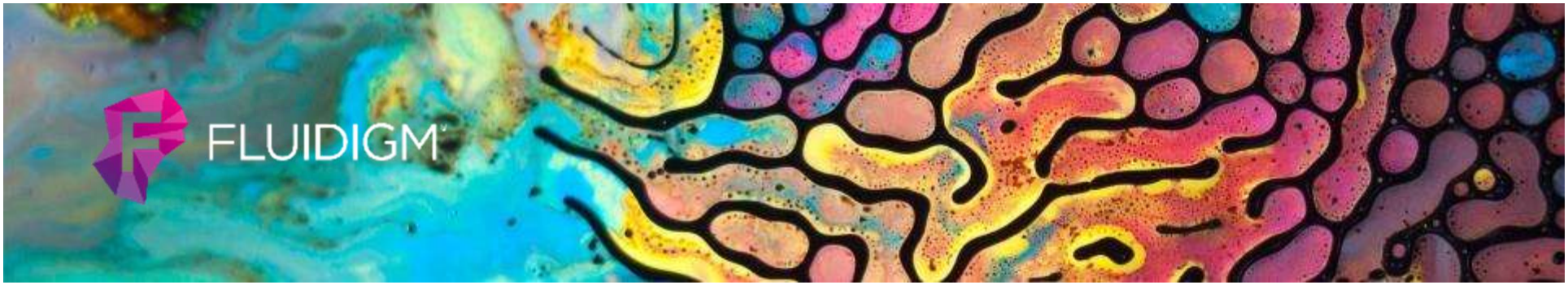
Single-Cell Genomics

Exploring new applications in microfluidics

Mark Lynch
April 10, 2019



Agenda

1. Microfluidics solutions for single-cell analysis
2. Single-cell total RNA sequencing
3. Single-cell high-throughput (HT) RNA expression and protein sequencing (REAP-seq)
4. Creating single-cell microenvironments

Microfluidic solutions for single-cell analysis

Cellular analysis cell by cell

The single-cell revolution is only beginning

Science special issue, A Fantastic Voyage in Genomics, 2017

Science, 2018 Breakthrough of the Year, published in December

Driving those advances are techniques for isolating thousands of intact cells from living organisms, efficiently sequencing expressed genetic material in each cell and using computers, or labeling the cells, to reconstruct their relationships in space and time. That technical trifecta 'will transform the next decade of research,' says Nikolaus Rajewsky, a systems biologist at the Max Delbrück Center for Molecular Medicine in Berlin.

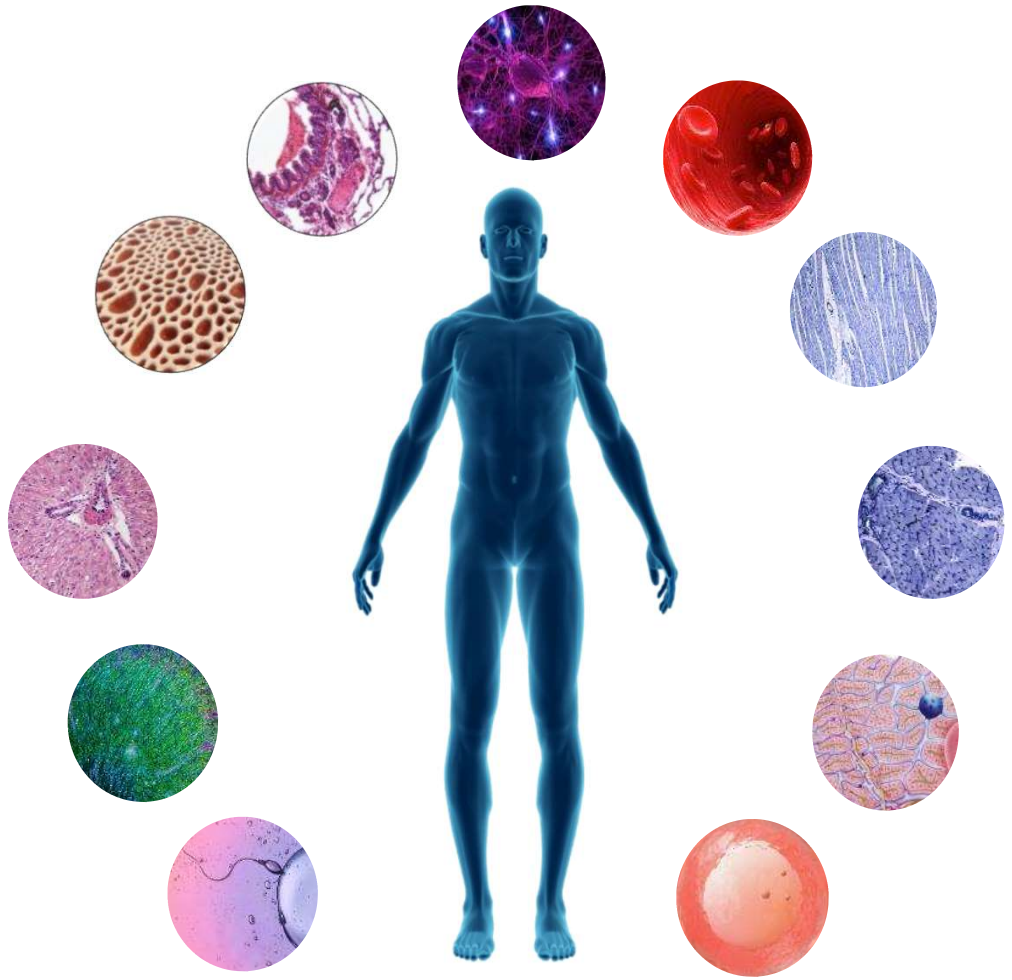
—Elizabeth Pennisi,
writing in 'Science' about the 2018 Breakthrough of the Year

Creating a cell atlas

The only way to identify and understand all cells in a tissue

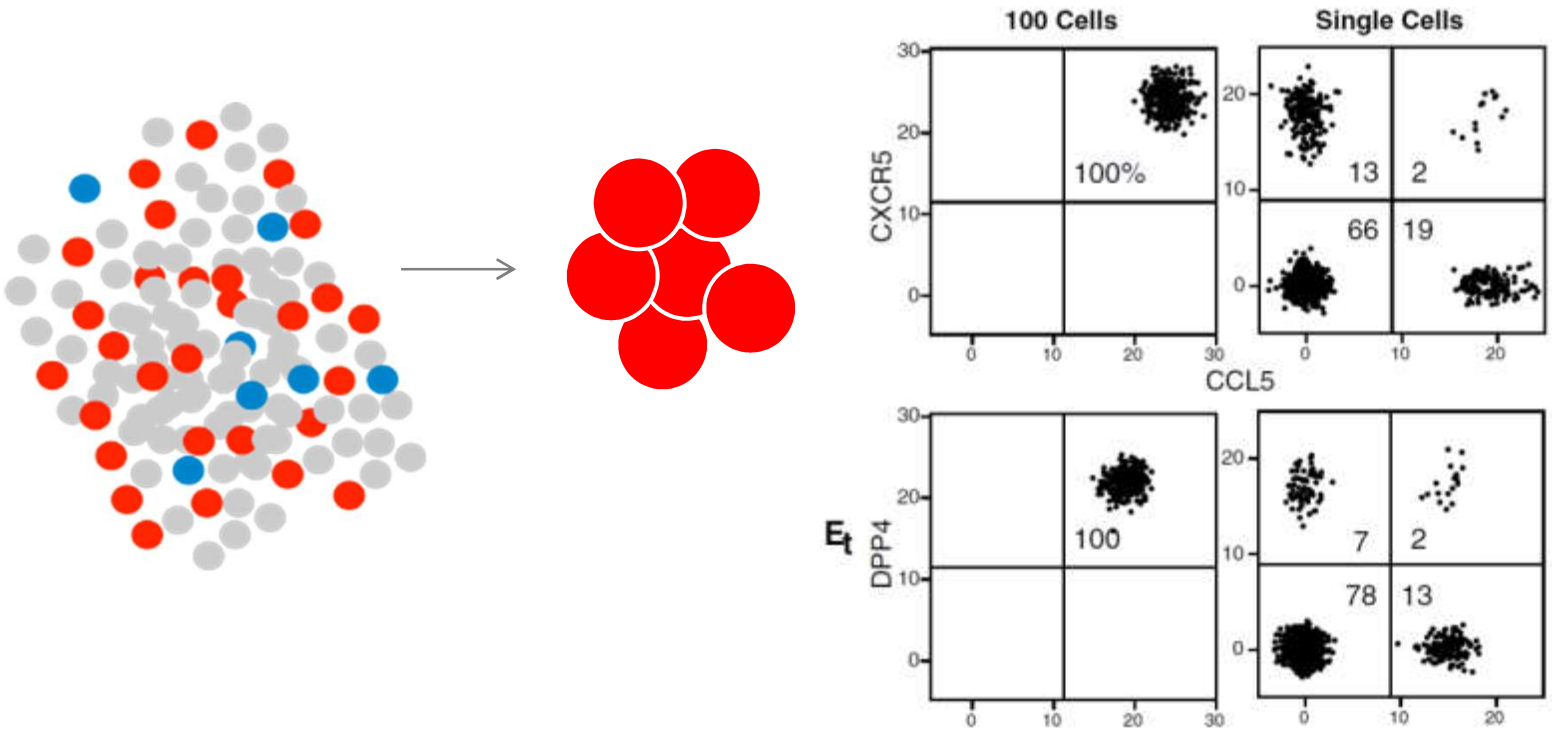
Three cell-based approaches:

1. Classification
2. Characterization
3. Context



The benefit

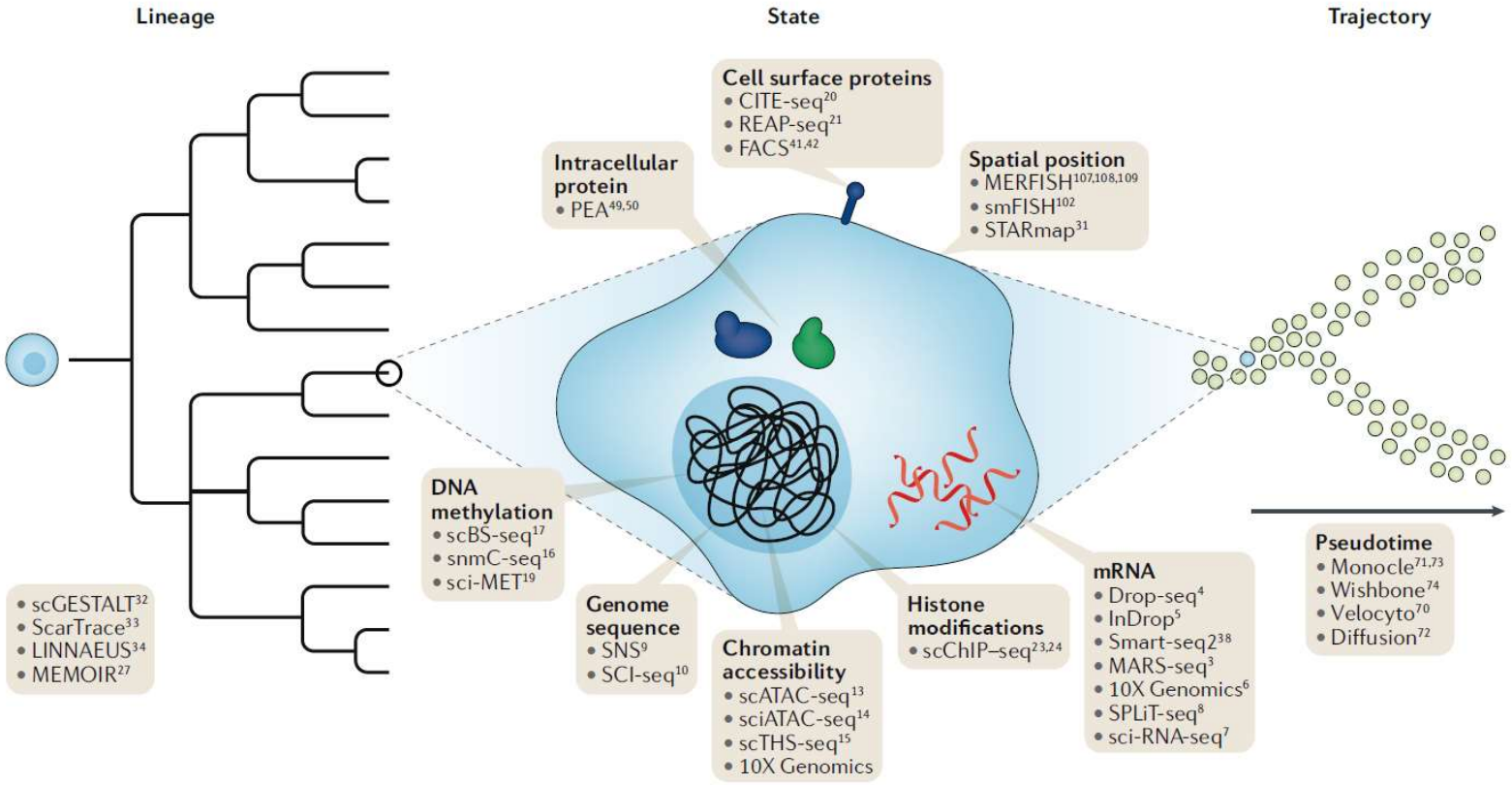
Individual cells behave differently from the average of many cells



Modified from Dominguez et al. *Journal of Immunological Methods* (2014)

Cell characterization

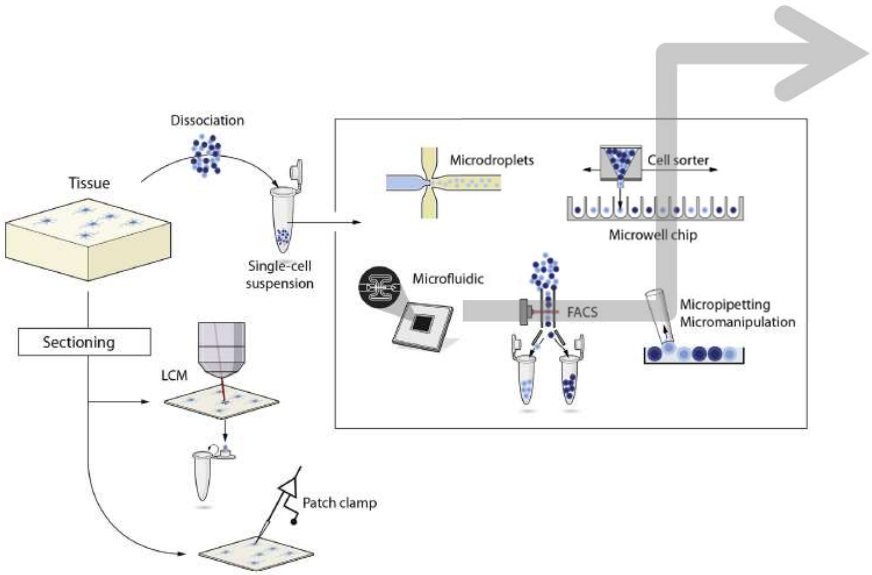
Deep profiling of cells to study multiple modes



Stuart and Satija. *Nature Reviews Genetics* (2019)

Microfluidics

Integrated fluidic circuit (IFC) technology to capture, verify, process and perturb individual cells



Modified from Hedlund and Deng.
Molecular Aspects of Medicine (2017)

