Frederick National Laboratory
for Cancer Research

sponsored by the National Cancer Institute

Single Cell RNA-Seq at CCR-SF: Sample prep and best practices using 10X Genomics

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DEPARTMENT OF HEALTH AND HUMAN SERVICES • National Institutes of Health • National Cancer Institute

Frederick National Laboratory is a Federally Funded Research and Development Center operated by Leidos Biomedical Research, Inc., for the National Cancer Institute

CCR Sequencing Facility

Core facility dedicated to providing Next-Generation Sequencing (NGS) services to Center for Cancer Research investigators, operating one Illumina HiSeq 4000, two HiSeq 2500, three NextSeq 500, one MiSeq, one PacBio RSII / Sequel, 10X Chromium and BioNano Irys system.

DNA/RNA-Protein Interaction

Transcript Profiling and Discovery

Illumina

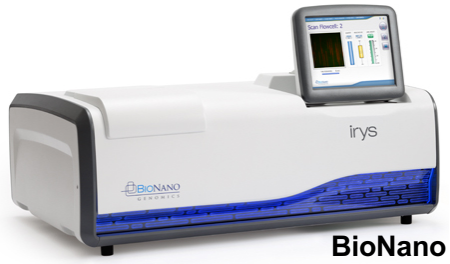
Genome-wide Methylation study

Targeted Exome sequencing

Whole Genome Structural Variants

Single Cell Gene Expression Profiles

10X Chromium



BioNano Irys

Next-Generation Mapping

De Novo Whole Genome Sequencing

Targeted Sequencing

PacBio RS II

Base Modifications

DNA Structure Variation analysis

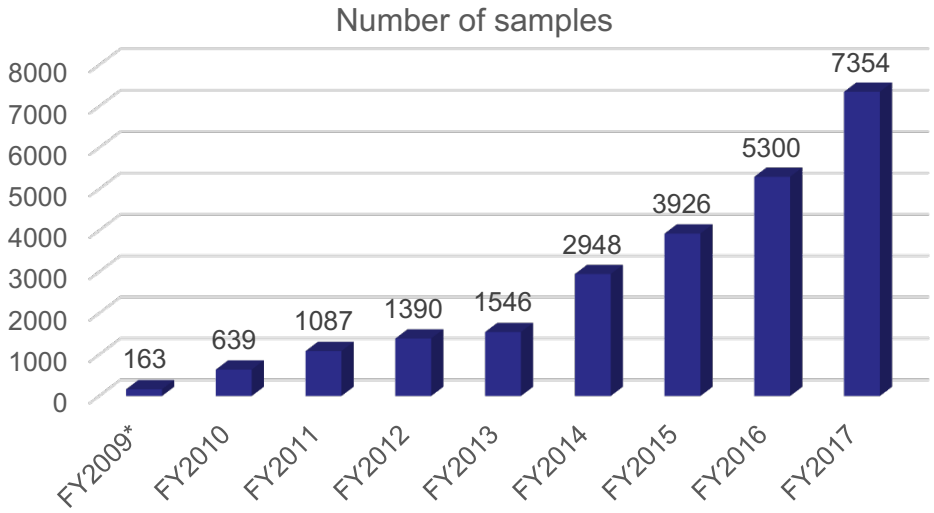
PacBio Sequel

Transcript Isoform Detection

CCR Sequencing Facility

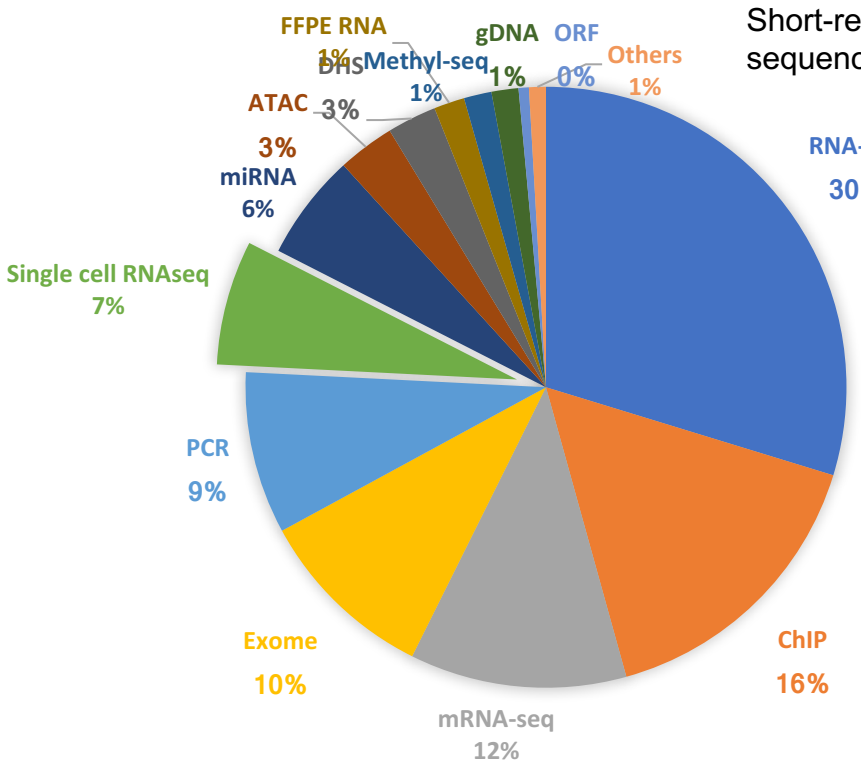
FY2017 Statistics:

- 120 NCI investigators
- More than 7300 samples.
- Delivered over 45 trillion bases



Sample types – FY2017

Short-read sequencing



Single cell RNA-Seq:

Fluidigm C1

10X Chromium

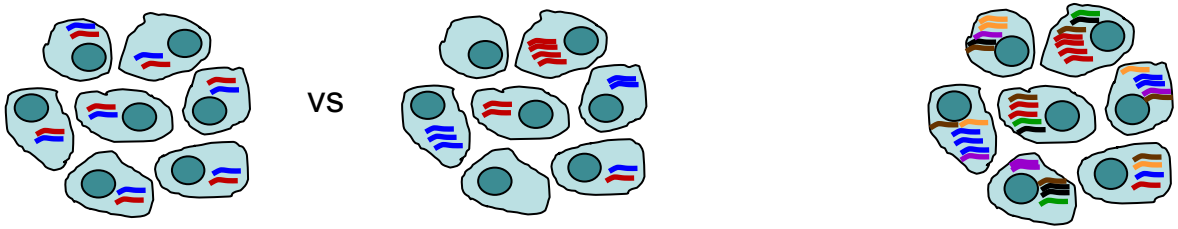
DropSeq

Others

FFPE Exome	MNase
RIP-Seq	10X gDNA
RiboSeq	shRNA
Drip	sgRNA
HiC	VirScan

Single-cell mRNA-seq

- Cell-to-cell variation in tissues and populations – normal, and important for biological functions, under normal and diseased states.
- Frequently manifested through changes in gene expression.
- Single-cell studies: Help to uncover the variation masked by the average of heterogeneous subpopulations, identification of rare populations.



.. for hundreds of genes

- Crucial in understanding cancer cells, stem cells, immune cells, cell lineages etc.

