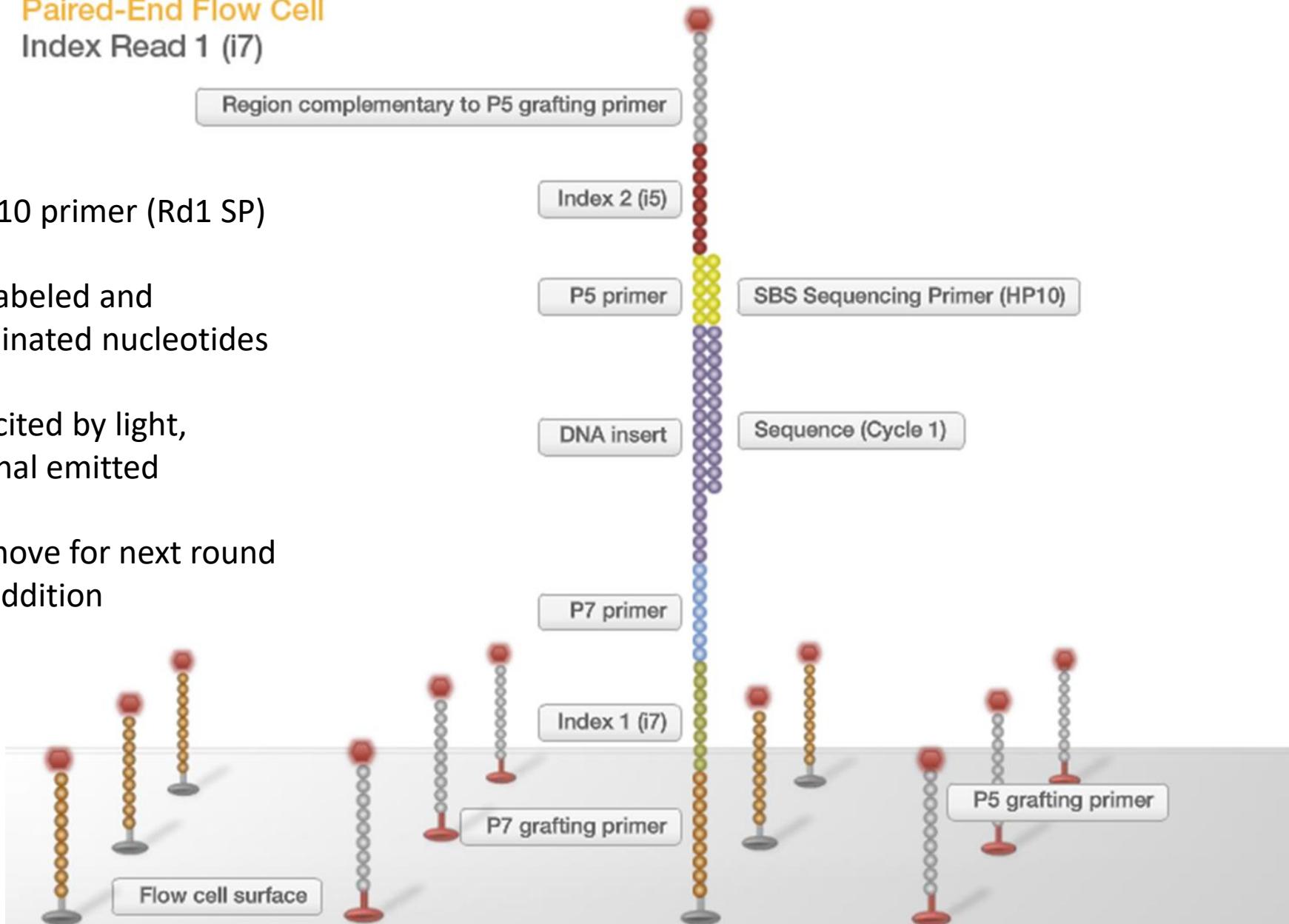
# Illumina Sequencing By Synthesis (SBS) – Read 1