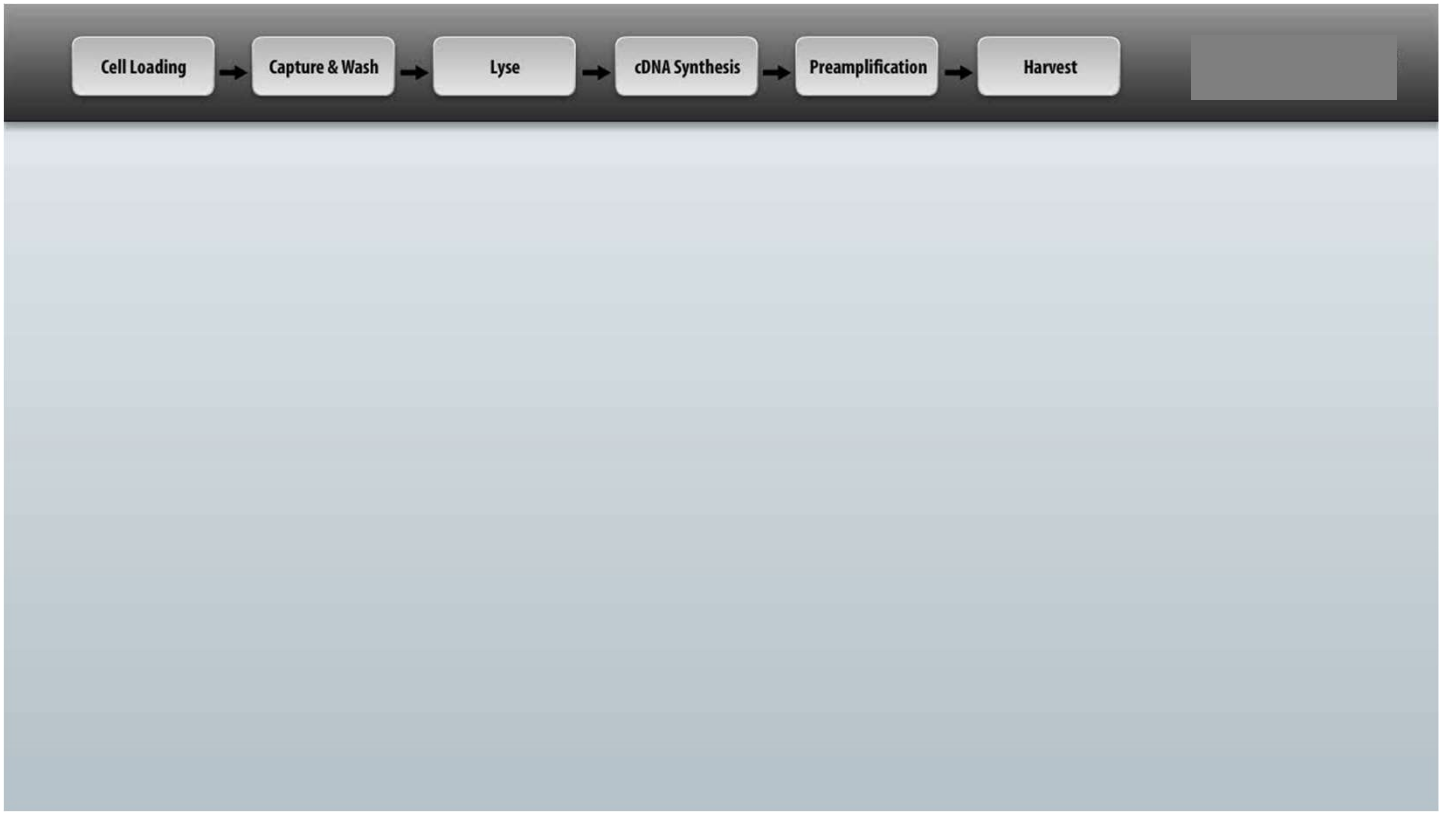
C1™ system

Biomark™ HD

Polaris™

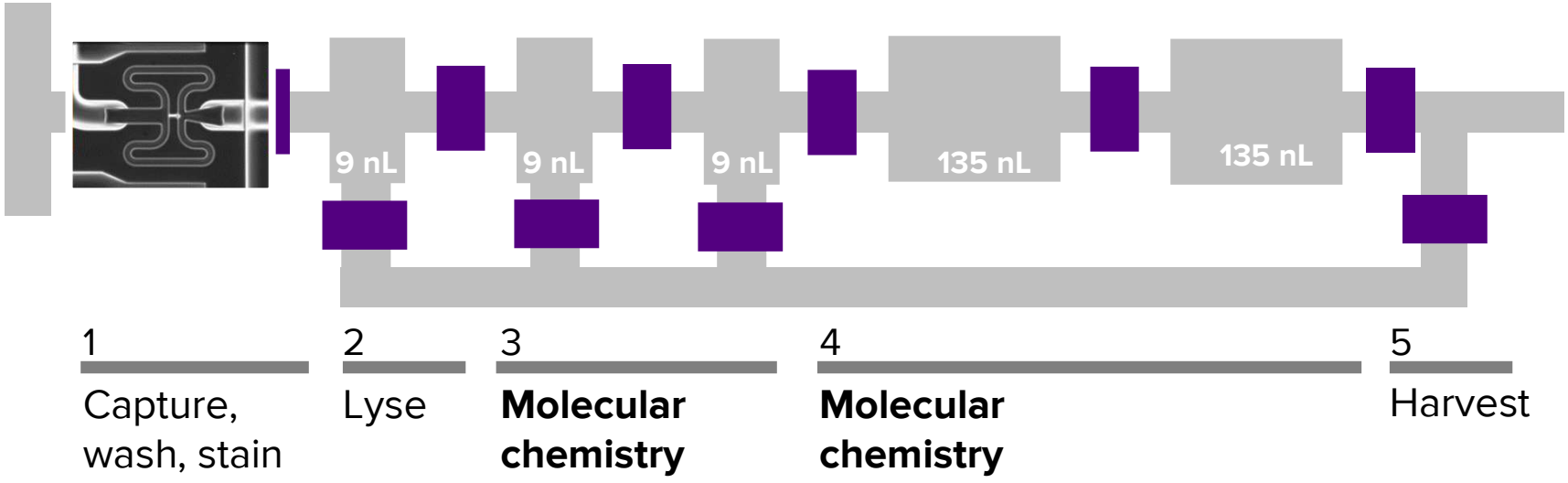
Microfluidics for single-cell genomics

C1 system and C1 IFCs



C1 IFC flexibility

To capture, verify, process and perturb individual cells



Processing cells using solid-state valve-formatted microfluidics enables a platform that is fully customized for flexibility.

C1 IFC

An IFC range to offer full flexibility

	Input	mRNA Sequencing Applications	Cell Size Formats	Compatible with C1 Script Builder™	Compatible with C1 Script Hub™ Applications
Capture and process up to 96 single cells	200–2,000 cells/µL	• Full length • Total • End counting	• Small • Medium • Large	○	○
Capture and process up to 800 single cells	2,000–5,000 cells/µL	• End counting • C1 REAP-seq	• Small • Medium	⊘	⊘

Formats:

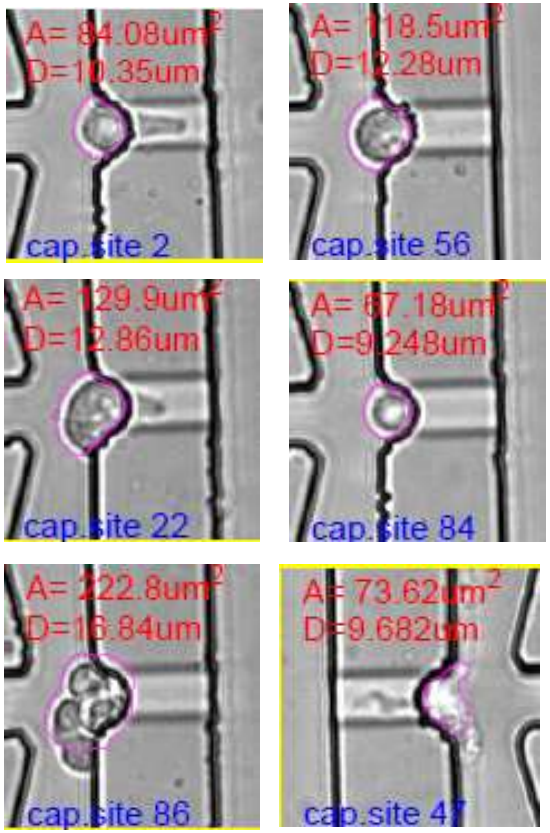
- Small captures cells from 5 to 10 µm.
- Medium captures cells from 10 to 17 µm.
- Large captures cells from 17 to 25 µm.

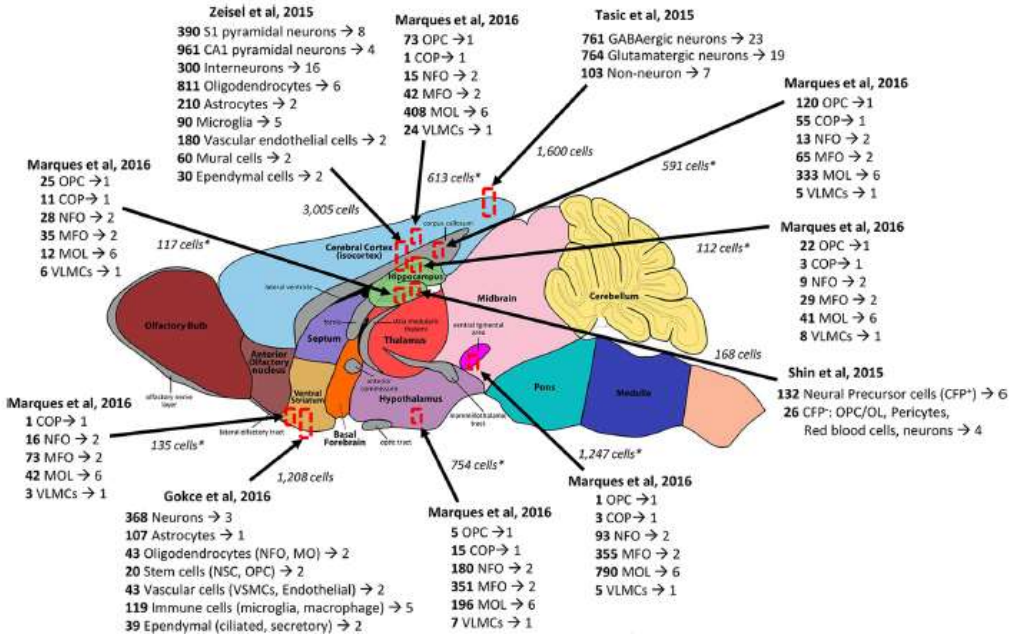
Visualizing cells using C1

Neuronal cells

Single cells

Debris

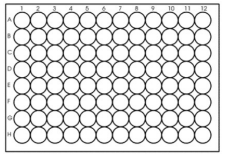




Single-cell total RNA sequencing

Benefits

Workflow, cost and deeper transcriptome characterization

	Total RNA Seq	SMART-Seq v4
On-IFC amplification	• Back-loading indexing PCR	• Universal amplification
Post C1 workflow	• No extra kit needed • Samples pooled after harvest • rRNA depletion and final PCR performed in a single tube 	• Nextera® XT, index kits required • Samples pooled after final PCR • Tagmentation through indexing PCR performed in 96-well plate 

With enhanced transcription data, C1 Total RNA Seq provides an easier and much cheaper workflow than SMART-Seq® v4.

ncRNA biotypes

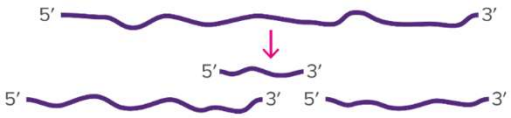
Abbreviation	Name	Function
lincRNA	Long intergenic noncoding RNA	Gene regulation, splicing, translation
eRNA	Enhancer RNA	Gene regulation
snRNA	Small nuclear RNA	Splicing
snoRNA	Small nucleolar RNA	Splicing, translation
miRNA	MicroRNA	Translation
rRNA	Ribosomal RNA	Translation
tRNA	Transfer RNA	Translation
tmRNA	Transfer-messenger RNA	Translation

Enabling study of these ncRNA biotypes at the single-cell level will allow for a more comprehensive understanding of cellular mechanisms.

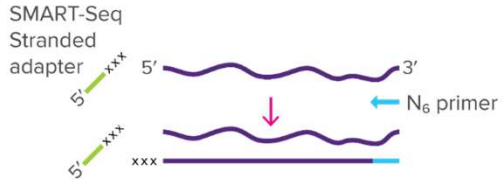
Stranded Total RNA Seq workflow

Automated steps on C1

1. RNA fragmentation

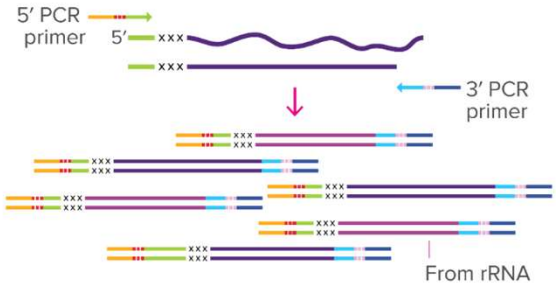


2. First-strand synthesis and tailing by RT



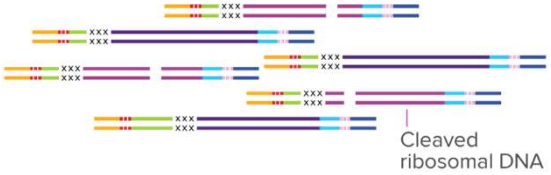
3. Template switching and extension by RT

4. **PCR 1:** Addition of Illumina® adapters with barcodes

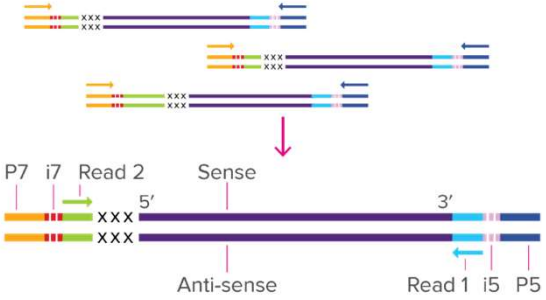


Single-tube library prep after C1

1. Cleavage of ribosomal cDNA



2. **PCR 2:** Enrichment of uncleaved fragments



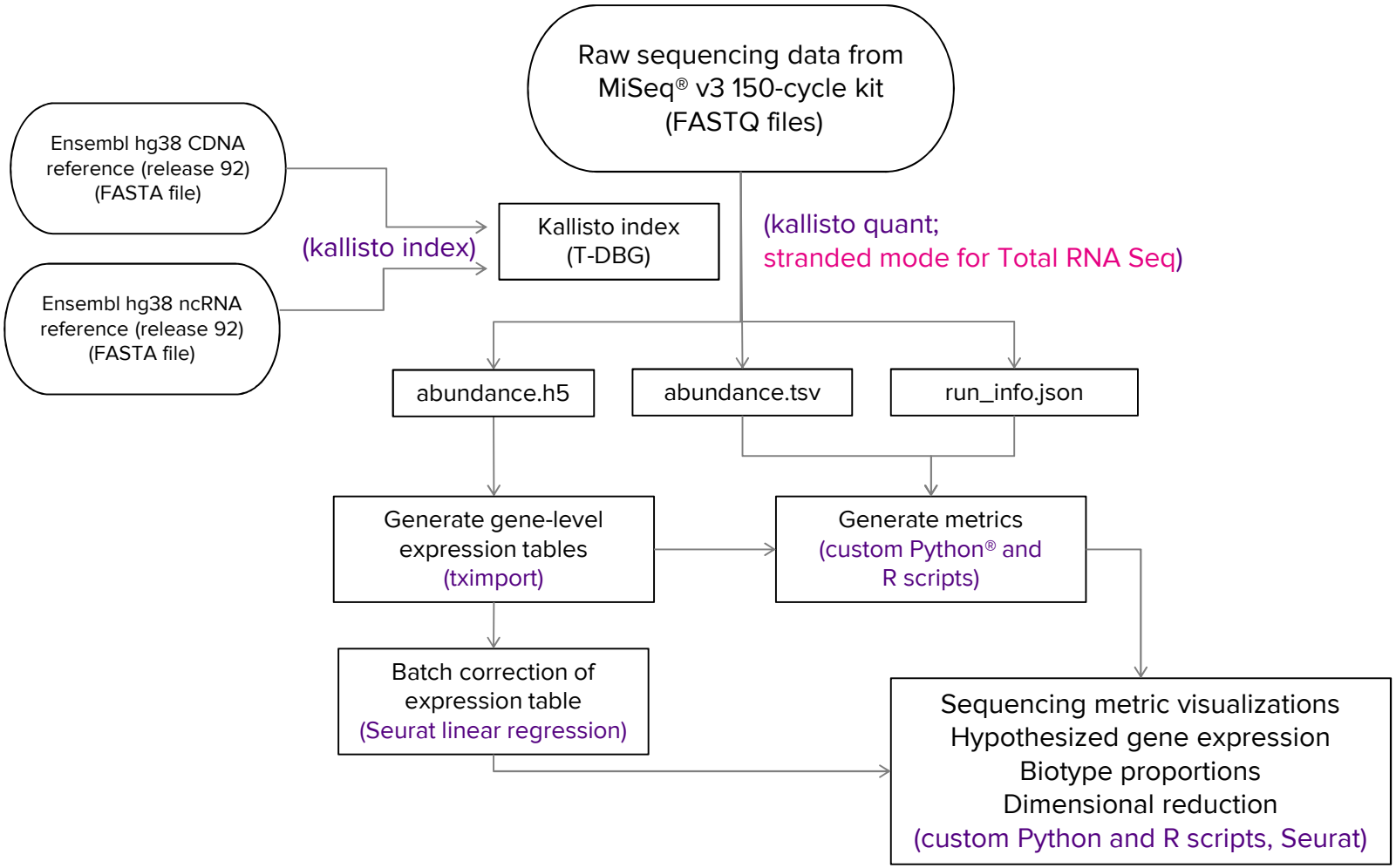
Adaptation of SMART-Seq Stranded Kit (Takara Bio)

Building the application

	K562 Cells	HL-60 Cells	Activated T Cells
Cell description	Myelogenous leukemia cell line, robust cell type	Leukemia cell line, fragile cell type	Primary cell, stimulated with anti CD3/CD28 beads
IFC size	Medium	Small	Medium
Total RNA Seq	3 IFCs*	3 IFCs*	2 IFCs
SMART-Seq v4	2 IFCs	2 IFCs	2 IFCs

* Initial Total RNA Seq experiments with K562 and HL-60 cells were run with the SMARTer® Stranded Total RNA Seq Kit v2 —Pico Input Mammalian. The remaining Total RNA Seq experiments were performed using the updated SMART-Seq Stranded Kit after its launch in May 2018.