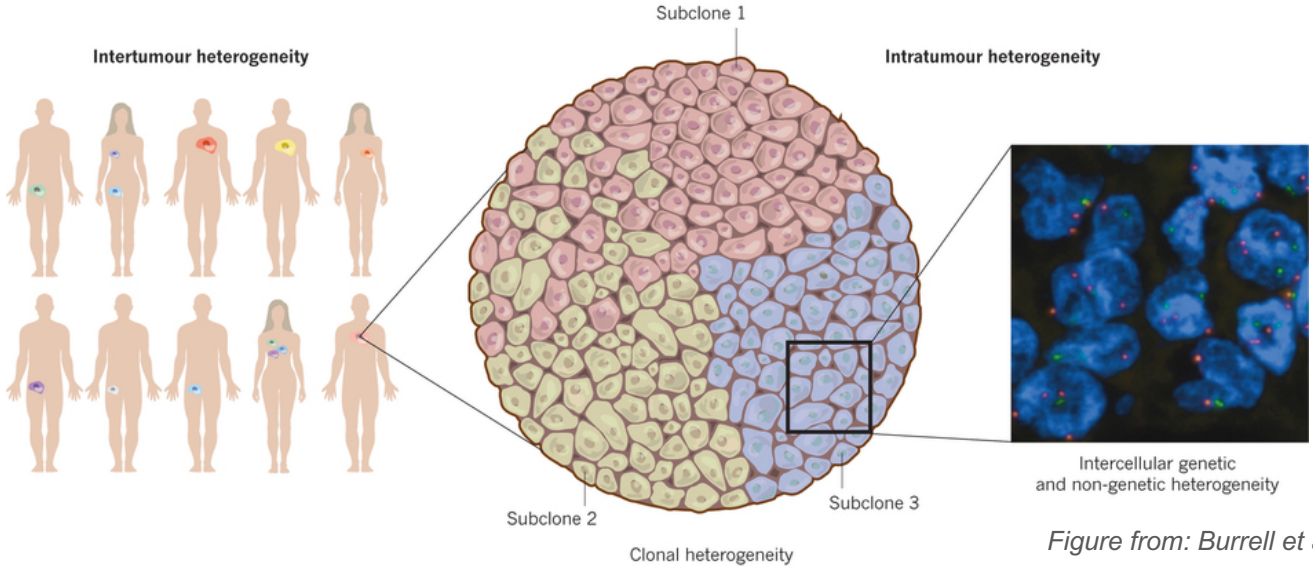
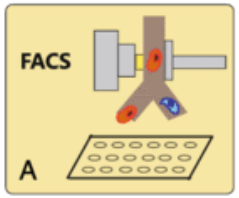
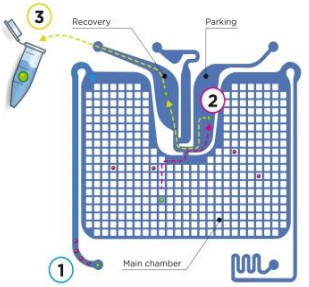


Figure from: Burrell et al., 2013. Nature, 501: 338

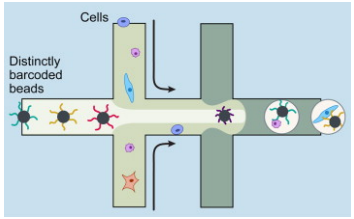
Single-cell mRNA-seq: Gene expression profiling at single cell level

A. Single cell-per-well protocols (low-throughput, full-length transcript analysis possible)
   A. FACS-based methods
   B. Fluidigm C1
   C. DEPArray





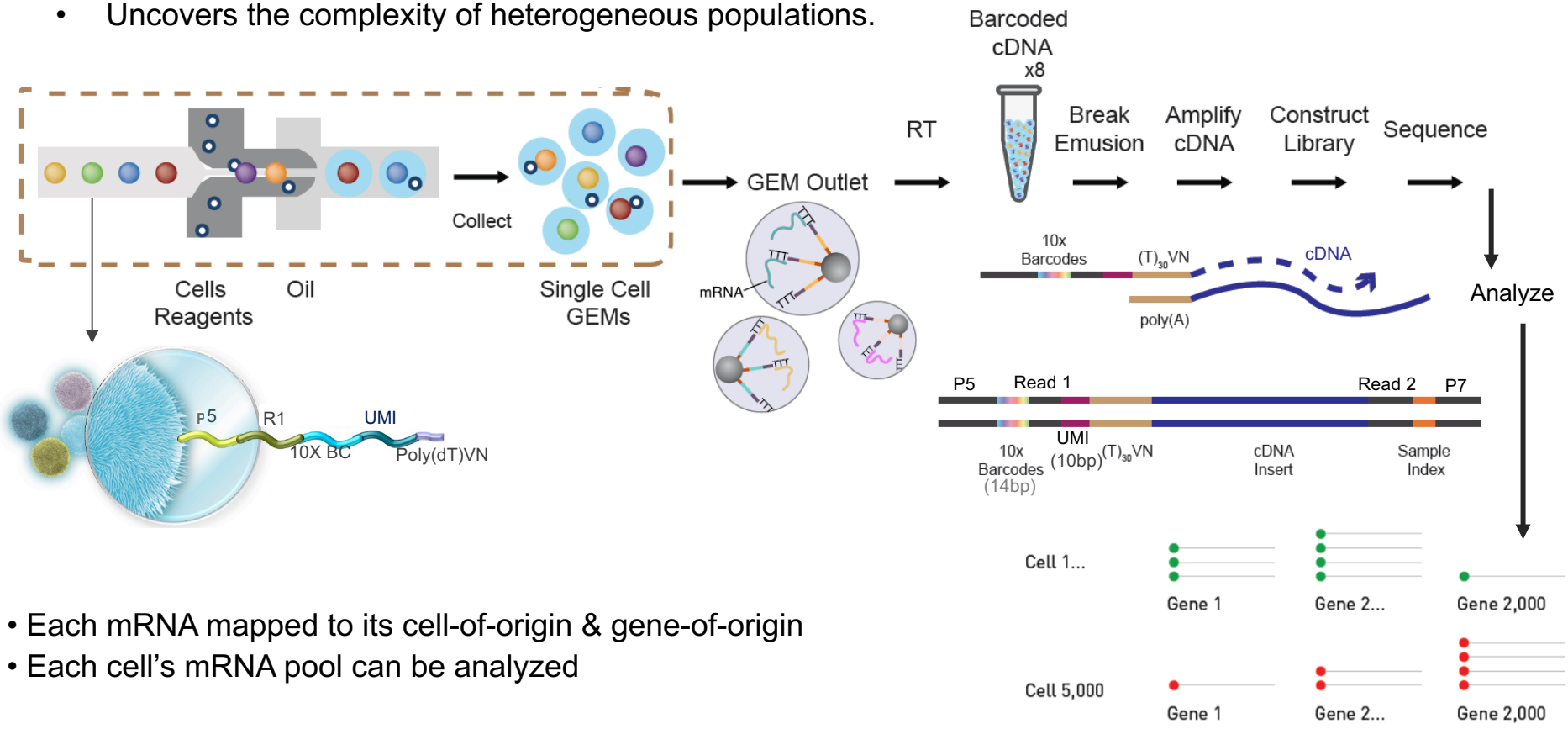
B. Droplet-based massively parallel protocols (high-throughput, expression analysis only)
   A. DropSeq
   B. 10X Genomics Chromium
   C. ddSeq
   D. BD Rhapsody



SF: lib prep/sequencing services for all approaches, end-to-end (sample to sequence) service provided for **10XChromium Single-cell RNAseq.**

Single cell 3’ mRNA-Seq using 10X Chromium

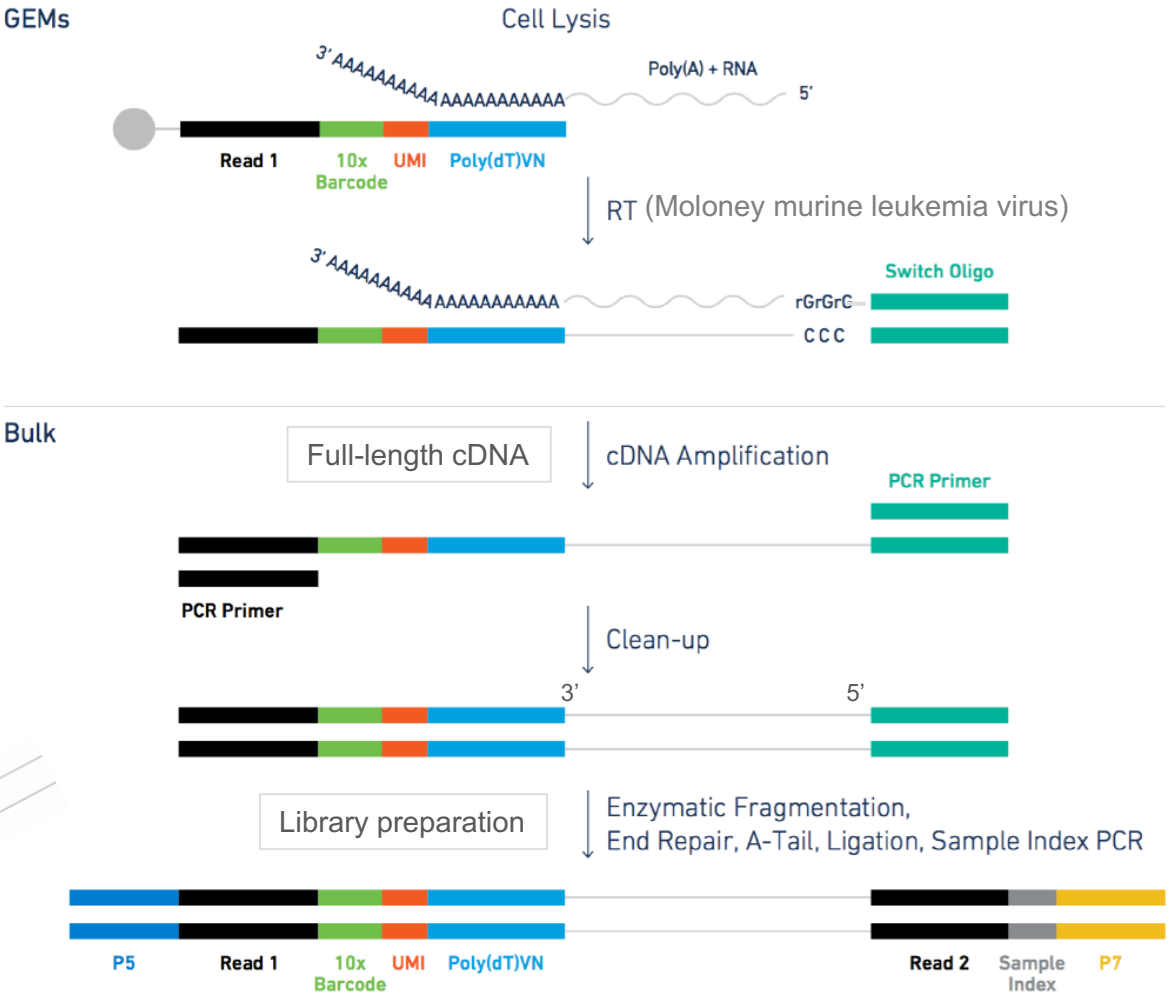
- Based on droplet microfluidics technologies such as Drop-Seq and inDrop
- Combines microfluidics with molecular barcoding to enable high-throughput single-cell RNA sequencing
- Transcriptional profiling of thousands of individual cells by partitioning into nanoliter-scale Gel Bead-In-Emulsions (GEMs)
- Uncovers the complexity of heterogeneous populations.