## Sequencing

### Paired-End Flow Cell

#### Index Read 1 (i7)

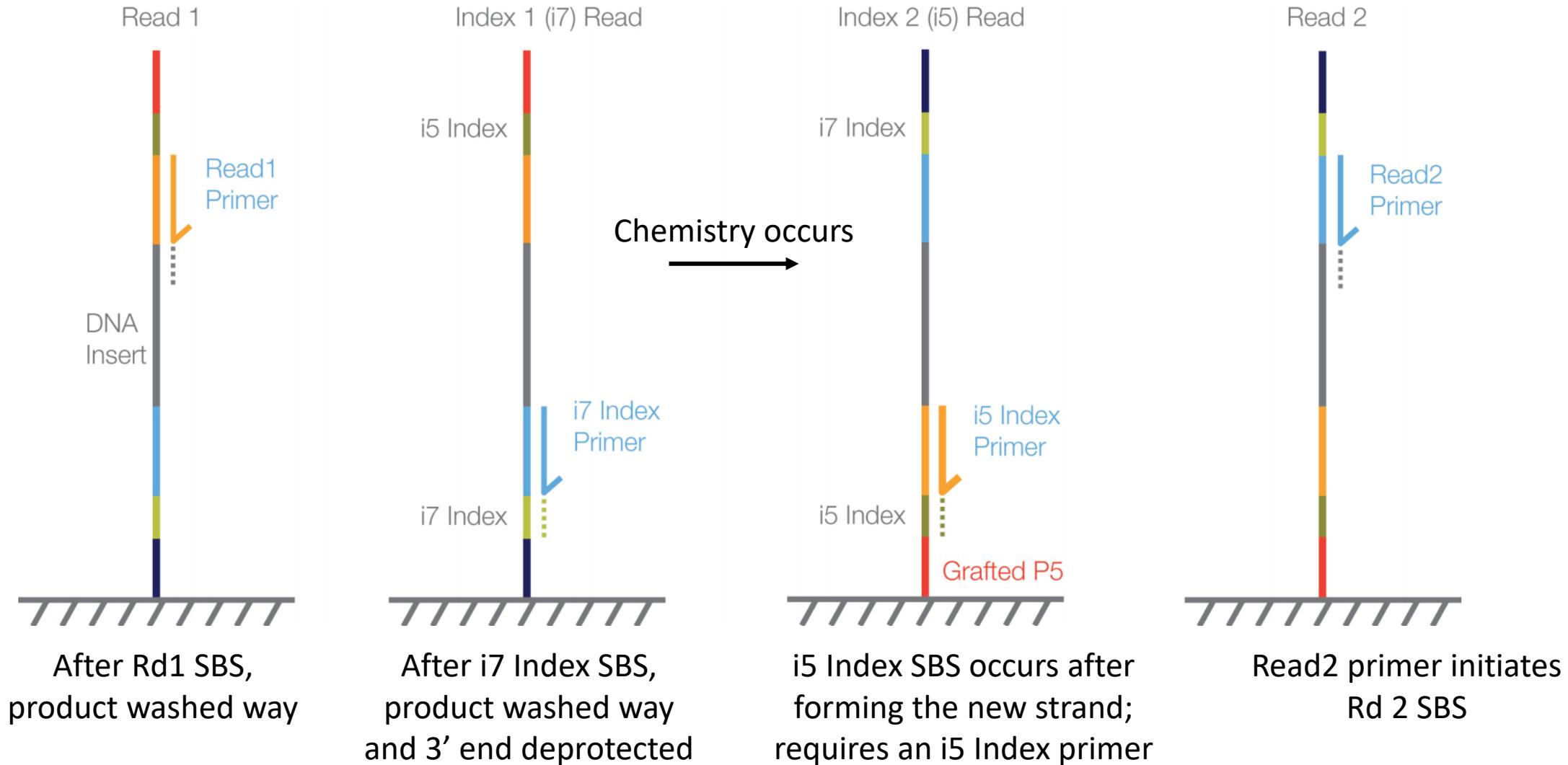
- Initiated by HP10 primer (Rd1 SP)
- Fluorescently labeled and reversibly terminated nucleotides
- Clusters are excited by light, fluorescent signal emitted
- Terminator remove for next round of nucleotide addition