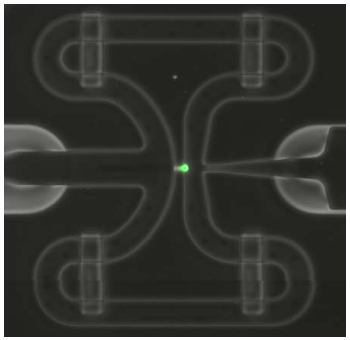
Bioinformatic pipeline



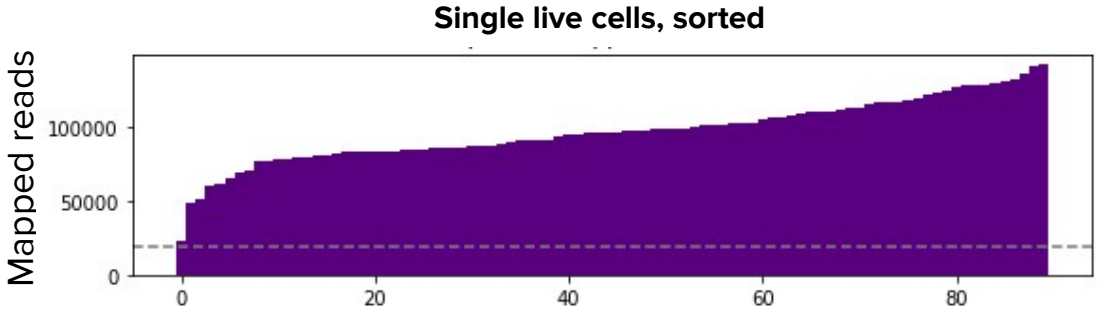
FASTQ files were also processed to visualize transcript coverage (Bowtie, Picard).

Processing live cells

Only cells with >20,000 mapped reads received secondary analysis



Single live cell



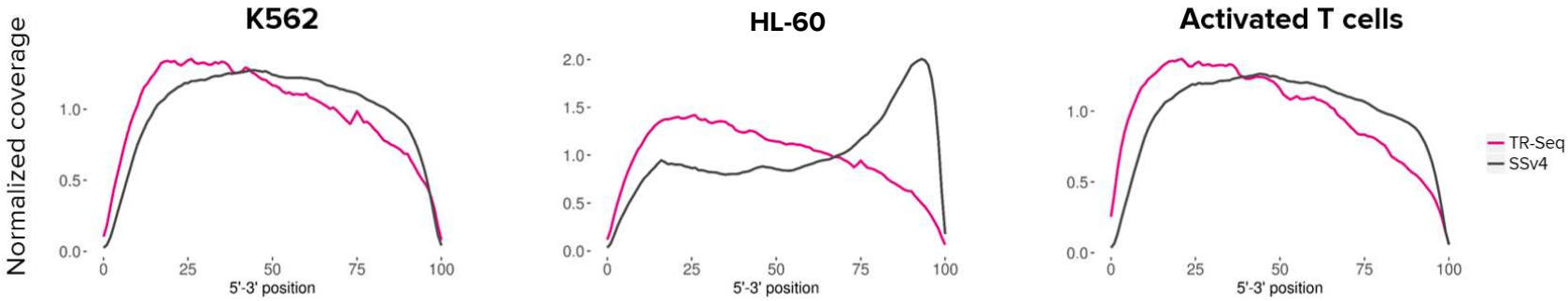
One IFC is shown as an example. Dashed line indicates 20,000 mapped reads.

	K562	HL-60	Activated T Cells
SMART-Seq v4	57 cells 90 cells	40 cells 42 cells	38 cells 24 cells
Total RNA Seq	58 cells 83 cells 76 cells	69 cells 45 cells 65 cells	38 cells 40 cells

A total of 765 cells passed filters for further analysis.

For K562 and HL-60, two IFCs each were run with an older version of Total RNA Seq chemistry before the current version was launched.

Total RNA Seq shows transcript coverage similar to or better than SMART-Seq v4



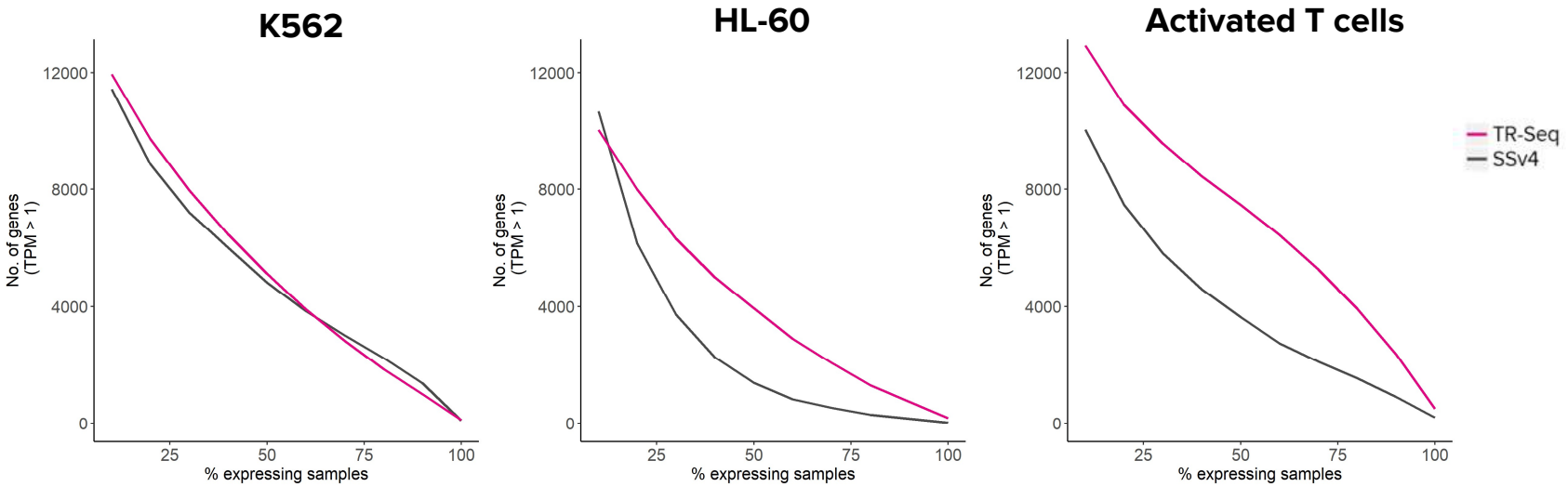
Total RNA Seq provides complete full-length mRNA sequencing with better coverage across transcripts in cell types that show high 3′ bias, which is important for characterizing the complete transcriptome.

Total RNA detects significantly more noncoding genes

Total RNA applications enable characterization of the full transcriptome including regulatory elements that control transcription.

Total RNA Seq

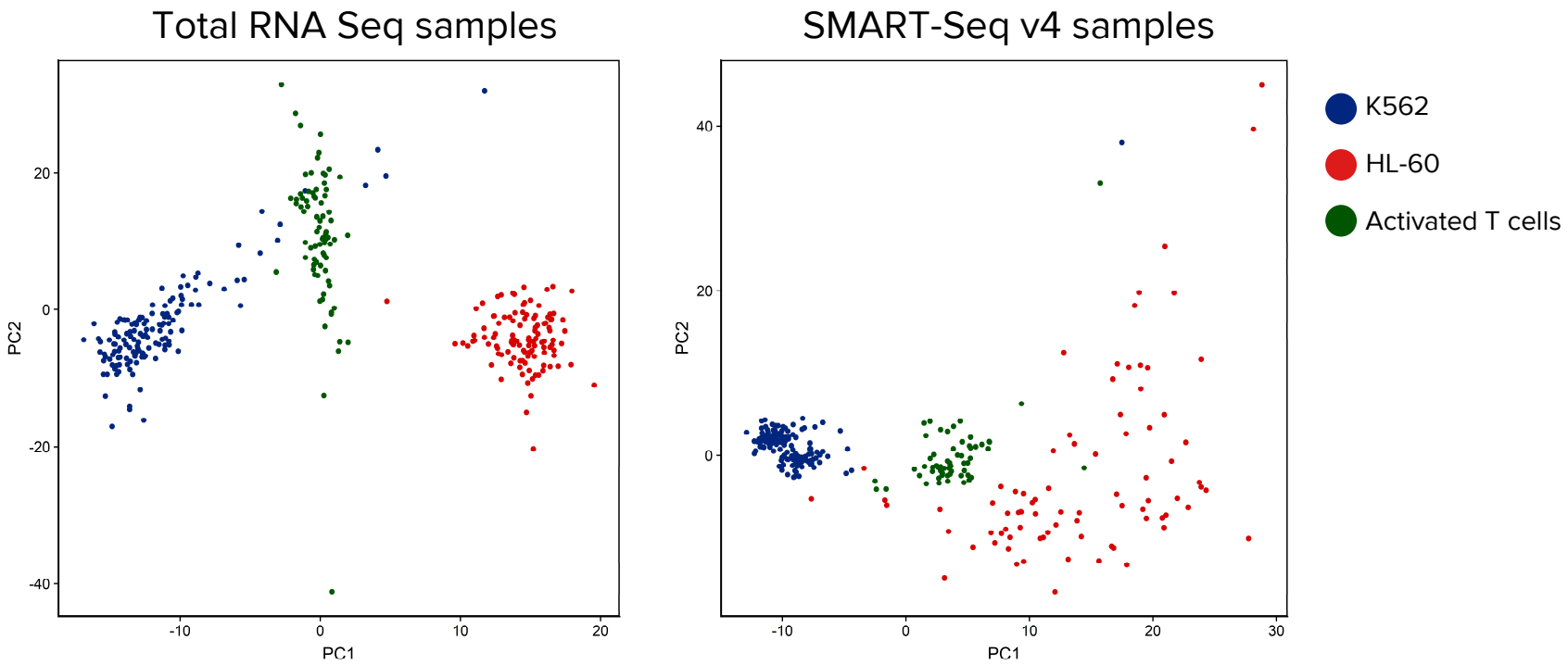
Shows gene expression similar to or more consistent than SMART-Seq v4



Within a cell type, Total RNA Seq detects genes with greater consistency across the full sample than SMART-Seq v4.

Total RNA Seq

Shows greater resolution when visualized by PCA



Total RNA Seq chemistry provides better resolution because more genes are detected, enabling complete transcriptome characterization.

PCA generated with Seurat

Total RNA Seq

Exhibits a greater detection of noncoding RNA

Total RNA Seq detects more ncRNA and a greater diversity of noncoding RNA biotypes.

Total RNA Seq methods can detect full-length nonpoly(A) isoforms

Method	Read Depth	Transcript Coverage	Poly(A) Transcript Isoforms	Nonpoly(A) Transcript Isoforms
Droplet-based methods	Low	3′ only	No	No
C1 high-throughput	Medium	3′ only	No	No
C1 96 (SMART-Seq v4)	High	Full-length	Yes	No
C1 96 Total RNA Seq	High	Full-length	Yes	Yes

Total RNA Seq
Summary

- Provides one of very few methods to sequence both poly(A) and nonpoly(A) RNAs in single cells
- Simpler workflow than most protocols, enabling on-IFC cell-indexing PCR and single-tube post-C1 prep (primarily ribosomal RNA depletion)
- Maintains full-length transcriptome coverage with little 5′–3′ bias in cell types where SMART-Seq v4 shows a strong 3′ bias
- Provides a method for researchers to perform deeper single-cell characterization by enabling analysis of novel noncoding RNA features

Download the C1 Total RNA Seq poster today.

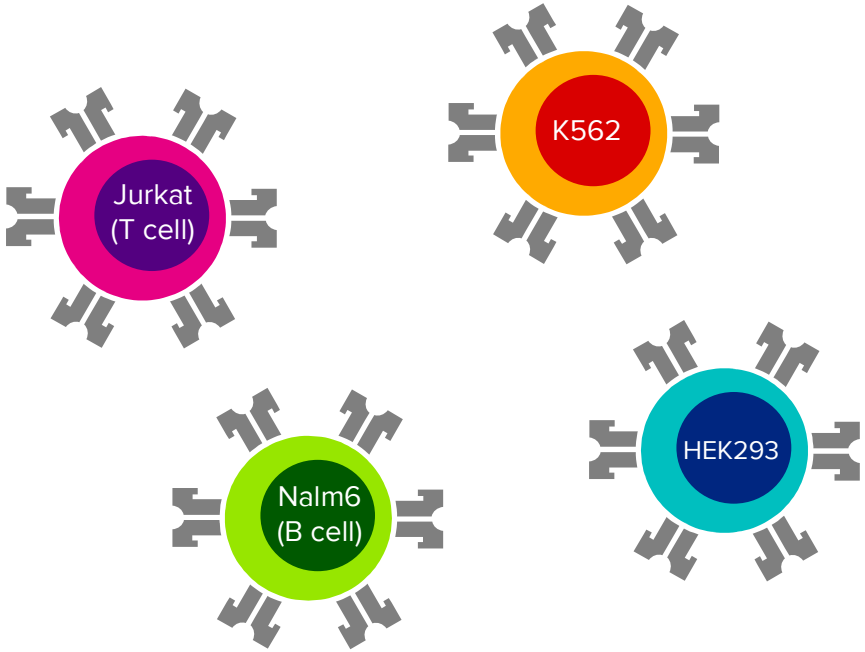
Single-cell high-throughput RNA expression and protein sequencing

What is REAP-seq?

- RNA expression and protein from the same isolated single cell
- With the enablement of REAP-seq on C1 Single-Cell mRNA Seq HT IFCs, researchers can simultaneously detect protein and gene expression from the same cell.
- Detecting both RNA and protein from the same cell eliminates the bioinformatic challenges of stitching datasets together.
- REAP-seq labels cells with antibodies conjugated to unique DNA sequences, circumventing the limitations of fluorescence and stable isotope-conjugated antibodies. Our method allows for more than 80 proteins to be detected per single cell.

Building the application

Antibody oligo conjugates are specific to the intended protein targets for transcriptome and protein marker detection

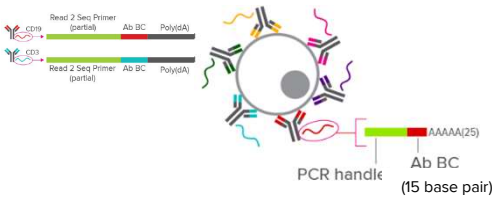


C1 REAP-seq workflow

1 Obtain barcoded antibodies.

2 Stain cells.

Preparation



3 Isolate antibody stained cells.

4 Image.

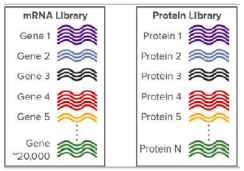
5 Perform REAP-seq chemistry followed by harvest.