- Each mRNA mapped to its cell-of-origin & gene-of-origin
- Each cell’s mRNA pool can be analyzed

http://www.10xgenomics.com/single-cell/

Generation of full-length cDNA using template switching and 3’ sequencing



Sequencing adaptors, Read 1 and Read 2, Barcodes and Sample Index

Single cell 3’ mRNA-Seq using 10X Chromium

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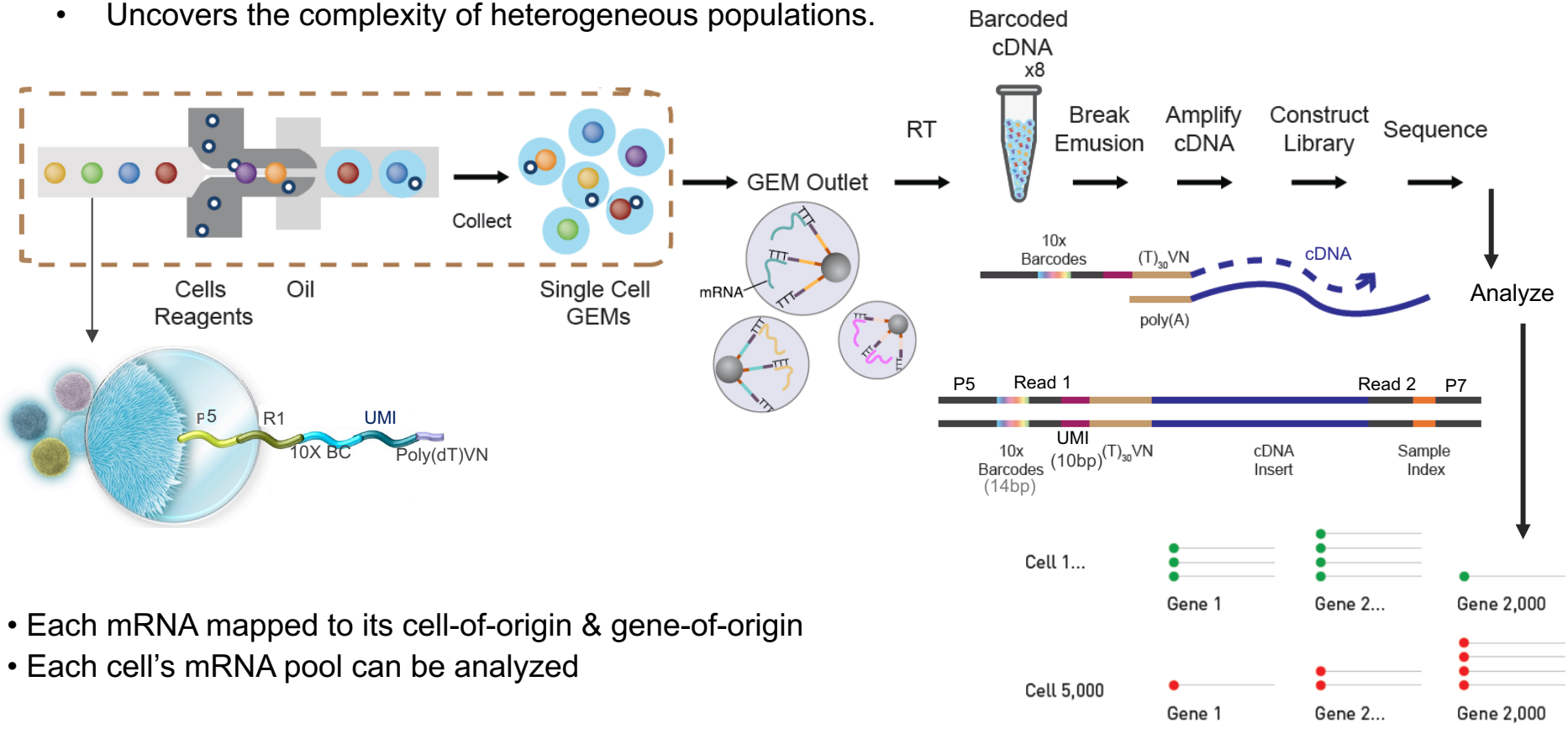

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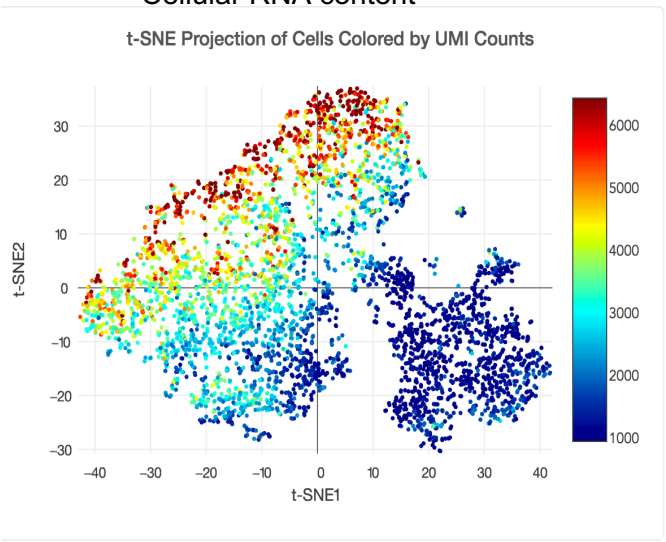
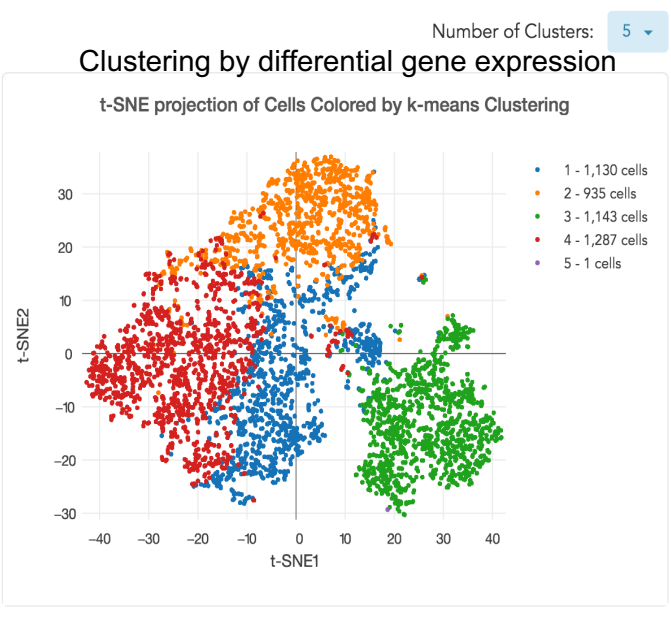
Single cell 3’ mRNA-Seq using 10X Chromium