# Illumina Sequencing By Synthesis (SBS) – Index(s) and Read 2

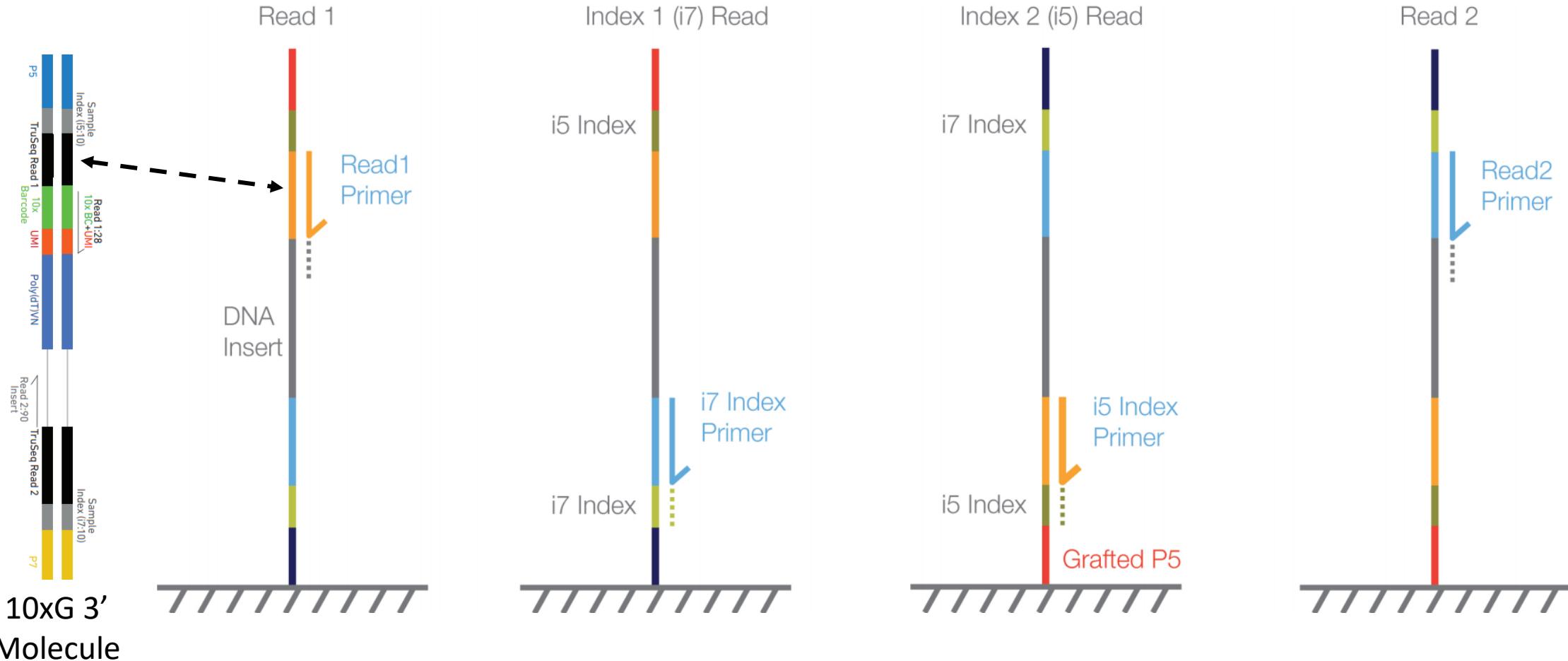
NovaSeq 6000 w/ v1.5 chem, MiniSeq w/ standard chem, NextSeq, HiSeq 4000, iSeq 100