C1 processing

6 Perform library preparation.

7 Sequence and analyze.

Data collection and analysis

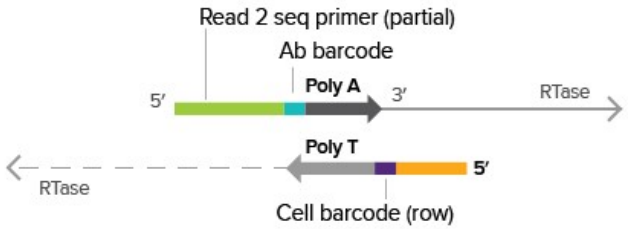


>500 bp ~193 bp

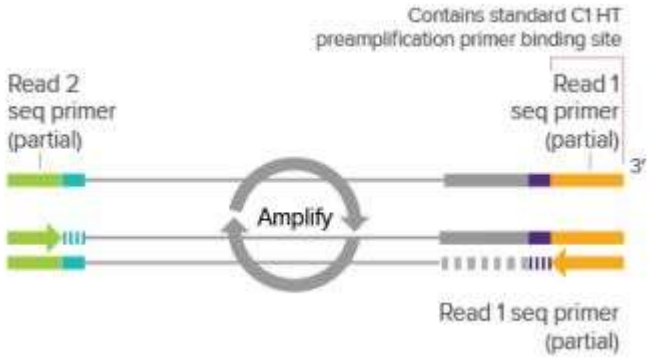
REAP-seq chemistry

Antibody barcode library prep

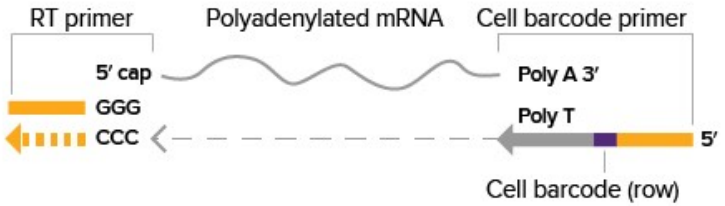
1. Reverse transcription

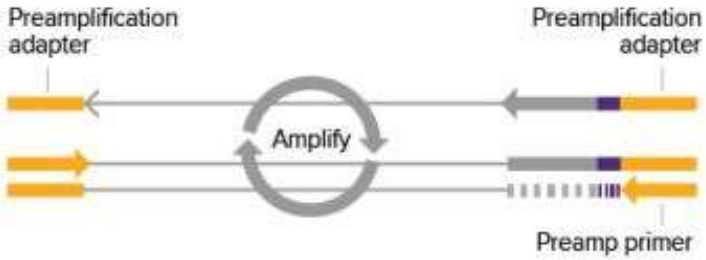


2. Preamplification



cDNA library prep



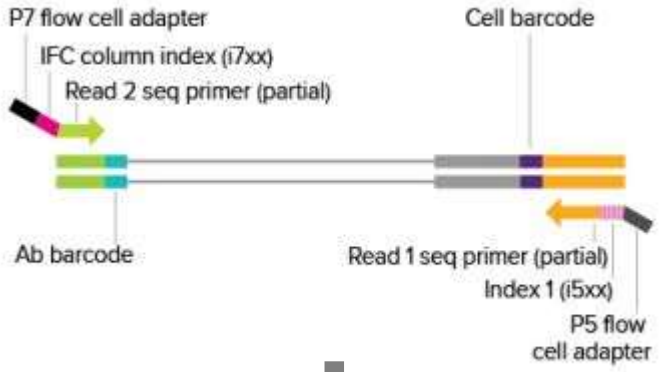


REAP-seq chemistry

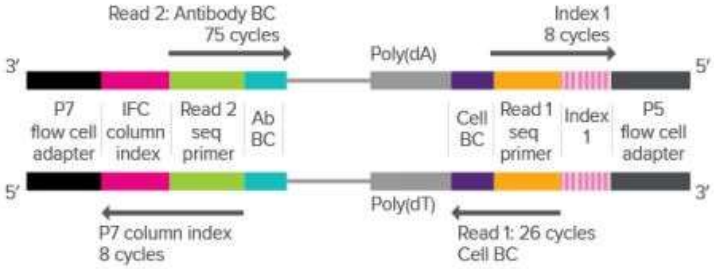
3. Harvest and clean up to separate and process cDNA and antibody (Ab) barcode (BC) library in parallel.

Antibody barcode library prep

Antibody library barcoding and adapter addition by PCR

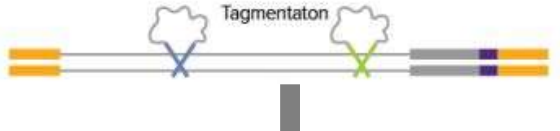


Final Ab BC library structure

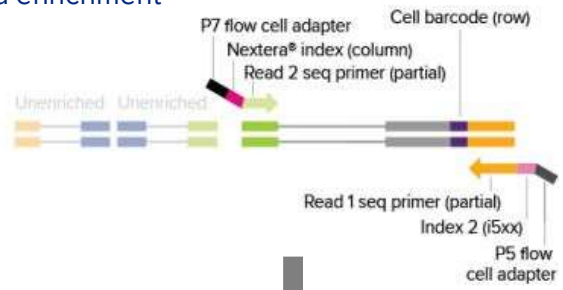


cDNA library prep

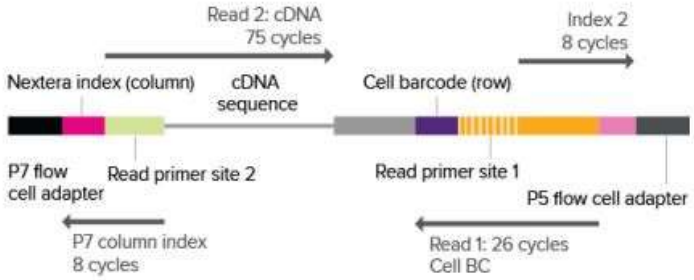
Library tagmentation



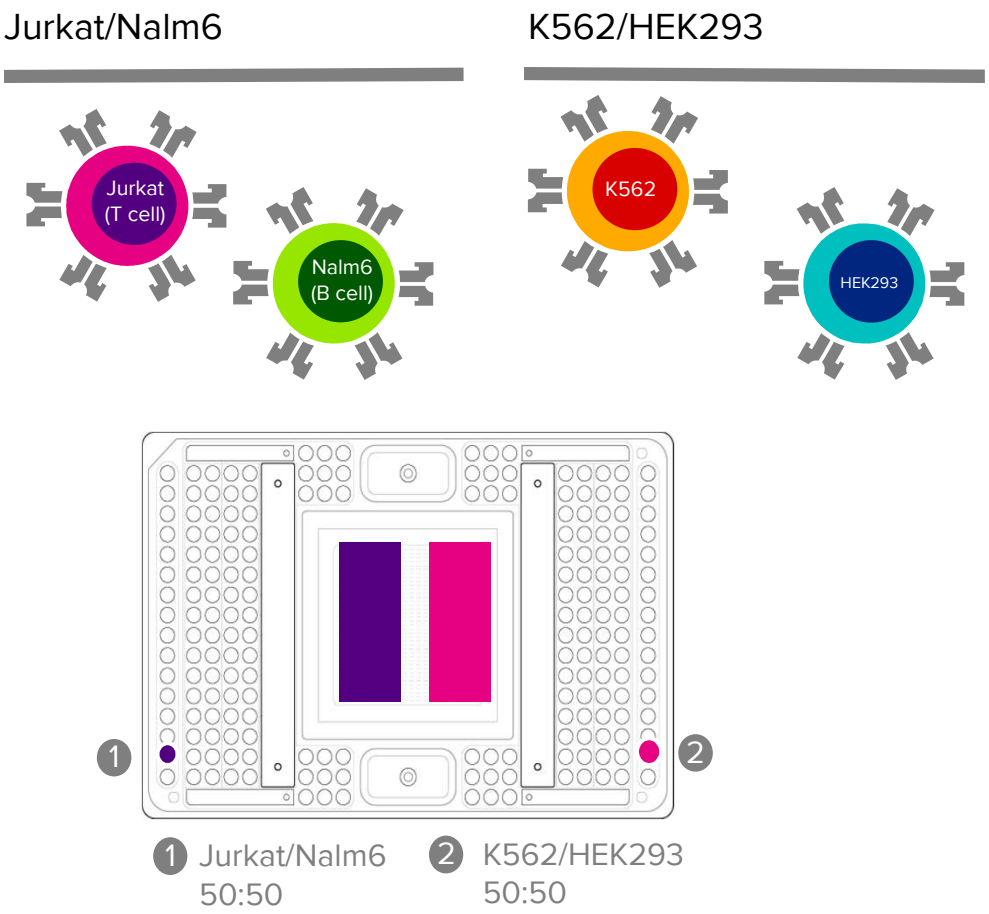
3′-end enrichment



Final cDNA library structure



Building the application



Antibody barcode

- 22 human Ab BCs targeting various protein markers are pooled before incubation.
- Well-characterized proteins of particular interest are CD10, CD19, CD3 and CD45RA.

Controls

- α-mouse and isotype controls to gauge nonspecific binding of antibody barcodes
- Performance monitoring in the K562/HEK293 quality control

Antibody barcode pool

Isotype Controls	Specificity	Clone	Reactivity	Barcode Sequence
Mouse IgG1, κ isotype Ctrl	N/A	MOPC-21	N/A	GCCGGACGACATTAA
Mouse IgG2a, κ isotype Ctrl	N/A	MOPC-173	N/A	CTCCTACCTAAACTG
Mouse IgG2b, κ isotype Ctrl	N/A	MPC-11	N/A	ATATGTATCACGCGA
Rat IgG1, κ Isotype Ctrl	N/A	RTK2071	N/A	ATCAGATGCCCTCAT
Rat IgG2a, κ Isotype Ctrl	N/A	RTK2758	N/A	AAGTCAGGTTCGTTT

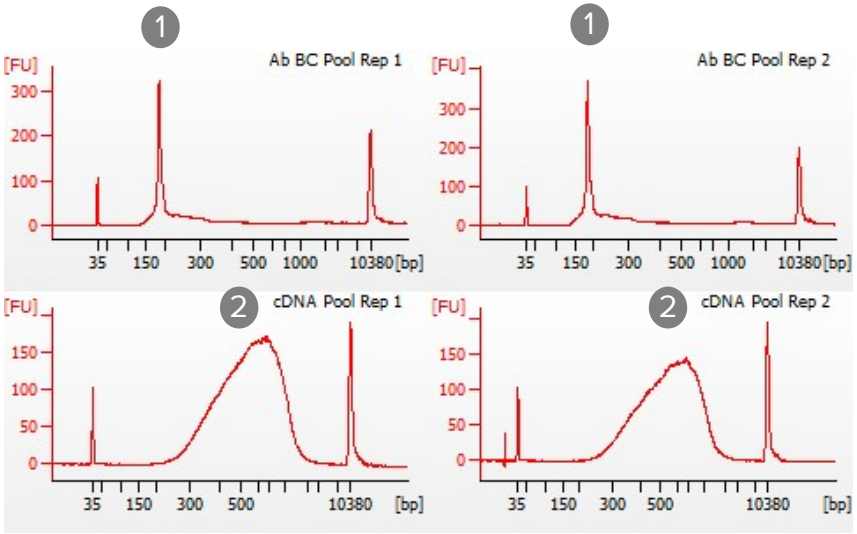
Mouse Antibodies	Specificity	Clone	Reactivity	Barcode Sequence
anti-mouse/rat CD29	CD29	HMβ1-1	Mouse, Rat	ACGCATTCCTTGTGT
anti-mouse CD24	CD24	M1/69	Mouse	TATATCTTTGCCGCA
anti-mouse CD106	CD106	429 (MVCAM.A)	Mouse	CGTTCCTACCTACCT
anti-mouse CD63	CD63	NVG-2	Mouse	ATCCGACACGTATTA
anti-mouse CD140a	CD140a	APA5	Mouse	GTCATTGCGGTCCTA

Human Antibodies	Specificity	Clone	Reactivity	Barcode Sequence
anti-human CD10	CD10	HI10a	Human	CAGCCATTCATTAGG
anti-human CD11c	CD11c	S-HCL-3	Human	TACGCCTATAACTTG
anti-human CD14	CD14	M5E2	Human	TCTCAGACCTCCGTA
anti-human CD16	CD16	3G8	Human	AAGTTCACTCTTTGC
anti-human CD19	CD19	HIB19	Human	CTGGGCAATTACTCG
anti-human CD195 (CCR5)	CD195 (CCR5)	J418F1	Human	CCAAAGTAAGAGCCA
anti-human CD197 (CCR7)	CD197 (CCR7)	G043H7	Human	AGTTCAGTCAACCGA
anti-human CD29	CD29	TS2/16	Human	GTATTCCCTCAGTCA
anti-human CD3	CD3	UCHT1	Human	CTCATTGTAACTCCT
anti-human CD34	CD34	581	Human	GCAGAAATCTCCCTT
anti-human CD4	CD4	RPA-T4	Human	TGTTCCCGCTCAACT
anti-human CD43	CD43	CD43-10G7	Human	GATTAACCAGCTCAT
anti-human CD45RA	CD45RA	HI100	Human	TCAATCCTTCCGCTT
anti-human CD45RO	CD45RO	UCHL1	Human	CTCCGAATCATGTTG
anti-human CD55	CD55	JS11	Human	GCTCATTACCCATTA
anti-human CD56 (NCAM) Recombinant	CD56 (NCAM) Recombinant	QA17A16	Human	TTCGCCGCATTGAGT
anti-human CD63	CD63	H5C6	Human	GAGATGTCTGCAACT
anti-human CD8A	CD8a	RPA-T8	Human	GCTGCGCTTTCCATT
anti-human CD9	CD9	HI9a	Human	GAGTCACCAATCTGC
anti-human CD95	CD95 (Fas)	DX2	Human	CCAGCTCATTAGAGC
anti-human CD98	CD98	MEM-108	Human	GCACCAACAGCCATT
anti-Human Podoplanin	Podoplanin	NC-08	Human	GGTTACTCGTTGTGT

Bioanalyzer quality control

cDNA and antibody barcode

- Determined quality and average base pair size of the cDNA and antibody barcode DNA-pooled libraries
- Pooled less than 20 columns from each IFC to increase reads per cell
- cDNA pool and antibody barcode curves look standard with average bp in the expected range.



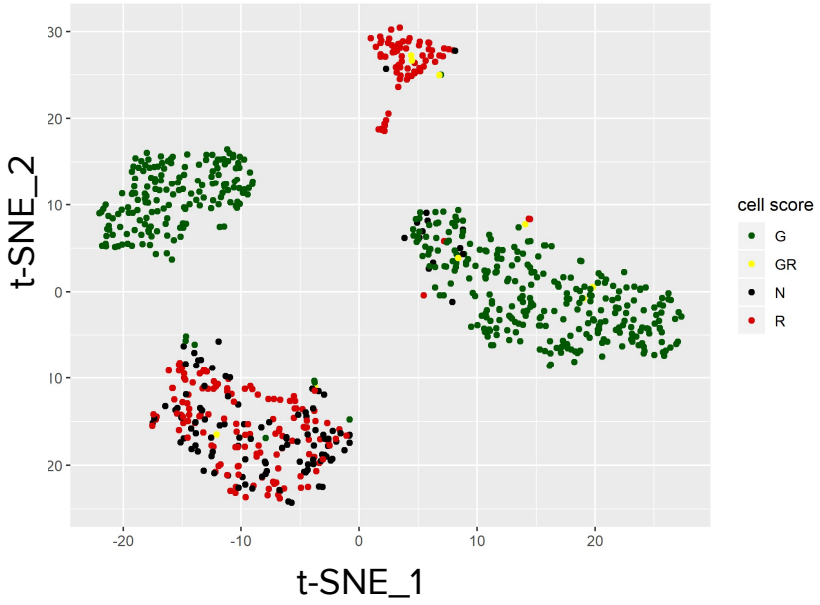
The average size of the antibody barcode pool was 186.8 bp and the average size of the cDNA pool was 602 bp.