Examples of clusters generated by 10X Chromium

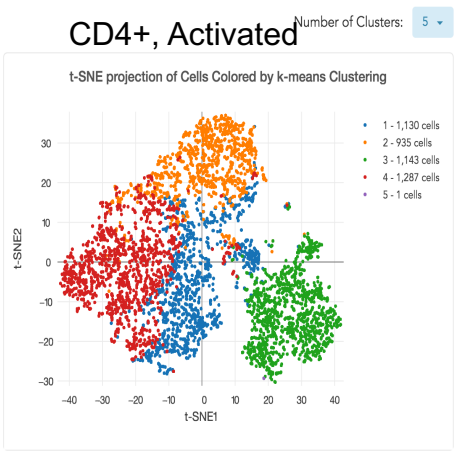
Cellular RNA content



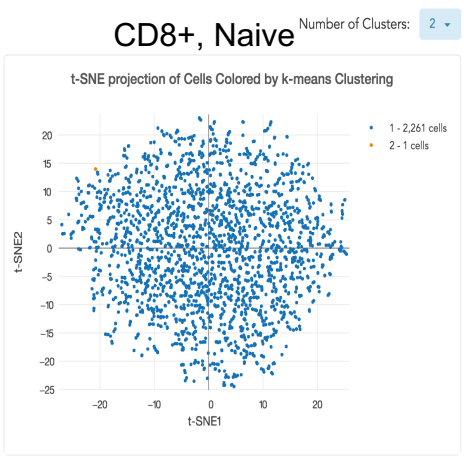
Clustering by differential gene expression



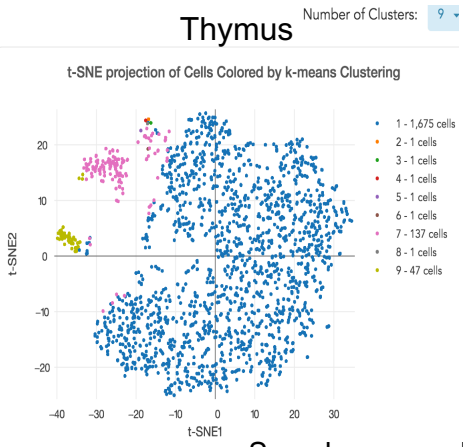
CD4+, Activated



CD8+, Naive



Thymus



Sample source: Dr. Remy Bosselut’s group

Single cell 3’ mRNA-Seq using 10X Chromium

Top differentially expressed genes per cell cluster (UMI counts/cell)

Gene name	Cluster 1	Cluster 2	Cluster 3
Abc1	28.52	0.03	14.7
Xyz2	4.56	8.33	30.85
Fgh3	8.94	17.44	1.27

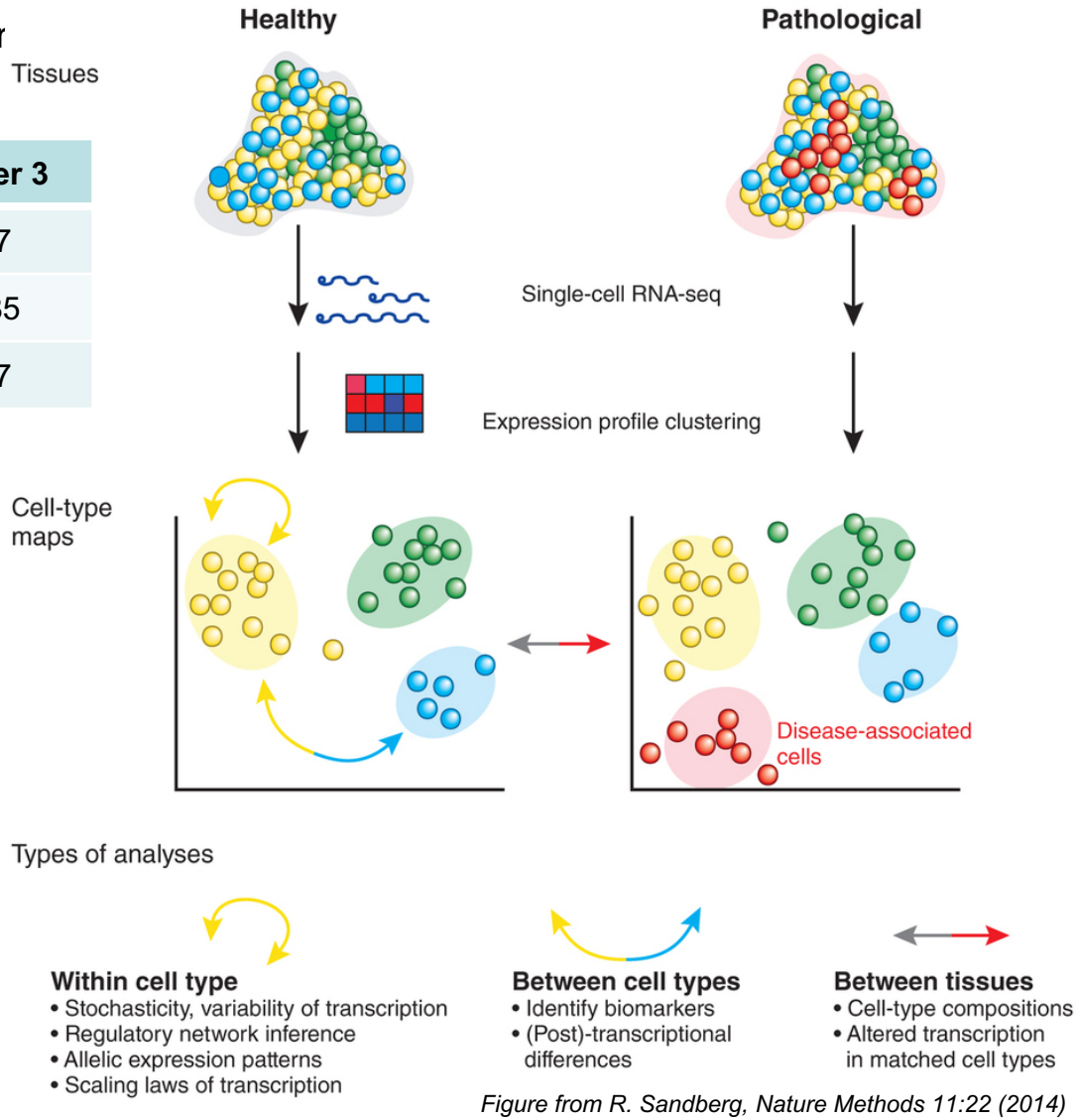


Figure from R. Sandberg, Nature Methods 11:22 (2014)

10X Chromium single-cell projects at CCR-SF

10X Chromium single cell projects at SF	
Number of labs	13
Total number of samples processed	123
Data delivered	101
Total number of cells sequenced	145,982
Median number of cells / sample	1020
Median genes / cell	1,475
Median transcripts / cell	3,650
Total genes detected / sample (Median)	14,914

- Data analyzed by CCR-SF IFX team

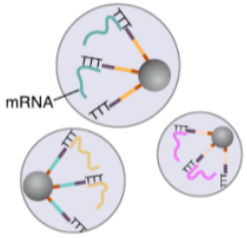
Different cell types processed at CCR-SF:

1. Human and mouse cell lines (HeLa cells and MEFs)
2. B cells (naïve and memory B cells)
3. Cells from the dermis layer of skin
4. T cells (naïve and activated, CD4+, CD8+, & thymus cells)
5. Stem cells (bone marrow cells)
6. Cutaneous MAIT (mucosal-associated invariant T) cells
7. CAR-T cells (cultured, activated)
8. Epithelial cells trypsinized from mouse skin
9. Tumor cells (Prostrate cancer) from organoid cultures
10. Cells from patient urine samples
11. Hepatic cancer patient-derived frozen tumor cells
12. Thymic epithelial cells from WT and mutant mice
13. Neuronal cells (cultured)
14. Single nucleus sequencing from frozen post-mortem brain tissues.

How many cells?