**Figure 3** Dual-Indexed Sequencing on a Paired-End Flow Cell (Workflow B)



# Illumina Sequencing By Synthesis (SBS) – Index(s) and Read 2

**Figure 3** Dual-Indexed Sequencing on a Paired-End Flow Cell (Workflow B)

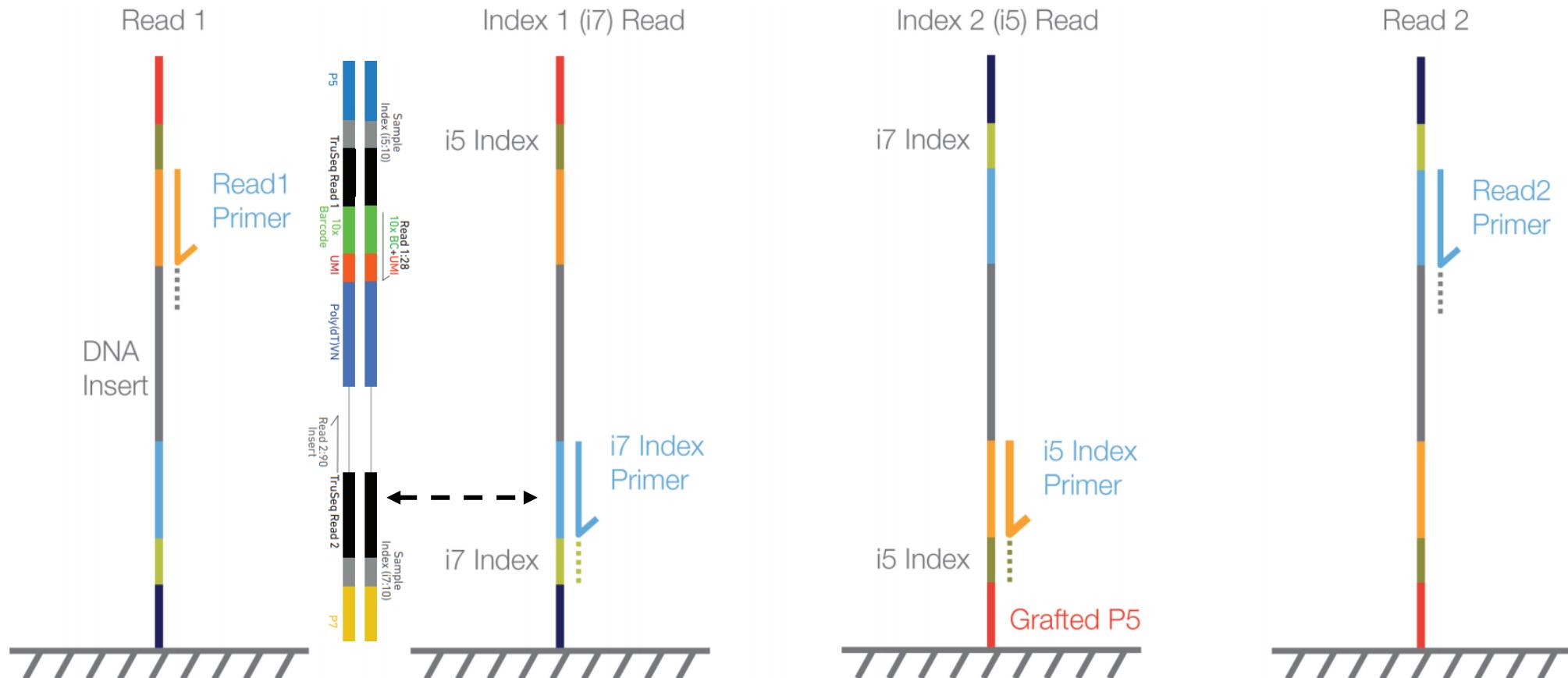


Run Recipe:

28 bp;