1 Antibody peak 2 cDNA peak

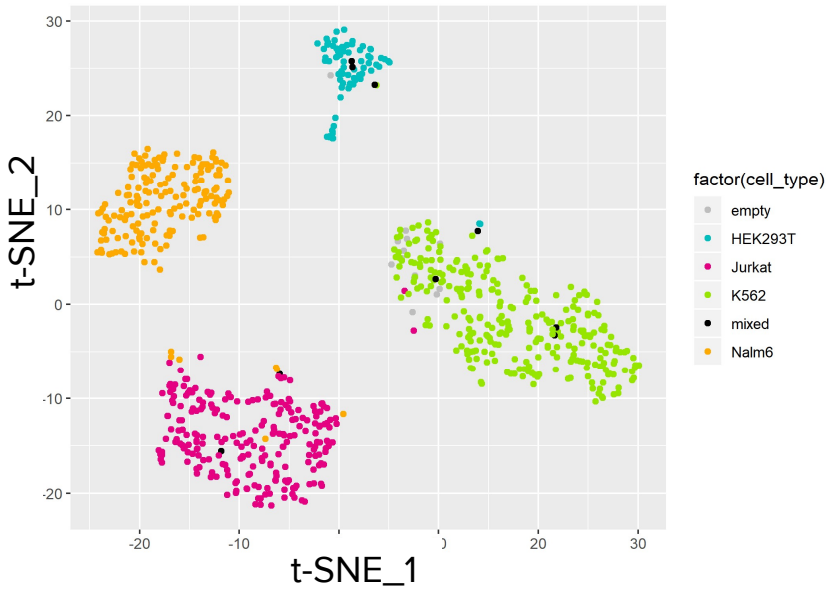
mRNA sequencing cluster analysis

Jurkat and Nalm6 cell mixing

Sample clustered by cell score

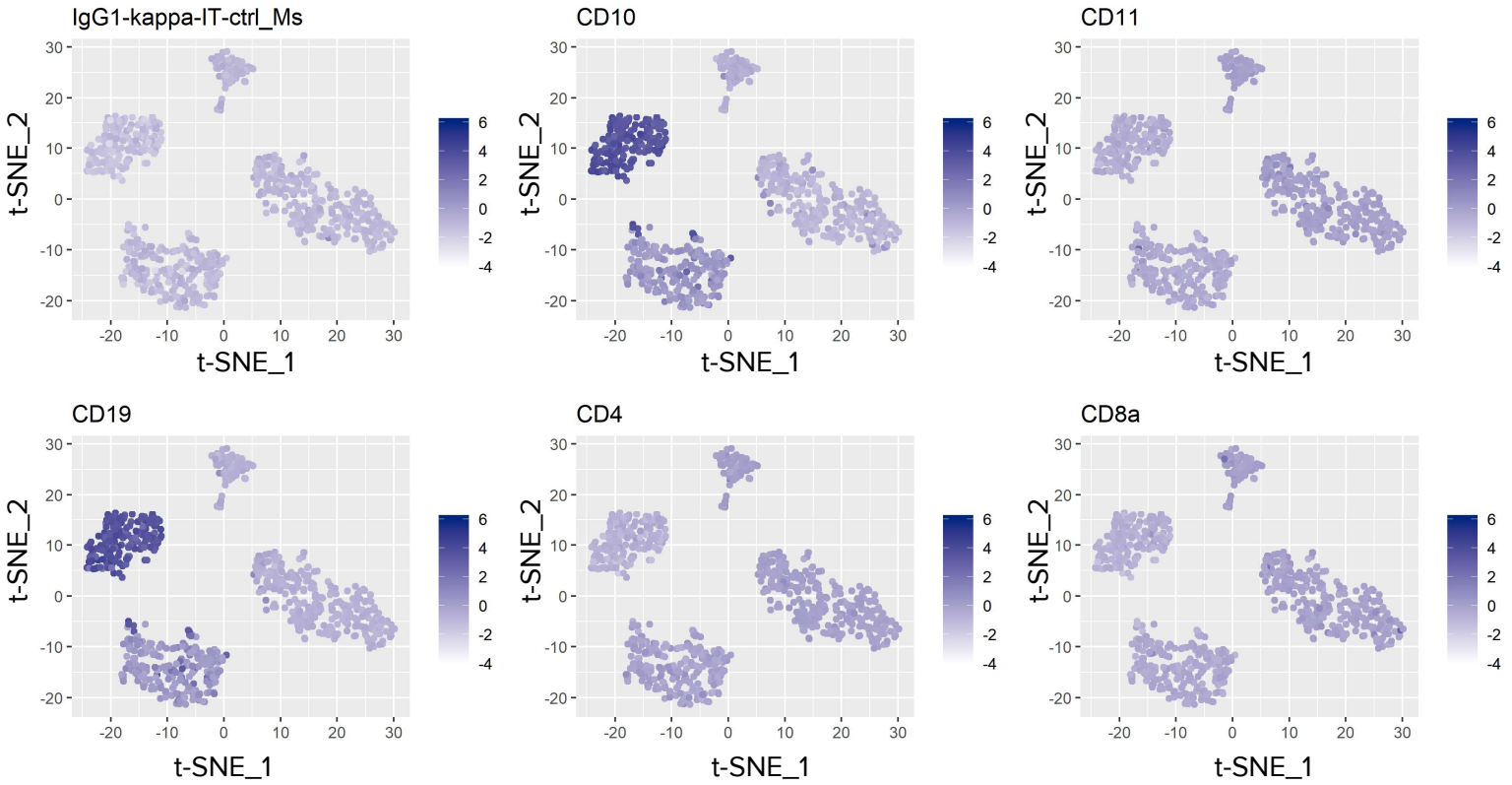


Sample clustered by cell type



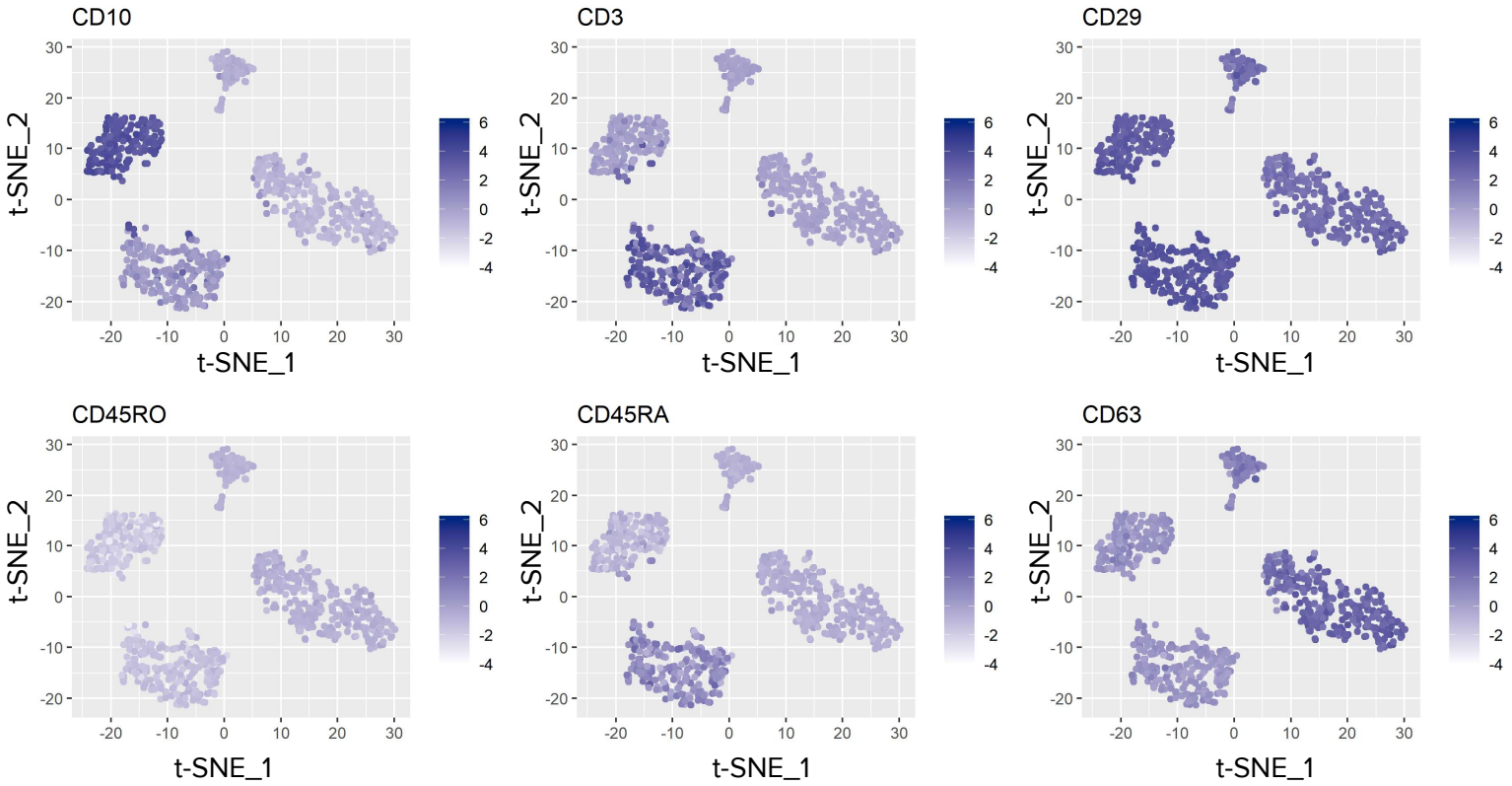
Samples cluster in concordance with cell sorting and cell type analysis, again illustrating good on-IFC performance for mRNA sequencing.

Protein sequencing cluster analysis



Samples cluster in concordance with cell sorting and cell type analysis, again illustrating good on-IFC performance for protein sequencing.

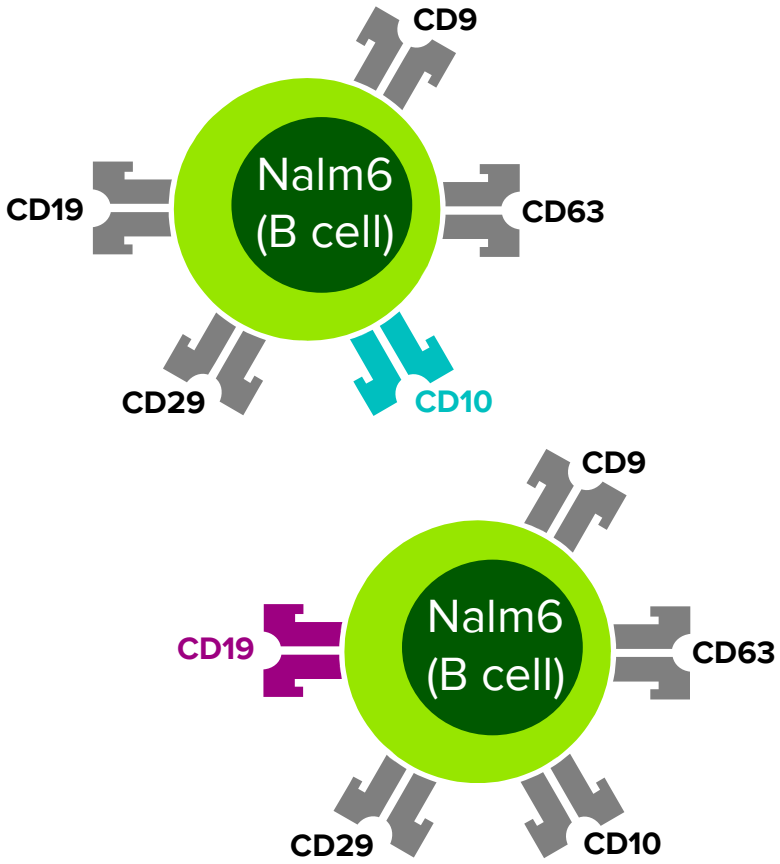
Protein sequencing cluster analysis



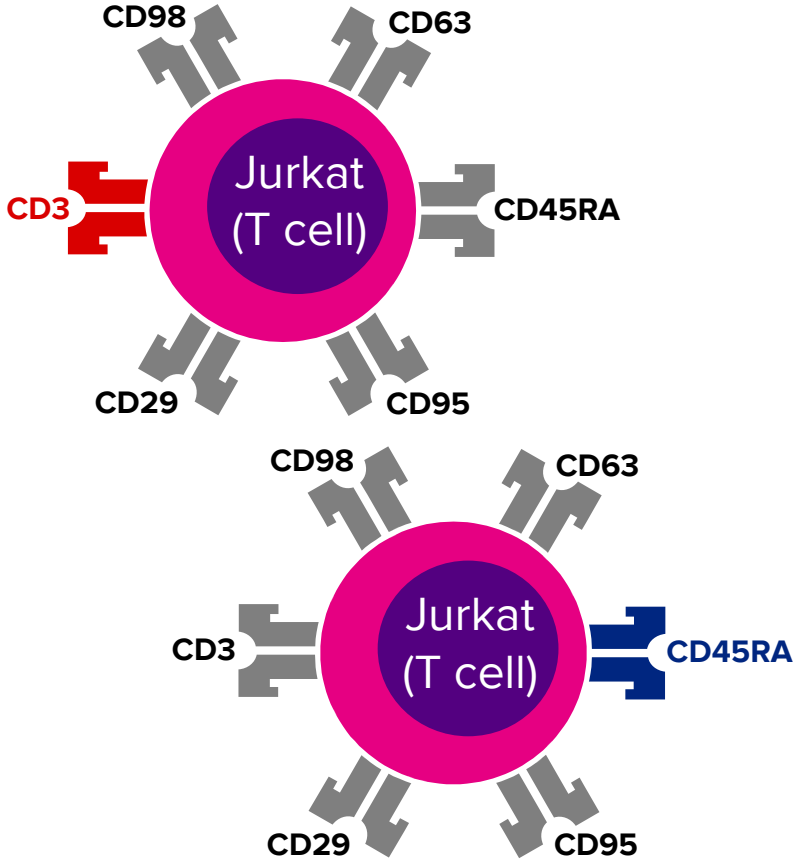
Samples cluster in concordance with cell sorting and cell type analysis, again illustrating good on-IFC performance for protein sequencing.

Qualifying protein expression specificity

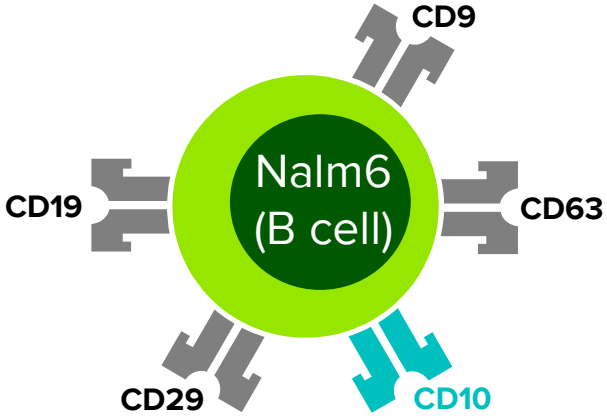
CD10 and CD19 on Nalm6

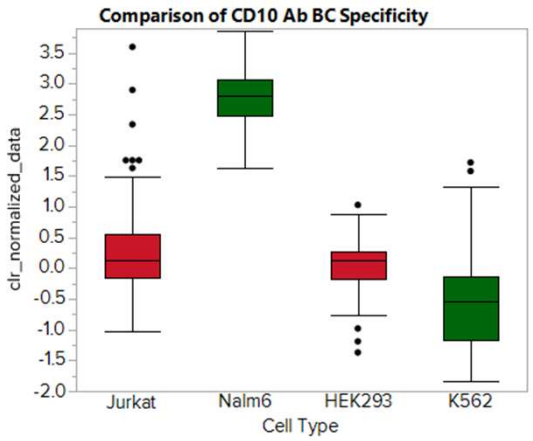


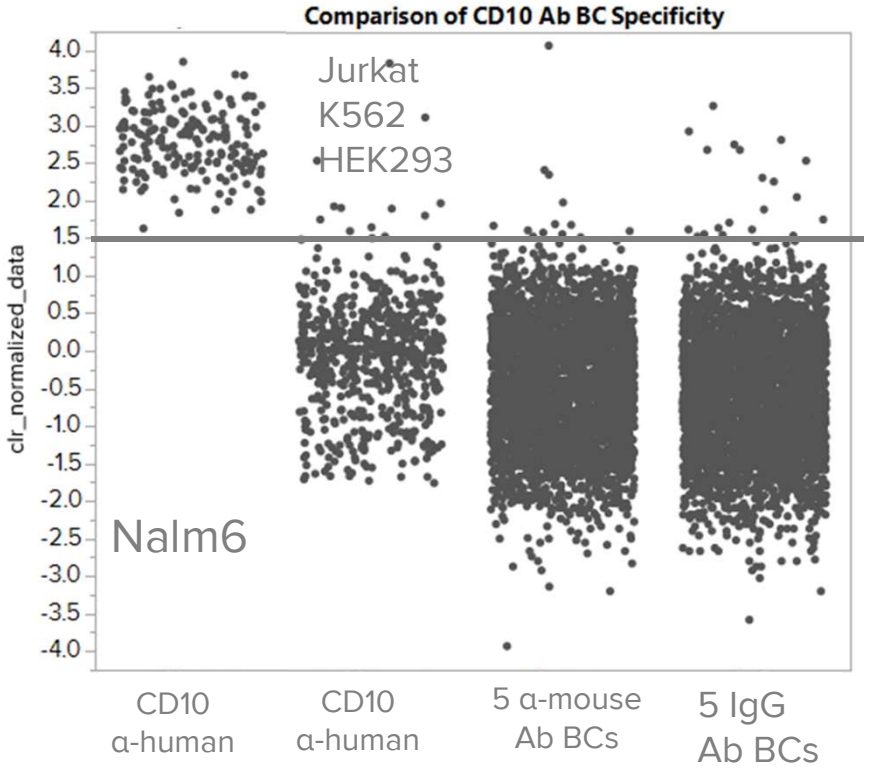
CD3 and CD45RA on Jurkat



CD10 protein expression and specificity

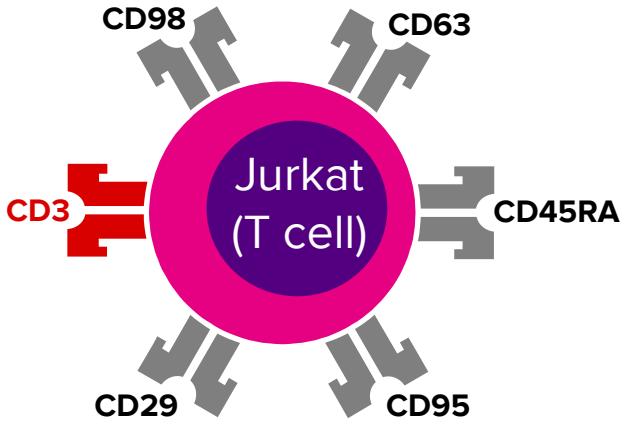


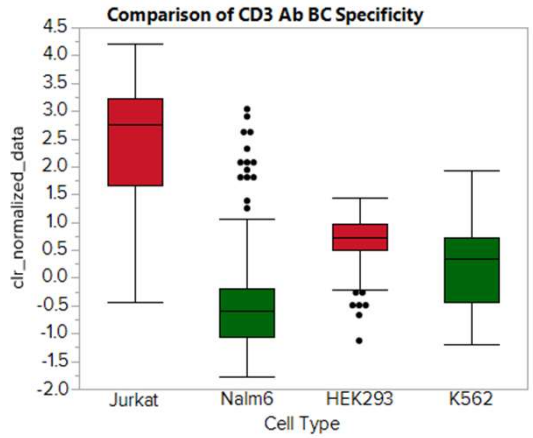


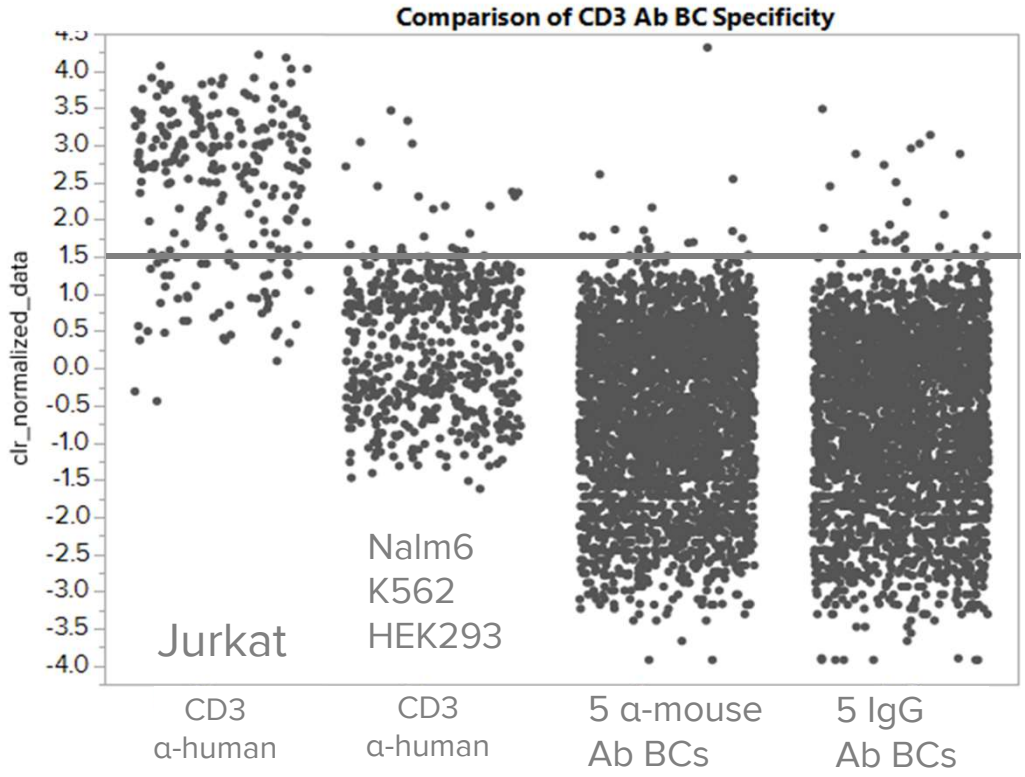


CD10 antibody barcode is expected to be specific to Nalm6. Comparing Jurkat, K562 and HEK293, we demonstrate that CD10 reads occur in Nalm6.