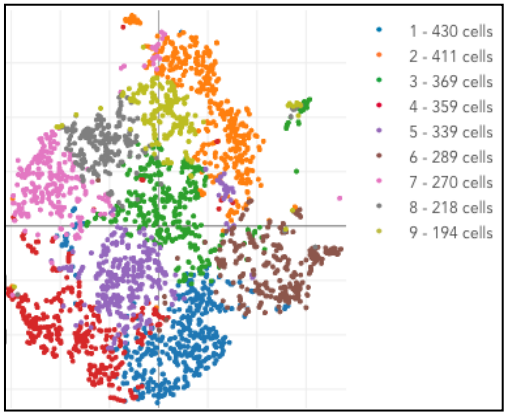
~ 10-15% transcripts captured per cell

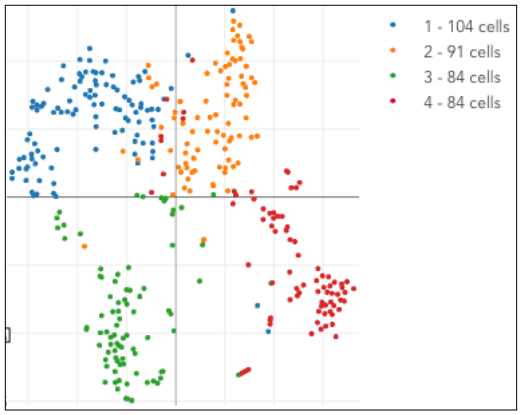
- PolyA capture
- RT conversion within the cell lysate



More cells – higher likelihood of more representative data

More cells – more confidence in heterogeneity analysis

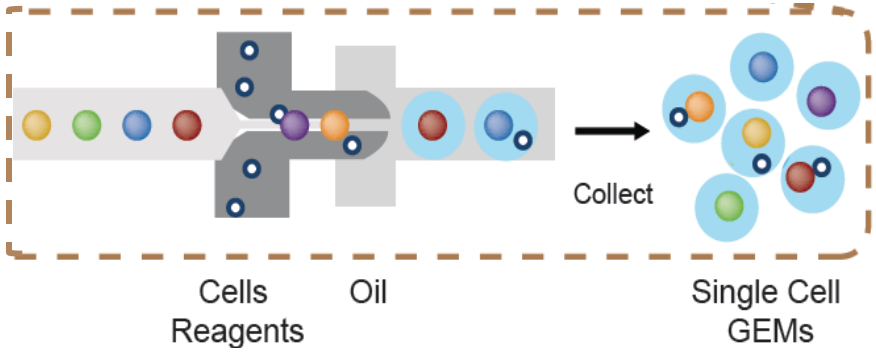




More cells – more probability of multiplets

10X Chromium:

- ~65% cell processing efficiency
- 500-10,000 cells per channel, ~0.4-7.6% doublet rate



How many cells?

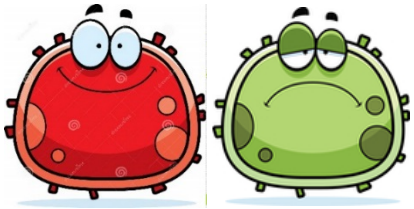
Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered*
~0.4%	~870	~500
~0.8%	~1700	~1000
~1.6%	~3500	~2000
~2.3%	~5300	~3000
~3.1%	~7000	~4000
~3.9%	~8700	~5000
~4.6%	~10500	~6000
~5.4%	~12200	~7000
~6.1%	~14000	~8000
~6.9%	~15700	~9000
~7.6%	~17400	~10000

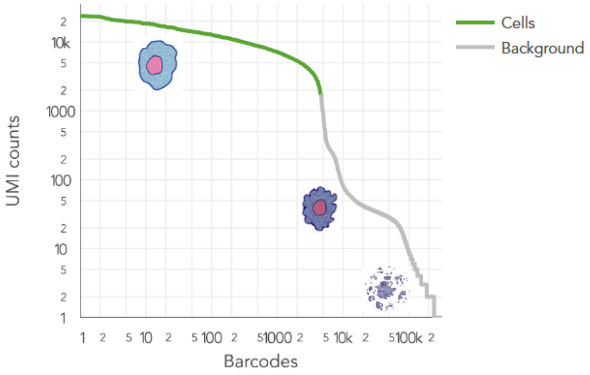
* For good quality samples

- Accurate cell count
- Single cell suspensions – no aggregates
- Enough cells for washes – removal of debris, nucleic acids, RT inhibitors

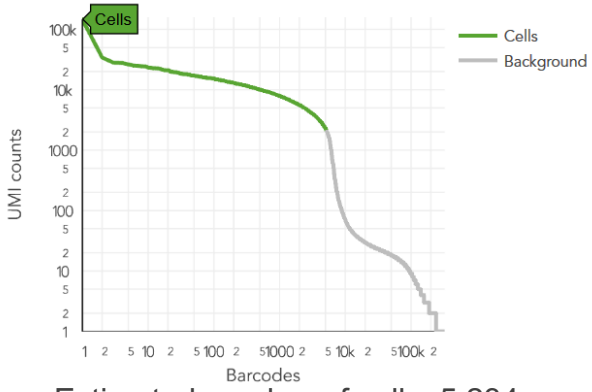
Quality matters!

- Low viability / dead cells
  - mRNA degraded, excluded from analysis – data from fewer cells
  - mRNA enters other GEMs – incorrect data
  - Unhealthy/dying cells may interfere with subpopulation analysis
- Ideal viability > 90%; >70% is good
- Fresh cells > Frozen; some cell types more susceptible to degradation
- Removal of dead cells, if enough number of cells available

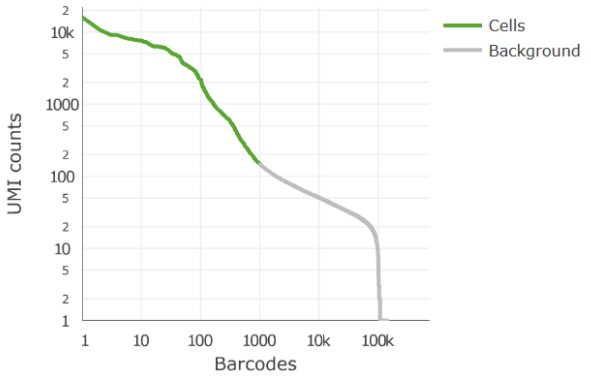




Estimated number of cells: 4,601
Estimated viability: ~87%



Estimated number of cells: 5,234
Estimated viability: ~90%



Estimated number of cells: 1,011
Estimated viability: ~20%

- Tips: Gentle handling of the cells; low speed spins, wide bore tips, maintain cells in ice, shorter processing time, no RT inhibitors

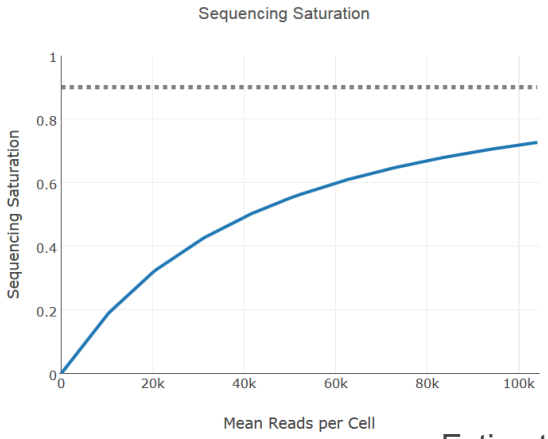
Sequencing Depth

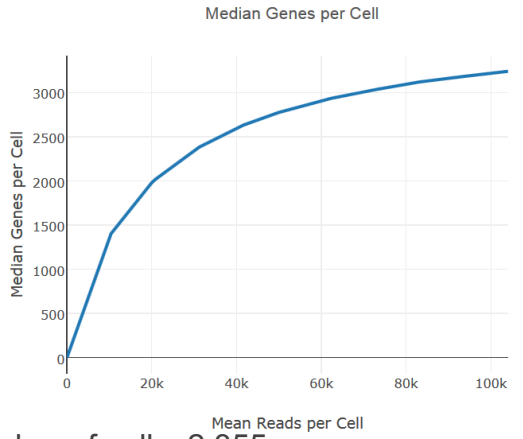
Depends on

- Cell type: More RNA vs less RNA per cell
- Cell number: More cells, more reads
- Cell quality: More healthy cells, more reads