# Illumina Sequencing By Synthesis (SBS) – Index(s) and Read 2

**Figure 3** Dual-Indexed Sequencing on a Paired-End Flow Cell (Workflow B)



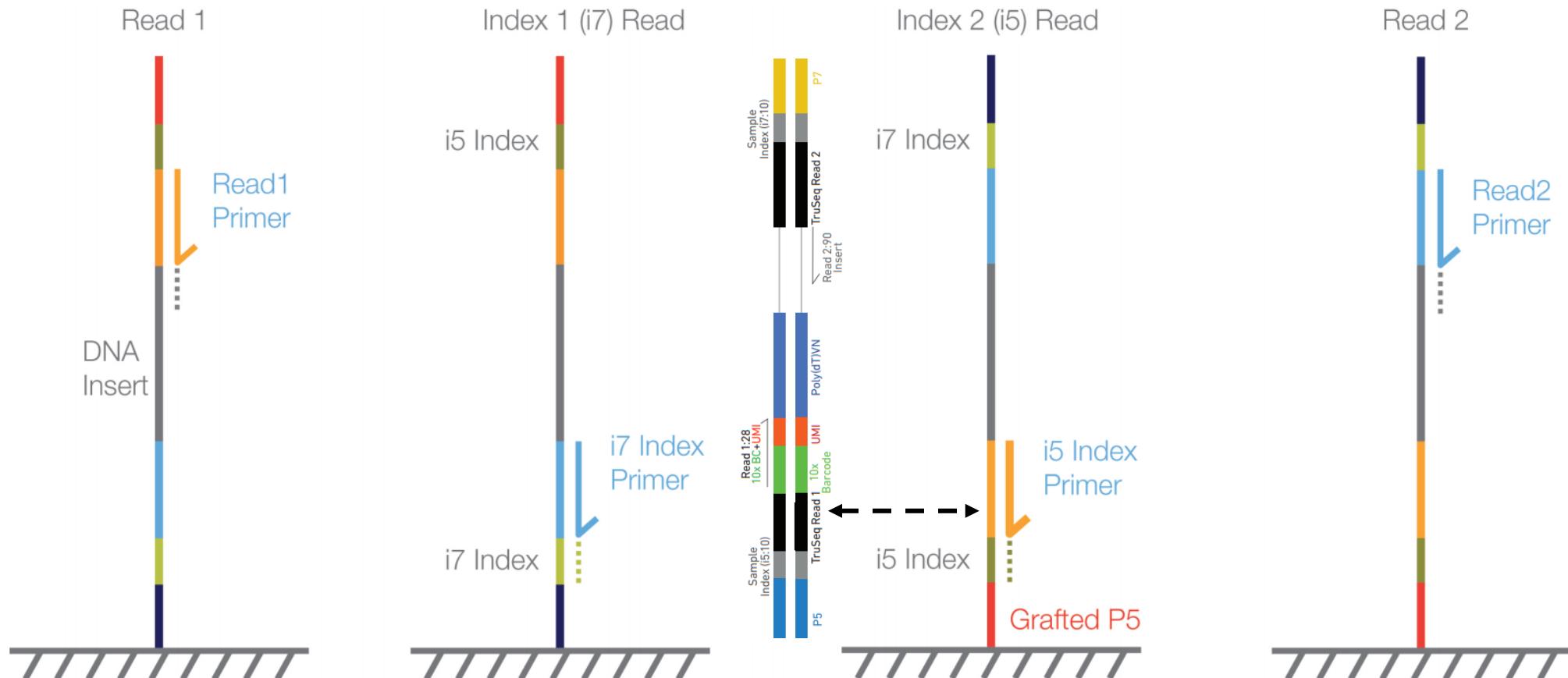
Run Recipe:

28 bp;

10 bp;

# Illumina Sequencing By Synthesis (SBS) – Index(s) and Read 2

**Figure 3** Dual-Indexed Sequencing on a Paired-End Flow Cell (Workflow B)



Run Recipe:

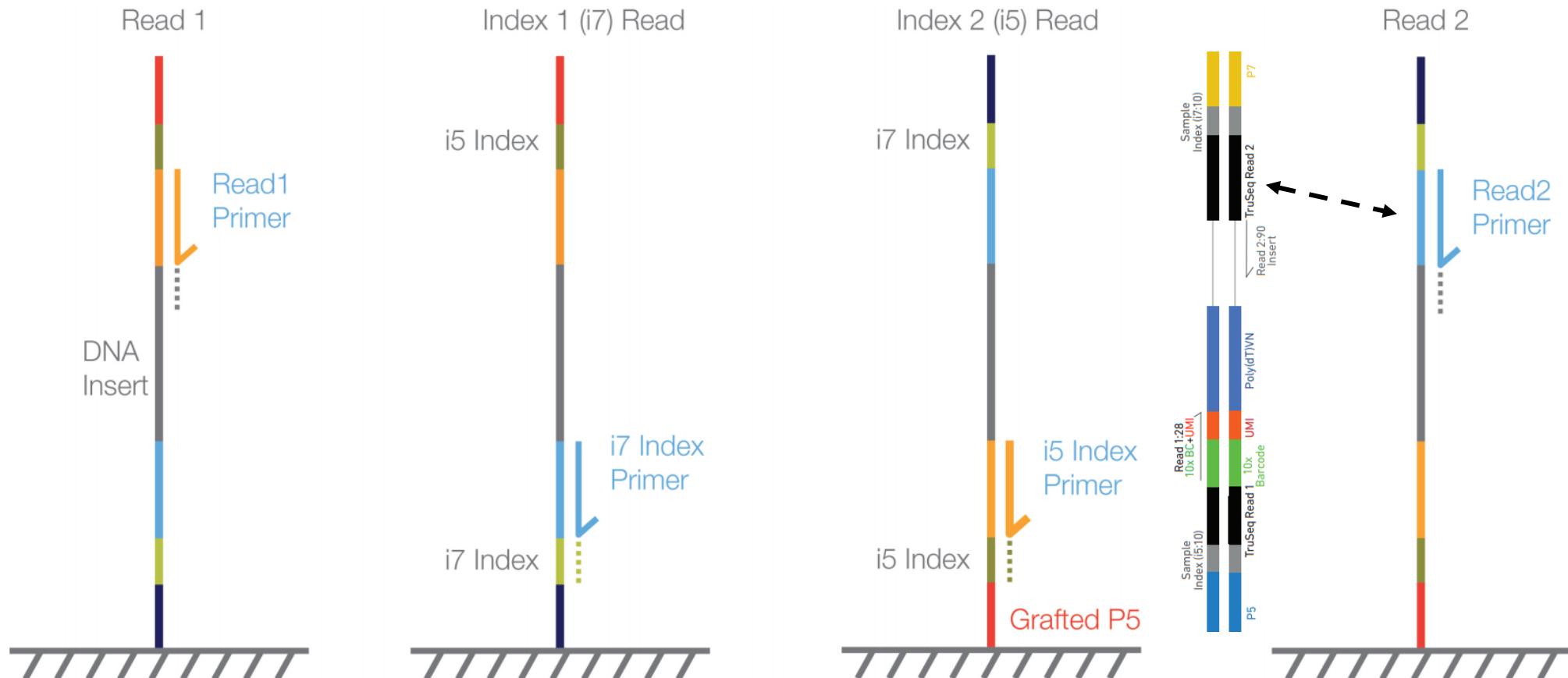
28 bp;

10 bp;

10 bp;

# Illumina Sequencing By Synthesis (SBS) – Index(s) and Read 2

**Figure 3** Dual-Indexed Sequencing on a Paired-End Flow Cell (Workflow B)



Run Recipe:

28 bp;

10 bp;

10 bp;

90 bp

# Why is Illumina Limited to Short-Read Lengths – SBS and Phasing

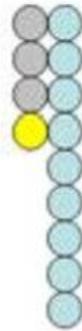
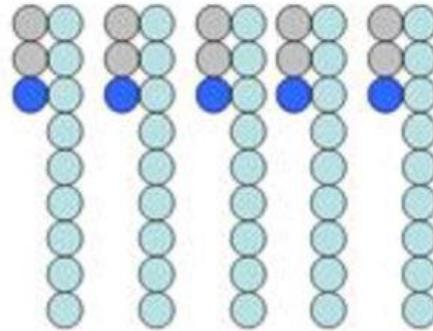
## Sequencing

- Read lengths are limited to < 300 bp with current Illumina SBS chemistry

Phasing



Prephasing



- Phasing is the rate at which single molecules within a cluster loose sync with each other during SBS
- Phasing is when molecules fall behind others in the cluster (can occur due to improper terminator cleavage)
- Pre-Phasing is when molecules are ahead of others in the cluster (can occur when nucleotides that do not contain terminators)
- Phasing and Pre-Phasing build over the course of SBS within a cluster, ultimately leading to unresolved signal-to-noise ratio