CD3 protein expression and specificity



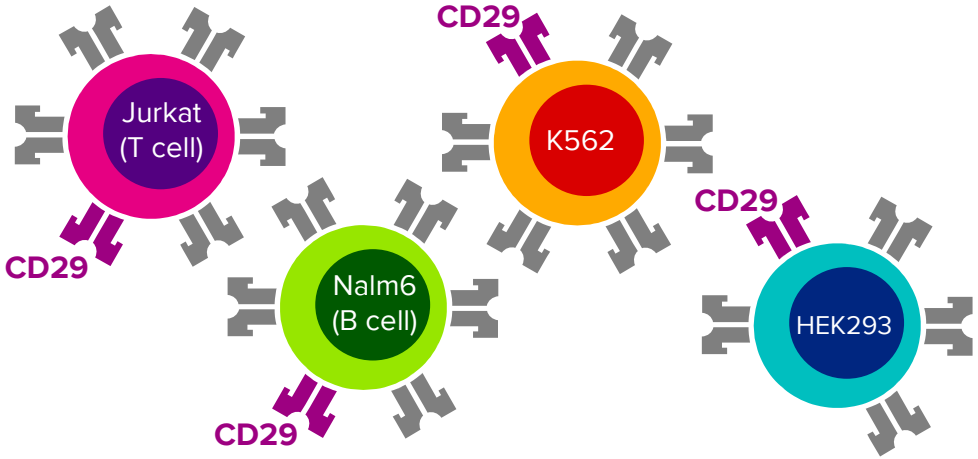


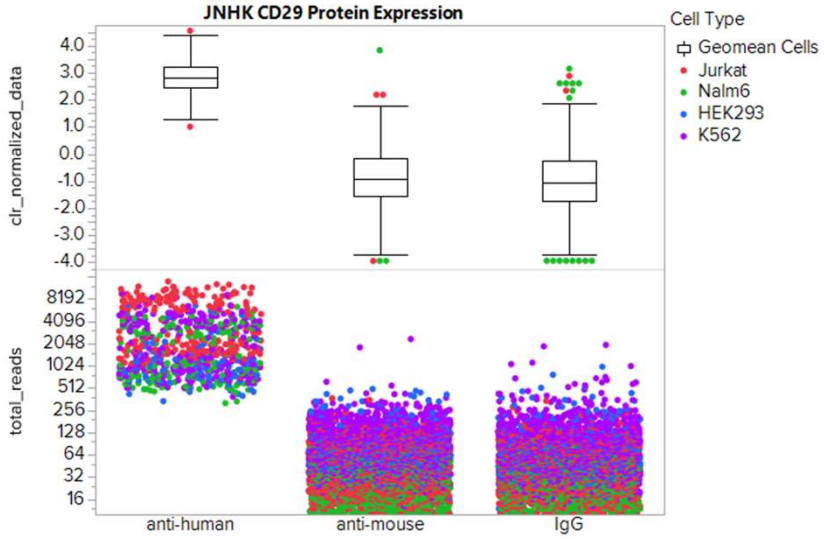


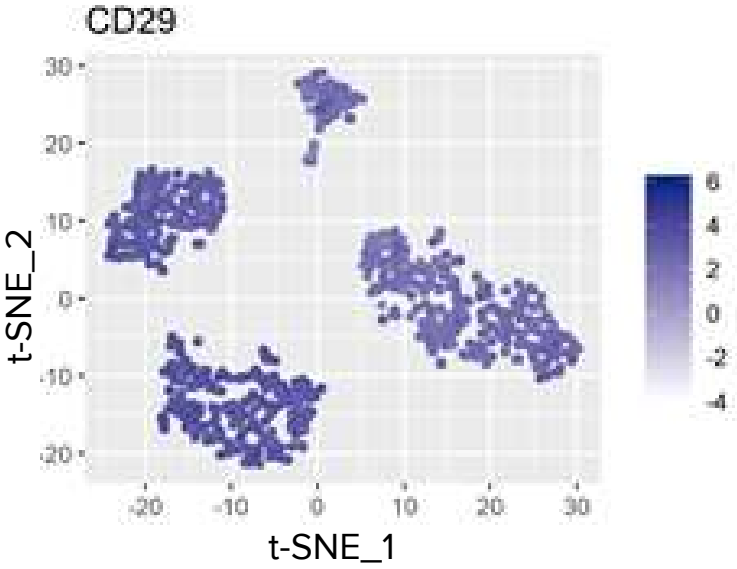
CD3 antibody barcode is expected to be specific to Jurkat. Comparing Jurkat, K562 and HEK293, we demonstrate that CD3 reads occur in Jurkat.

Protein expression results

CD29 co-expression total reads







CD29 was expressed in all cell lines as predicted. Expression of CD29 between all four cell lines was higher than the number of reads from α-mouse and IgG Ab BC controls.

REAP-seq
Summary

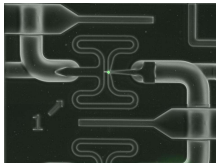
- Antibody barcode specificity used with C1 REAP-seq is shown to be very specific to the intended targets.
- Highly characterized proteins such as CD10, CD19, CD3 and CD45RA were differentially expressed by the appropriate cells as shown by clustering, flow cytometry and box plots.
- Total CD10, CD19, CD3 and CD45A reads were above the number of reads produced by α-mouse and IgG control antibody barcodes, as shown by the dot plots.

Download the C1 REAP-seq technical note today.

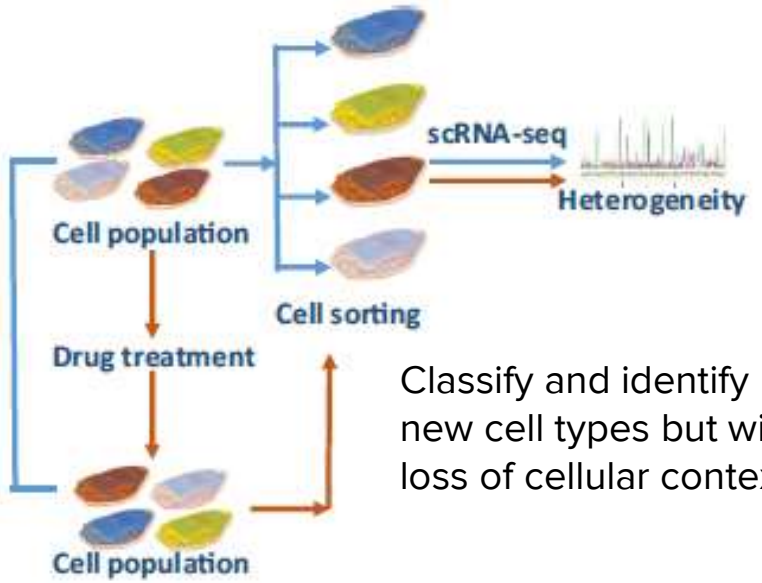
Creating a single-cell microenvironment

Single-cell omics and multi-omics

Single-cell processing

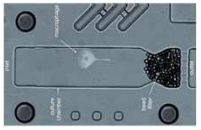


Single-cell omics and multi-omics

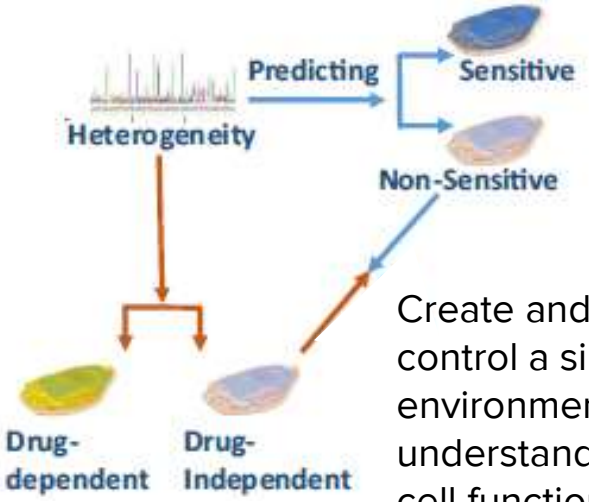


Classify and identify new cell types but with loss of cellular context.

Single-cell functional analysis



Single-cell microenvironment



Create and control a single-cell environment to understand single-cell function.

Modified from Wang et al., *Cell Biology and Toxicology* (2018)

Single-cell functional analysis

Microenvironment and single-cell interaction

Enable

- Single-cell culture with time course dosing, imaging and molecular readout
- Single-cell contextual analysis

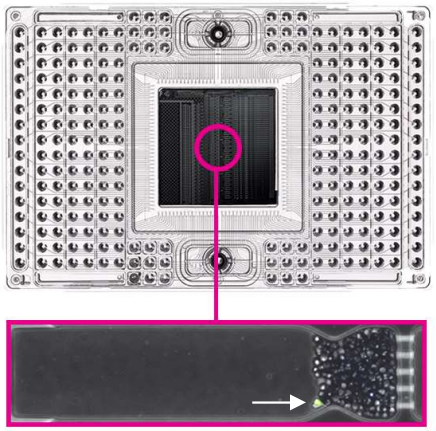
Discover

- Molecular mechanisms
- Drug efficacy and toxicity
- The correlation of single-cell mRNA sequencing with cell heterogeneity, response, interaction and phenotype using drug combinations

Cell suspension

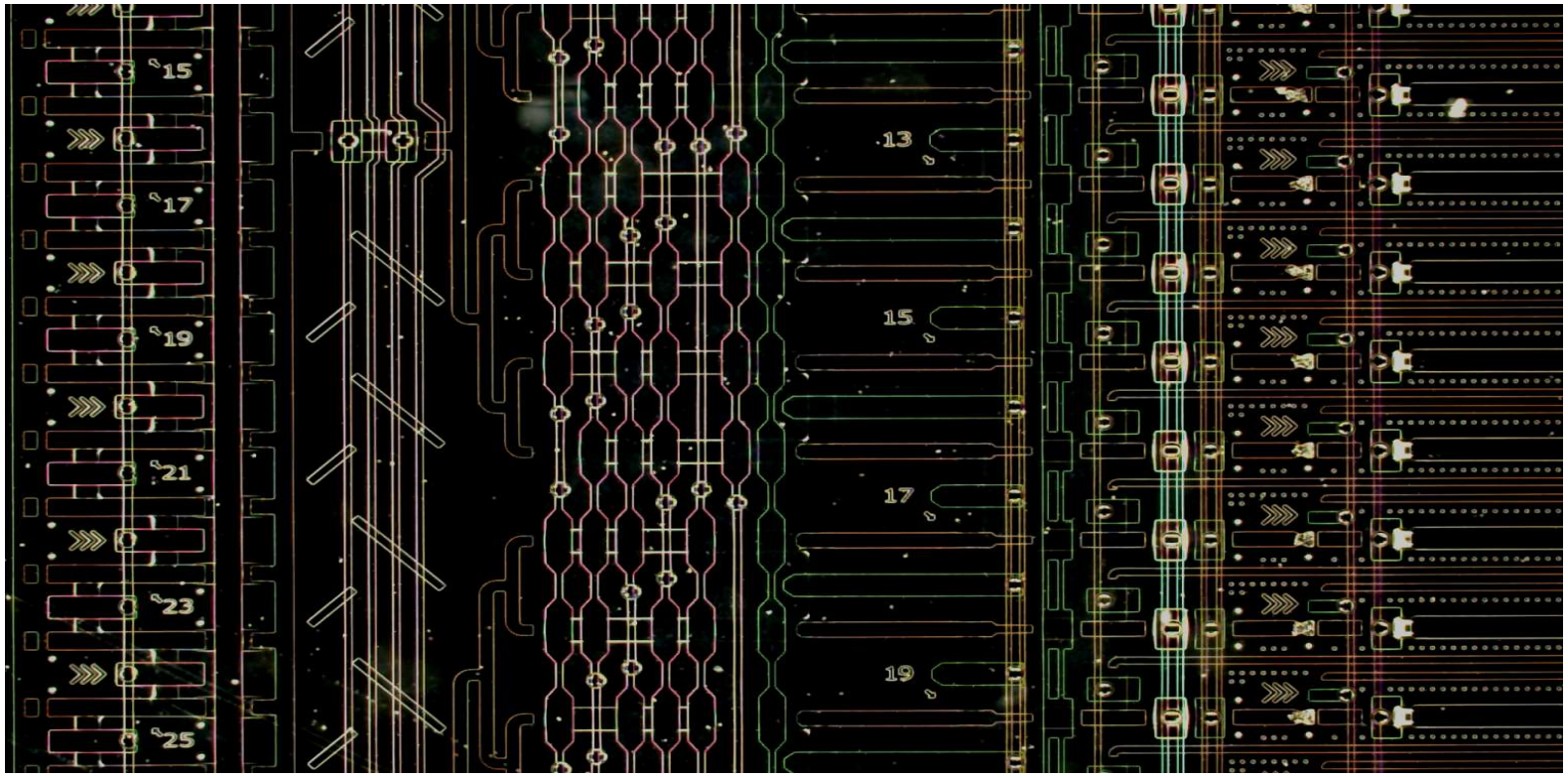
Polaris system

cDNA



Polaris IFC

Active single-cell selection and treatment correlating phenotype to molecular readout



64 single-cell partitions

Selection and dosing path

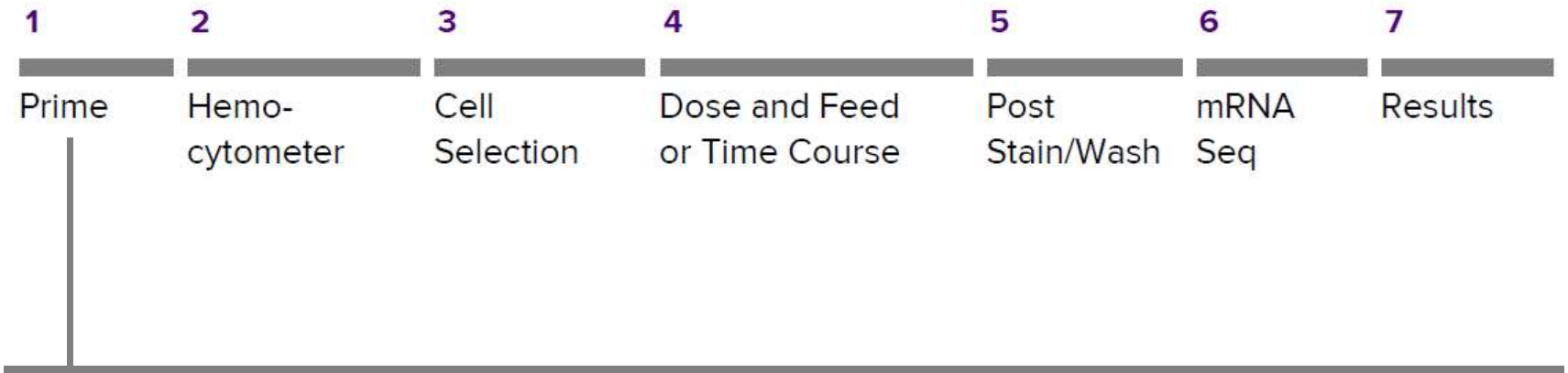
Cell culture chambers

Chemistry

Micrograph (4X magnification) of the cell selection, capture and culture/dose sections of the Polaris IFC.