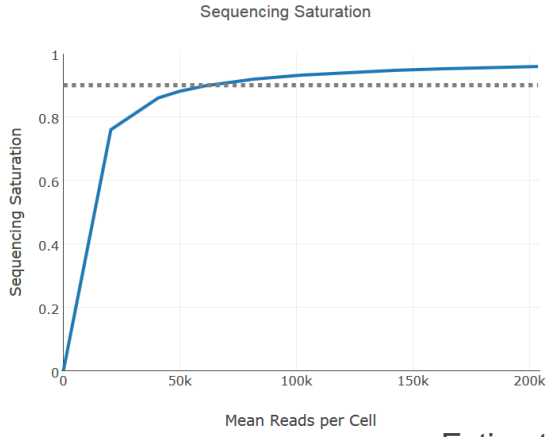
Typical: 100,000 reads per cell

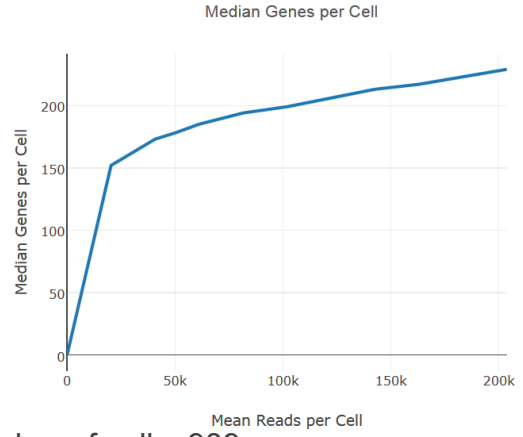
- 2-3 samples per NextSeq lane (SR)





Estimated number of cells: 2,355
Median genes per cell: 3,241





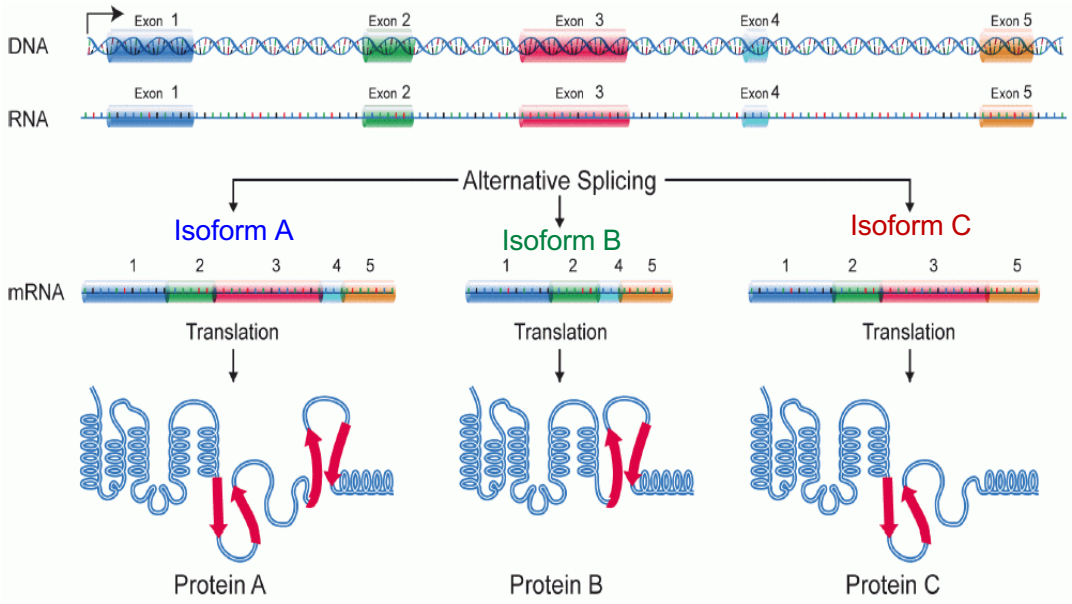
Estimated number of cells: 989
Median genes per cell: 484

Tips

- Cells should have high viability
- Remove dead/unhealthy cells, if possible
- Cell number: enough to enable at least a couple of washes and cell counting before loading
- Cell handling: as gentle as possible. Use low speed spins, wide bore tips
- Minimize cell stress: maintain cells in ice
- Processing time: as short as possible. Capture as quickly after extraction as possible.
- If delays are inevitable, keep cells in the medium they are happy in
- Fresh cells better than frozen.
- Single cell suspensions should be free from cell aggregates and debris
- No RT inhibitors in the cell suspensions
- Accurate cell count

Coming soon – Full-length RNA-seq from single cells

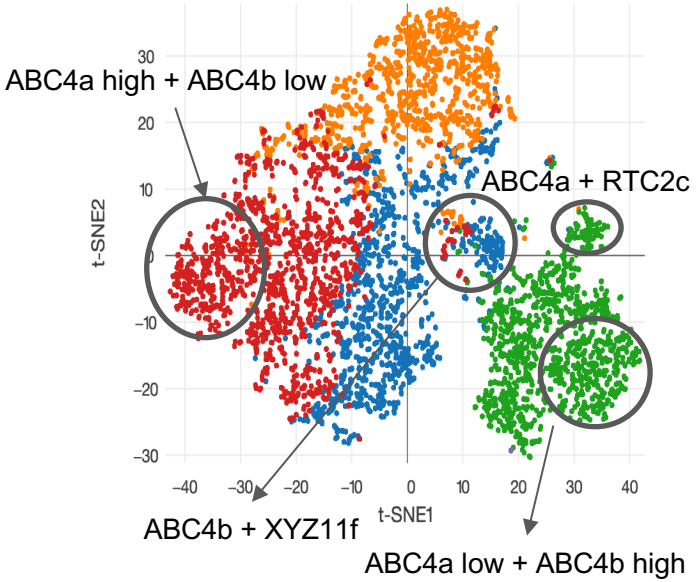
Alternate Splicing: Increases informational diversity, regulates various cellular processes, commonly dysregulated in cancer, thought to result in functional diversity of most cancer-relevant genes.



Subpopulation 1 (20% cells): *ABCa* + *ABCb*

Subpopulation 2 (50% cells): *ABCa* + *ABCc*

Subpopulation 3 (30% cells): *ABCb*

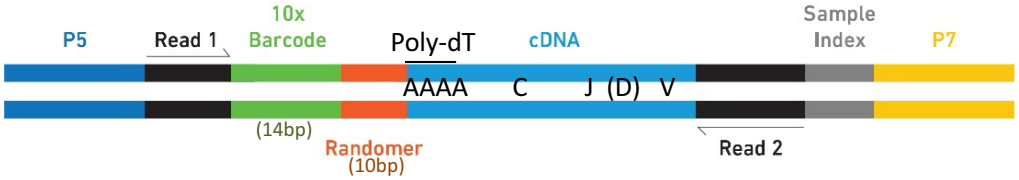


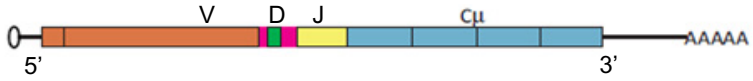
- Using long-read sequencing technology to obtain full-length sequence and isoform information from single cells

Coming soon – Analysis of V(D)J rearrangement in B- and T-cells

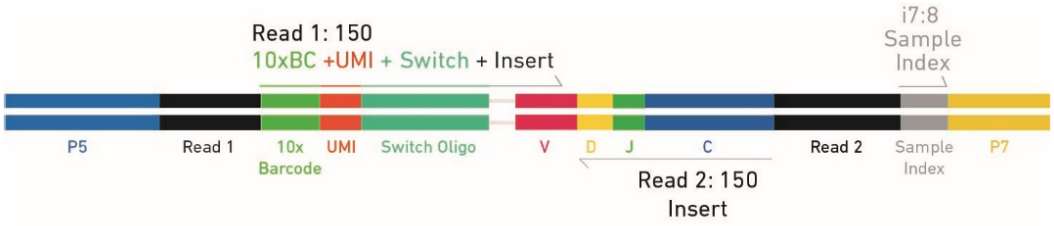
- Single cell sequencing to reveal the true clonality and diversity of the immune repertoire
- Simultaneous analysis of a single sample for cellular heterogeneity and phenotype, as well as T-cell receptor and B cell immunoglobulin repertoires.

3' Gene Expression Library Structure:

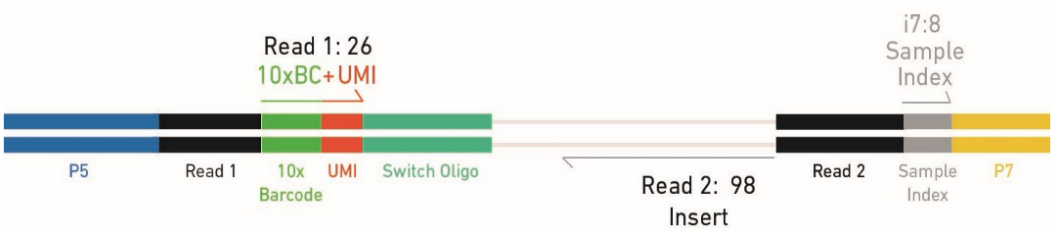




V(D)J Enriched Library Structure:

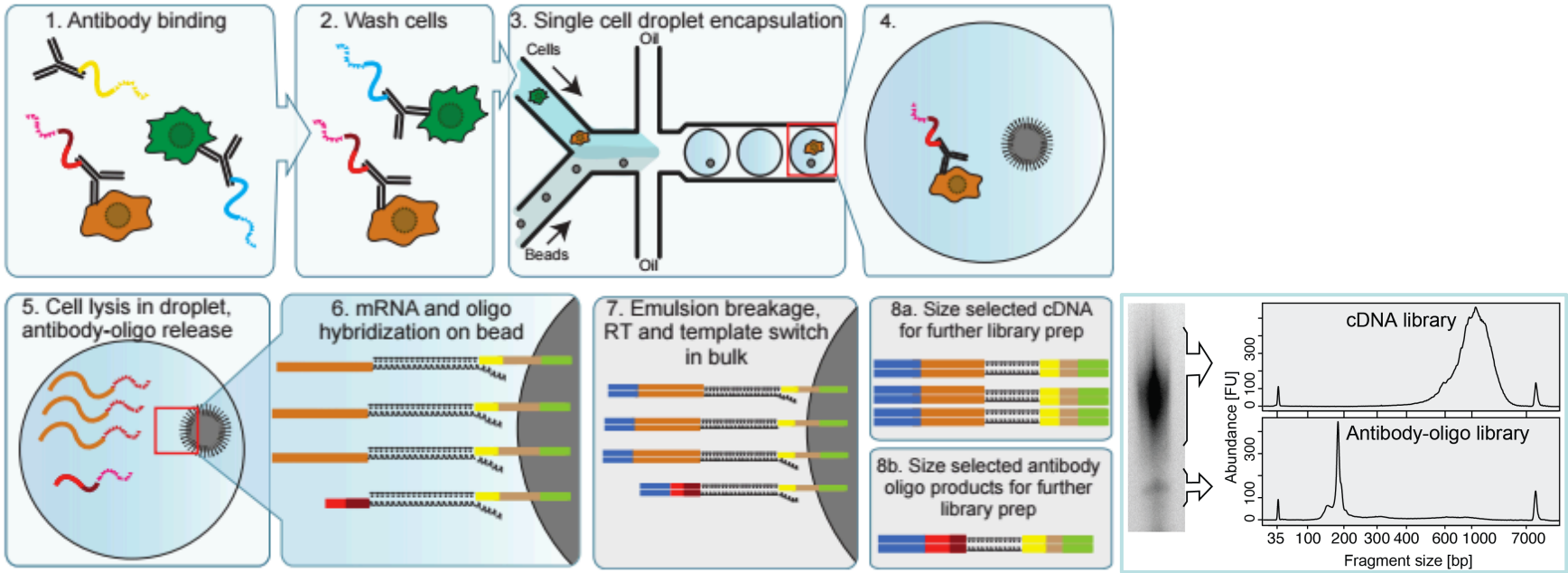


5' Gene Expression Library Structure:



Coming soon – CITE-seq (Cellular Indexing of Transcriptomes and Epitopes by sequencing)

- Couples measurement of surface protein markers on thousands of single cells with simultaneous mRNA sequencing - NYGC
- Correlation between mRNA and protein levels
- Clustering of cells with similar transcriptional profiles

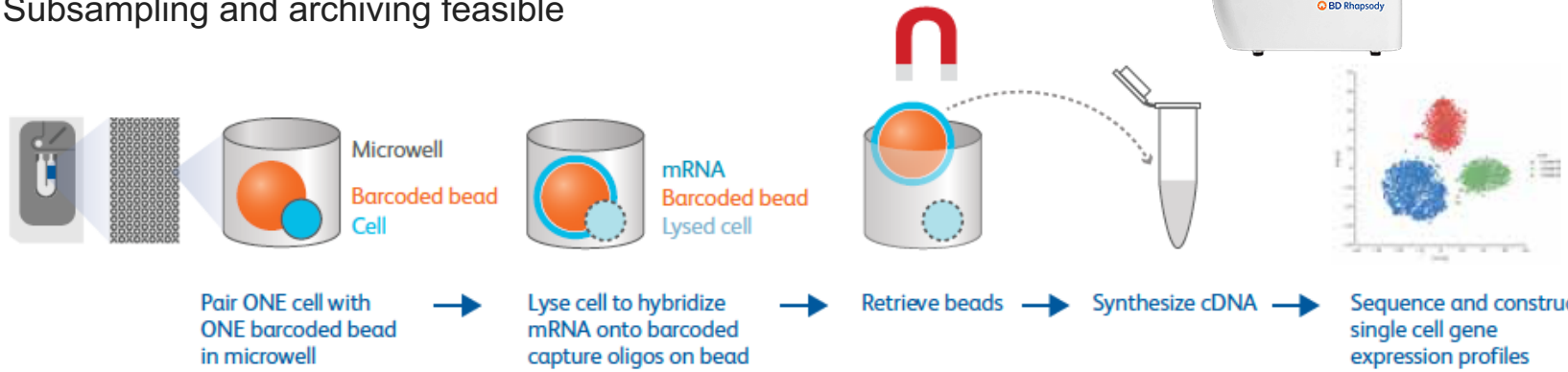


Stoeckius et al, 2017, Nature Methods **14**: 865

Coming soon – targeted RNAseq from single cells

Targeted RNAseq from single cells using BD Rhapsody system

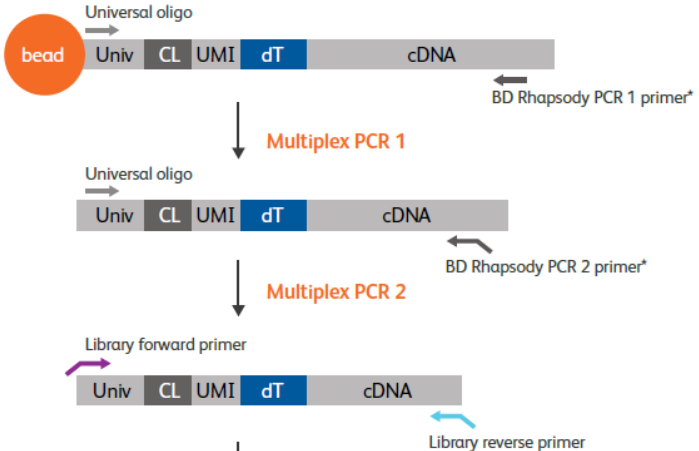
- Analysis of low abundance transcripts
- Less sequencing required, cheaper
- Subsampling and archiving feasible



- Panels – immune cell panels available, custom option in future.

Whole Genome Sequencing from single cells

Amplification of cDNA using targeted primer panels



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Xin Wang
Francis McMahon
Yasmine Belkaid
Ramiro Iglesias-Bartolome
KathyKelly
Jairaj Acharya
Chris Nagao
Tae-Wook Chun
Jeffrey Kopp

Thank you!