Polaris workflow

Prime



- Prepares the control lines for microfluidic control
- User prepares chambers for culturing conditions:

  Suspension: No extracellular matrix (ECM) deposited on culture chambers

  Adherent: Deposit ECM on culture chambers (for example, fibronectin, 25 ng/µL)

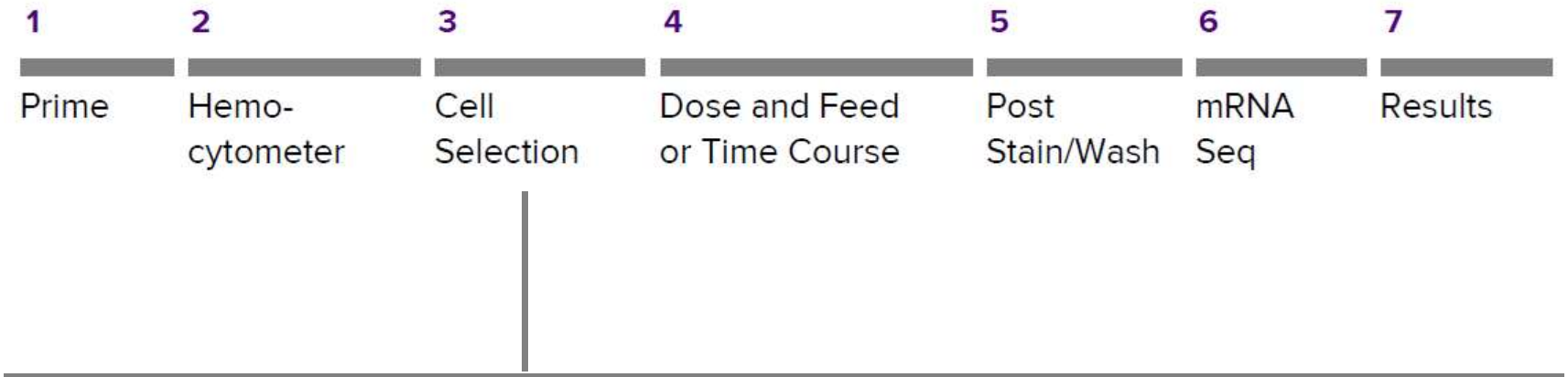
Prime

Extracellular matrices tested on Polaris IFCs

- Fibronectin
- Laminin
- Vitronectin
- Collagen
- Untreated PDMS

Polaris workflow

Cell Selection



- Select 48 target single cells (3% to 100% of total cell population) across 3 fluorescence channels.
- Two-population selection feature allows for study of two samples: control vs. treatment study design, etc.
- Low cell input numbers needed. Start from an input of 300 to 8,000 cells per inlet (5 inlets/25 µL total).
- Size-independent selection of desired cells based on fluorescent markers
- Gentle path to capture chamber suits delicate cells

Example of cell types tested

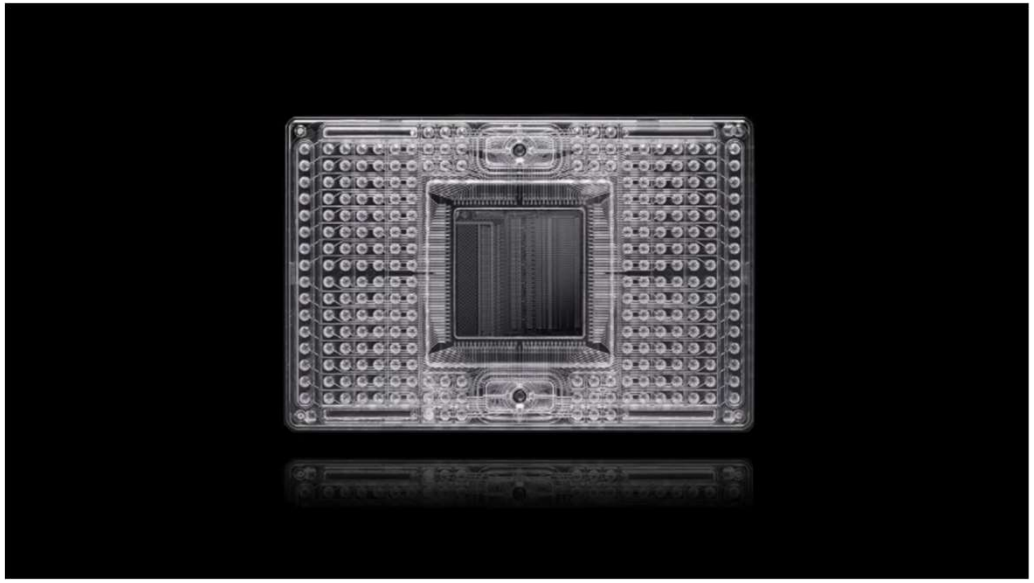
Cell Selection

Cell Type	Suspension/Adherent
A431	Adherent
A549	Adherent
BJs	Adherent
Neurons	Adherent
PC3	Adherent
Basophils	Suspension
CTCs	Suspension
HL-60	Suspension
K562	Suspension
PBMC	Suspension
T cells	Suspension
CD34+	Suspension

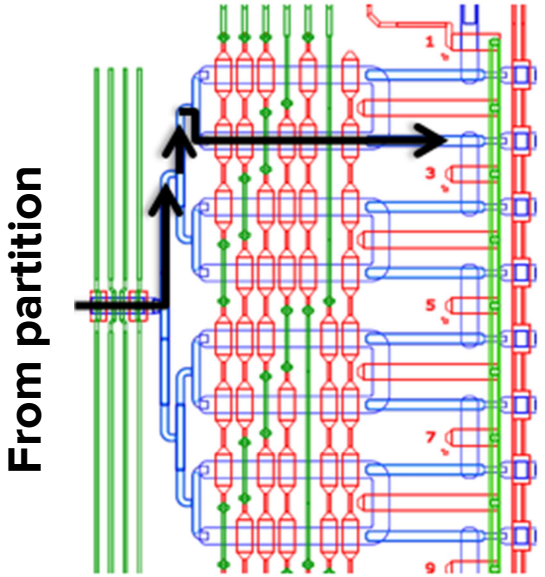
Single-cell selection

Cells in partition during flow

Cells in partition when flow stops



Qualified single cells redirected to capture site



Cells flowed through multiplexer to capture site.

Imaging confirms arrival and viability of single cell.

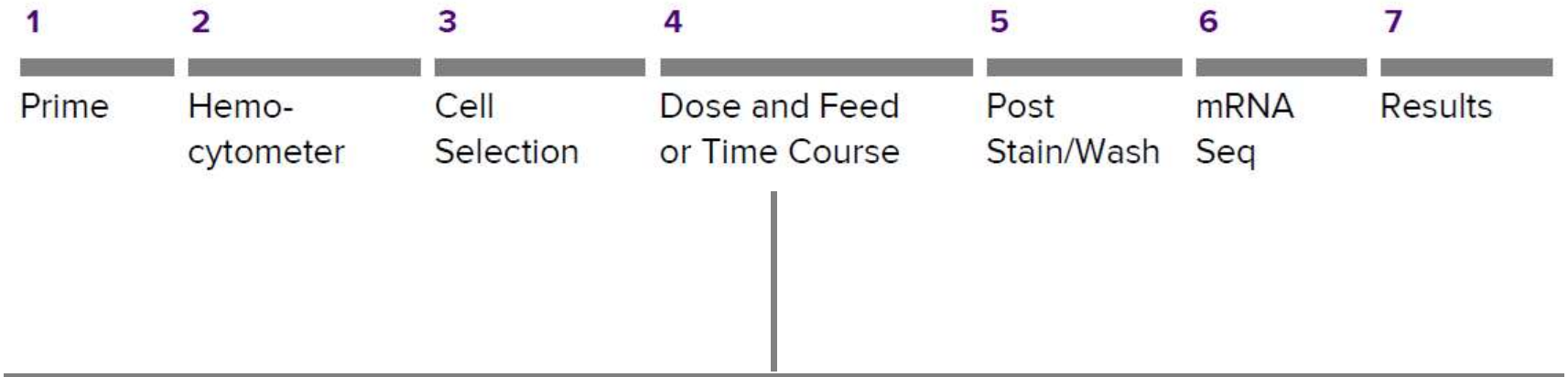
Single-cell selection

Supported wavelengths

Channel Name	Excitation (nm)	Emission (nm)	Suggested Purpose
Zombie	438/28	570/30	Viability assessment (used in Post Stain only)
FAM™	475/40	525/25	Selection marker
VIC®	530/20	570/30	Universal marker
Cy5®	632/28	700/30	Selection marker

Polaris workflow

Dose and Feed or Time Course



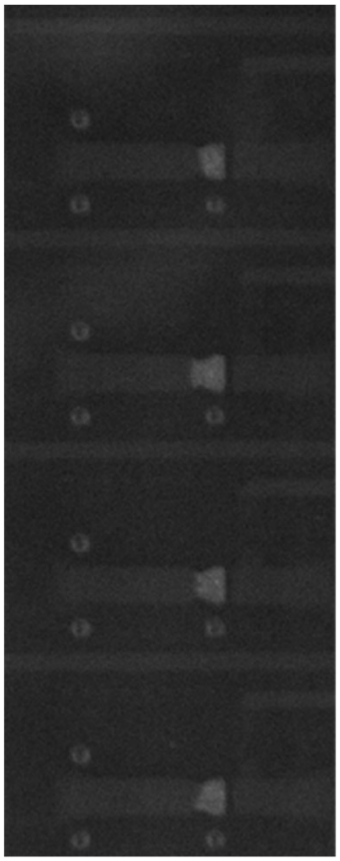
The following options are presented at this step:

- **Dose and Feed:** Different reagent groups treated at the same time points
- **Time Course:** Same reagent treated for all cells at different time points
- **No Treatment:** Skip treatment of single cells and proceed straight to chemistry.

Single-cell culture

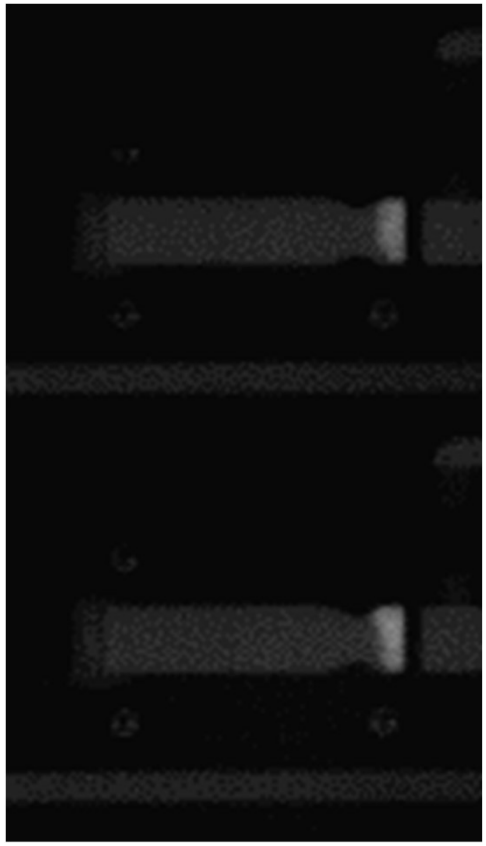
Dose and Feed

- Flexible dosing routines to expose cells to a variety of extracellular compounds
- Up to 8 treatment groups of 6 single cells in each group
- All cells dosed at same times with a maximum duration of 24 hours
- Fluorescence imaging every hour



Single-cell culture

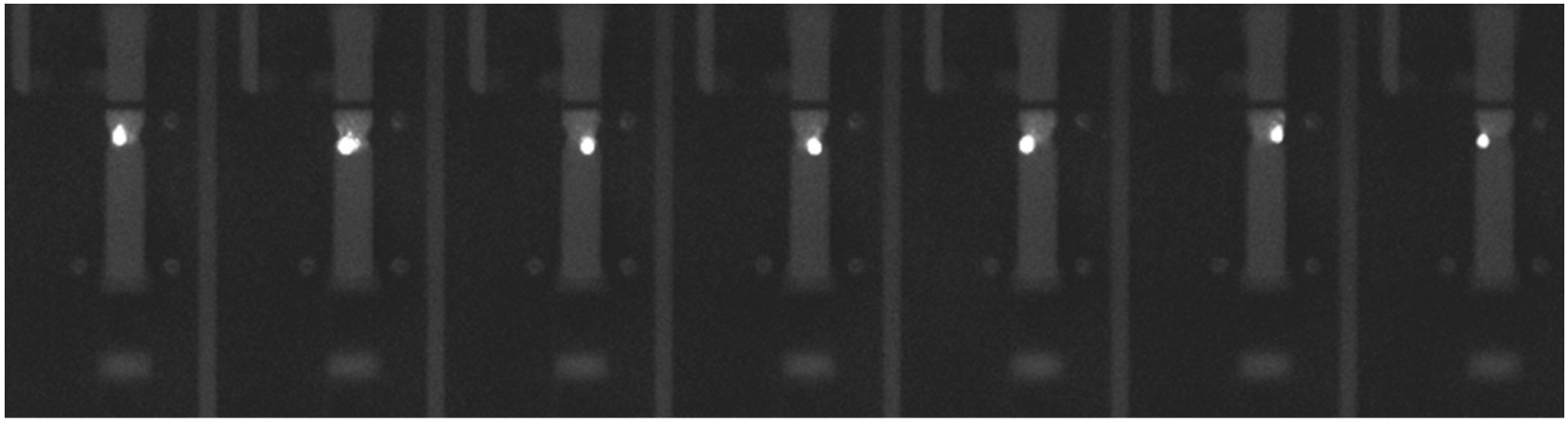
Dose and Feed



K562 cells transfected with
nGFP mRNA (>15 hr)

Single-cell culture
Time Course

- Same reagent for all cells, provided at different time points
- Fixed at 6 groups of 8 cells per time point with a maximum duration of 24 hours
- Fluorescence imaging every hour

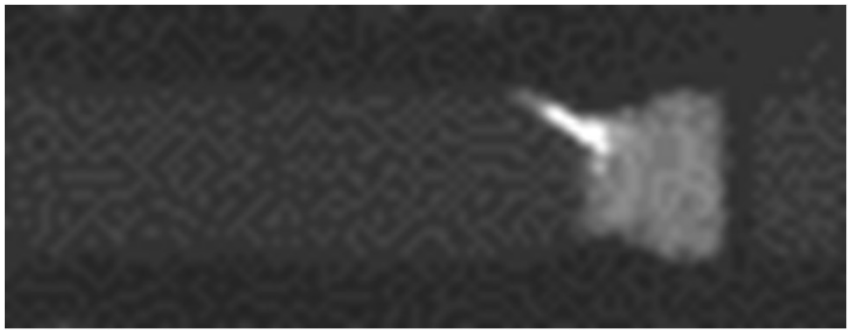


19 hr of feeding (K562)

Single-cell culture

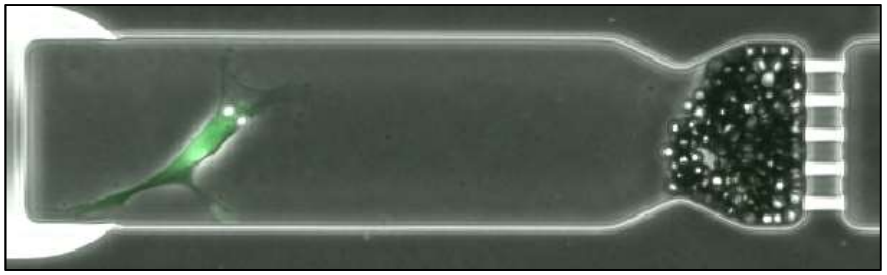
Cell adheres, spreads and migrates with time