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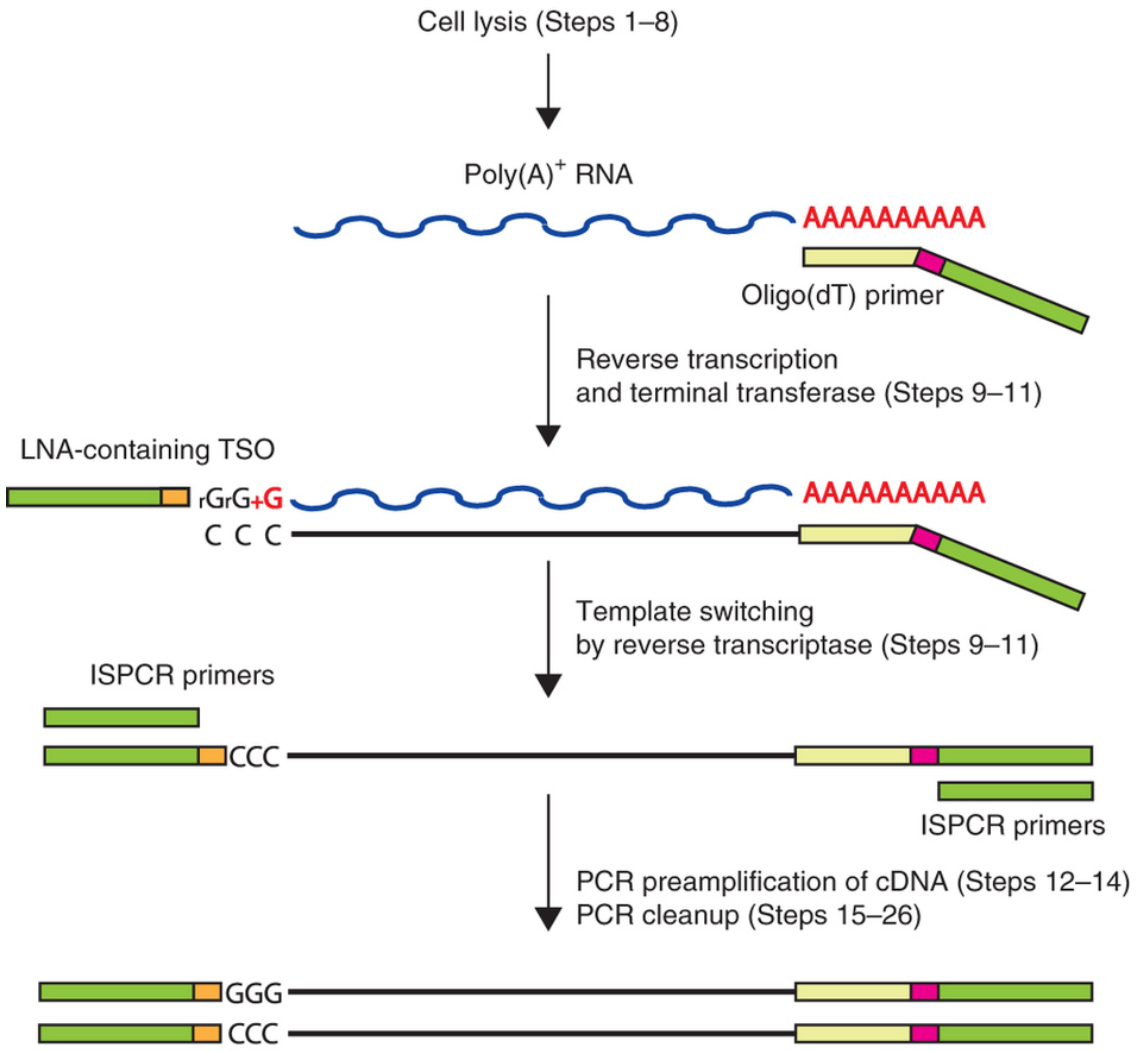

Cell lysis (Steps 1–8)
Poly(A)+ RNA
AAAAAAAAAA
Oligo(dT) primer
Reverse transcription
and terminal transferase (Steps 9–11)
LNA-containing TSO
rGrG+G
C C C
AAAAAAAAAA
Template switching
by reverse transcriptase (Steps 9–11)
ISPCR primers
CCC
ISPCR primers
PCR preamplification of cDNA (Steps 12–14)
PCR cleanup (Steps 15–26)
GGG
CCC

Short-read Sequencing : Sequencing by Synthesis

Illumina Sequencers (MiSeq, NextSeq, HiSeq)

- Max read length: 300bp
- Variety of sequencing applications: genome assembly, transcriptome analysis, SNP detection, DNA methylation analysis, metagenomic studies.
- High level multiplexing of samples possible.
- Big advantage: scale.

- Limitations of short read sequencing – genome assembly, isoform analysis, structural variation, haplotypes and SNP phasing.

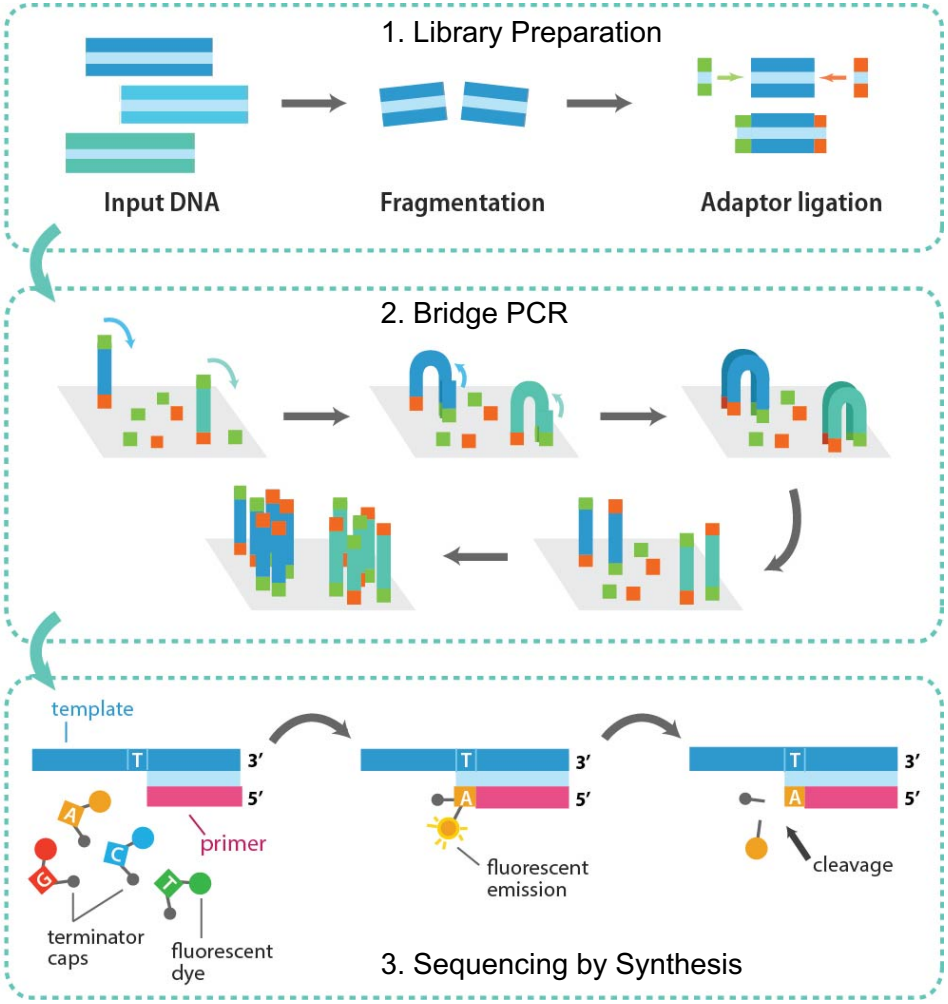
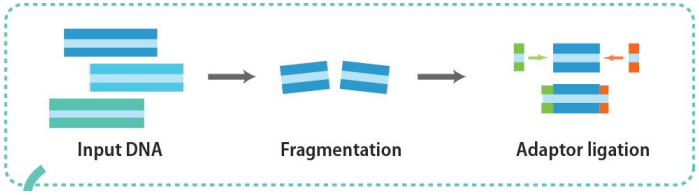


Figure from: https://www.abmgood.com/Next-Generation-Sequencing-Service.html

Library preparation for Short-read Sequencing



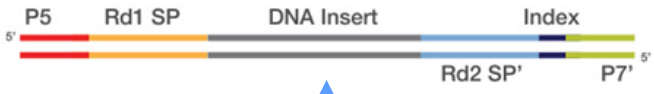
Single cells

Total RNA

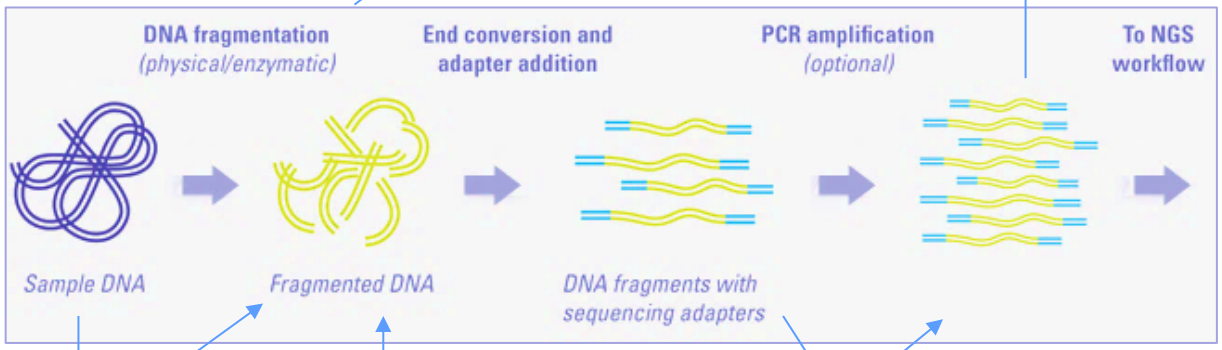
Lysates → mRNA enrichment rRNA depletion ← **FFPE RNA**

cDNA synthesis
(**Gene expression, whole transcriptome, miRNA, lncRNA**..)

Bisulfite conversion
(Methyl-Seq)



Genomic DNA



Barcoded amplicons
(SV analysis by 10XGenomics)

ChIP DNA

Target selection
(**Exome-Seq** etc)