Selected BJ fibroblast



Capture site imaged on Polaris to confirm placement

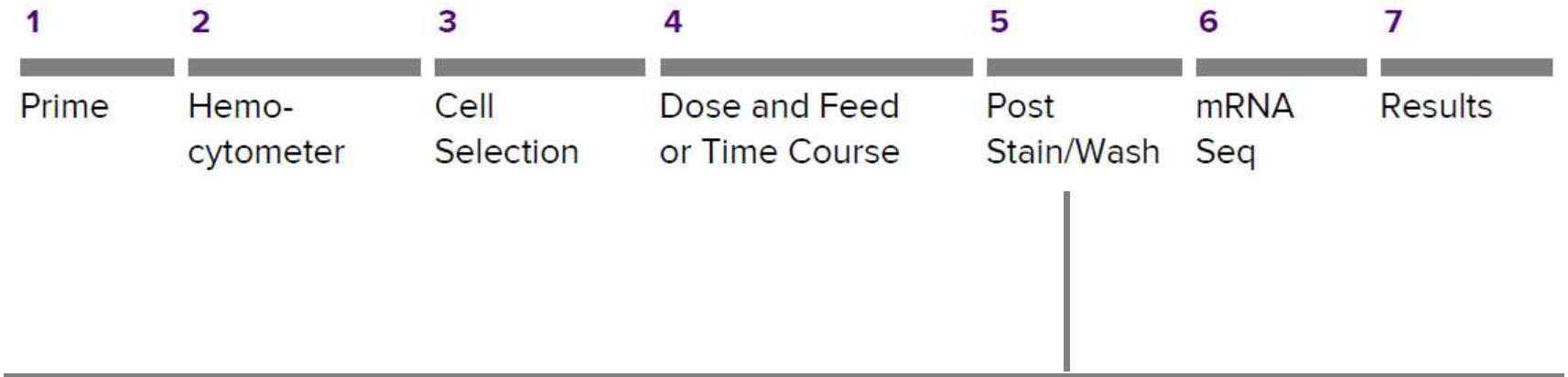
After 20 hours in culture



20X high-resolution image on inverted microscope

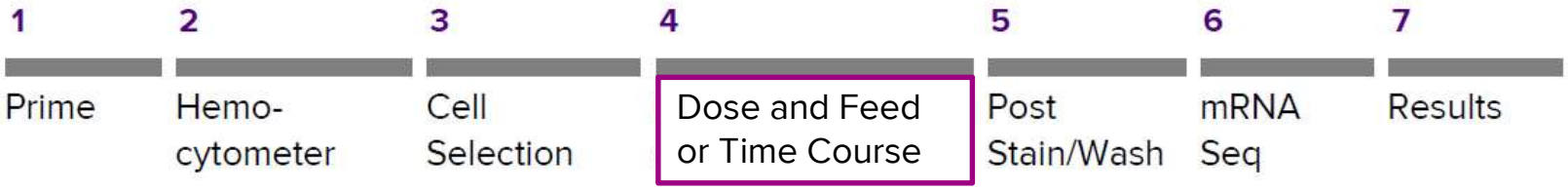
Polaris workflow

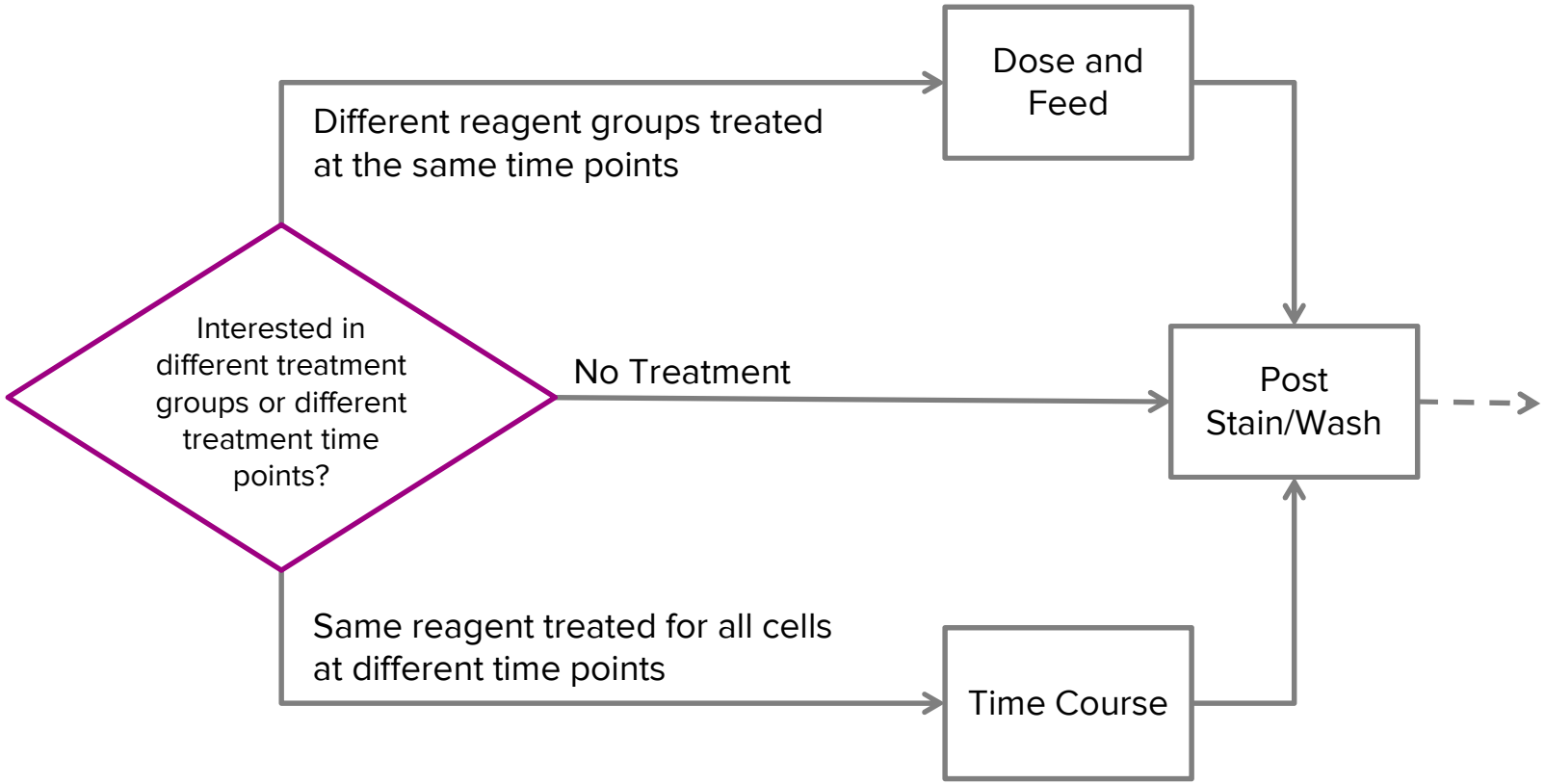
Post Stain/Wash



- Provides the option to assess viability with staining (for example, Zombie stain) before initiating chemistry
- Default incubation time is 10 minutes and maximum incubation time is 1 hour.
- Images recorded before and after the incubation period.

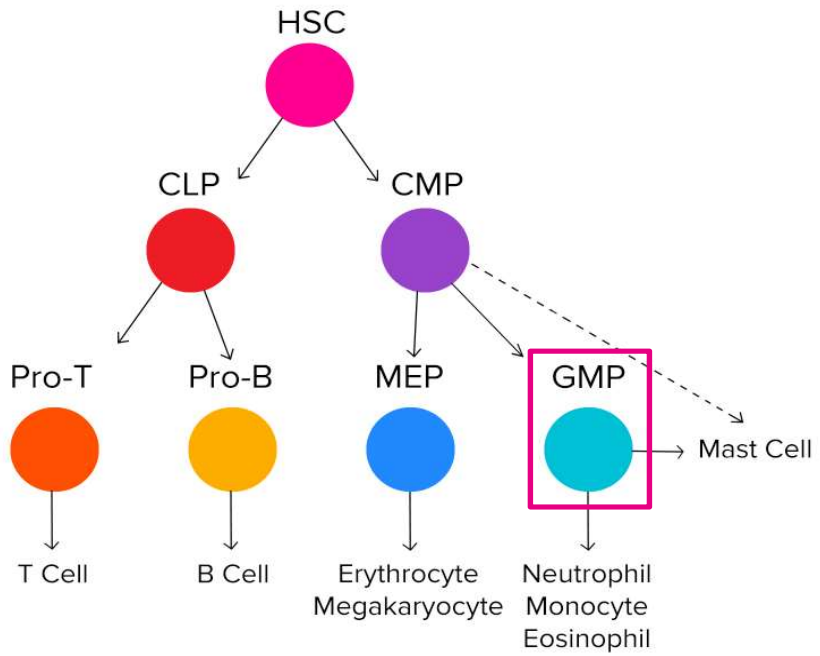
Workflow options





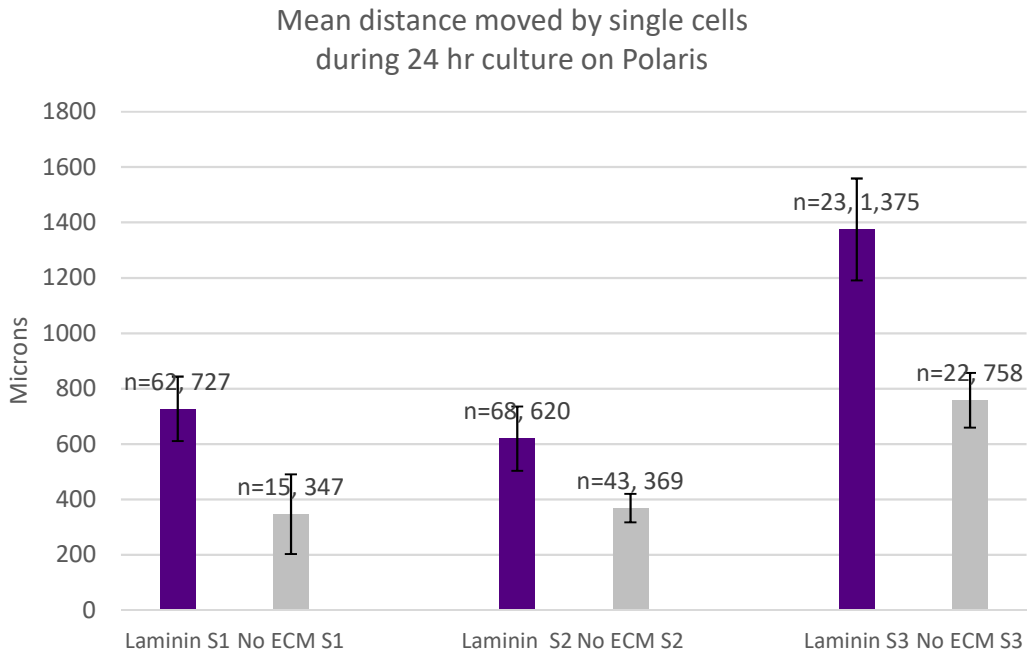
Polaris enables study of extracellular matrix effect on hematopoietic stem cells (HSCs)

- Granulocyte-macrophage progenitors (GMP) are a subset of CD34+ hematopoietic stem/progenitor cells (HSPCs).
- Polaris single-cell culture allows evaluation of **ECM's effect on CD34+ cells**, without intercellular signaling interference.
- Polaris features enable study of **migratory phenotype and differential gene expression** following mRNA-seq chemistry.

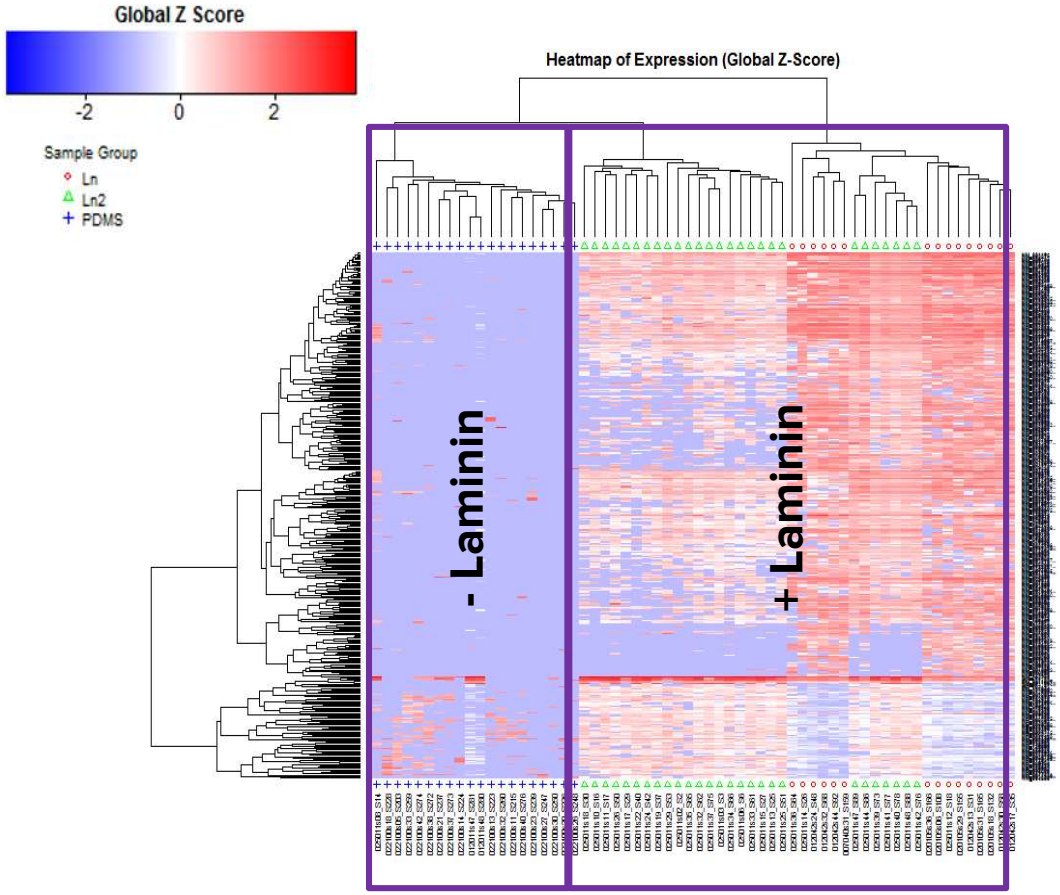


Polaris imaging revealed that laminin increases motility of CD34+ HSCs

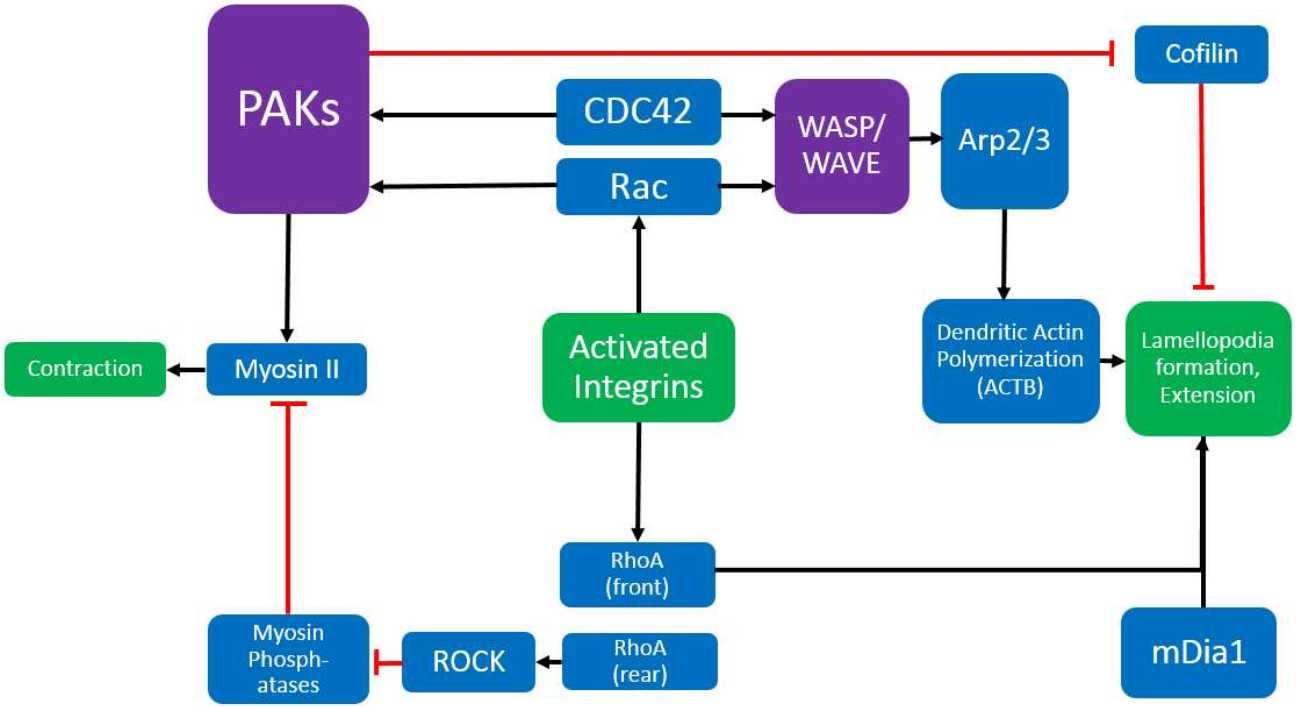
Relative to untreated culture chambers, motility increased ~2-fold on laminin (24-hour single-cell culture on Polaris with imaging).



Polaris mRNA Seq chemistry reveals that motility was integrin-driven



Gene expression outlines motility triggered by integrin via small Rho GTPases



Differentially expressed between Ln and PDMS
Not observed in this dataset
Events related to motility

Polaris enables study of activity responses to agonist application on neural stem cells

RESEARCH ARTICLE

‘Multimodal Single-Cell Analysis Reveals Physiological Maturation in the Developing Human Neocortex’

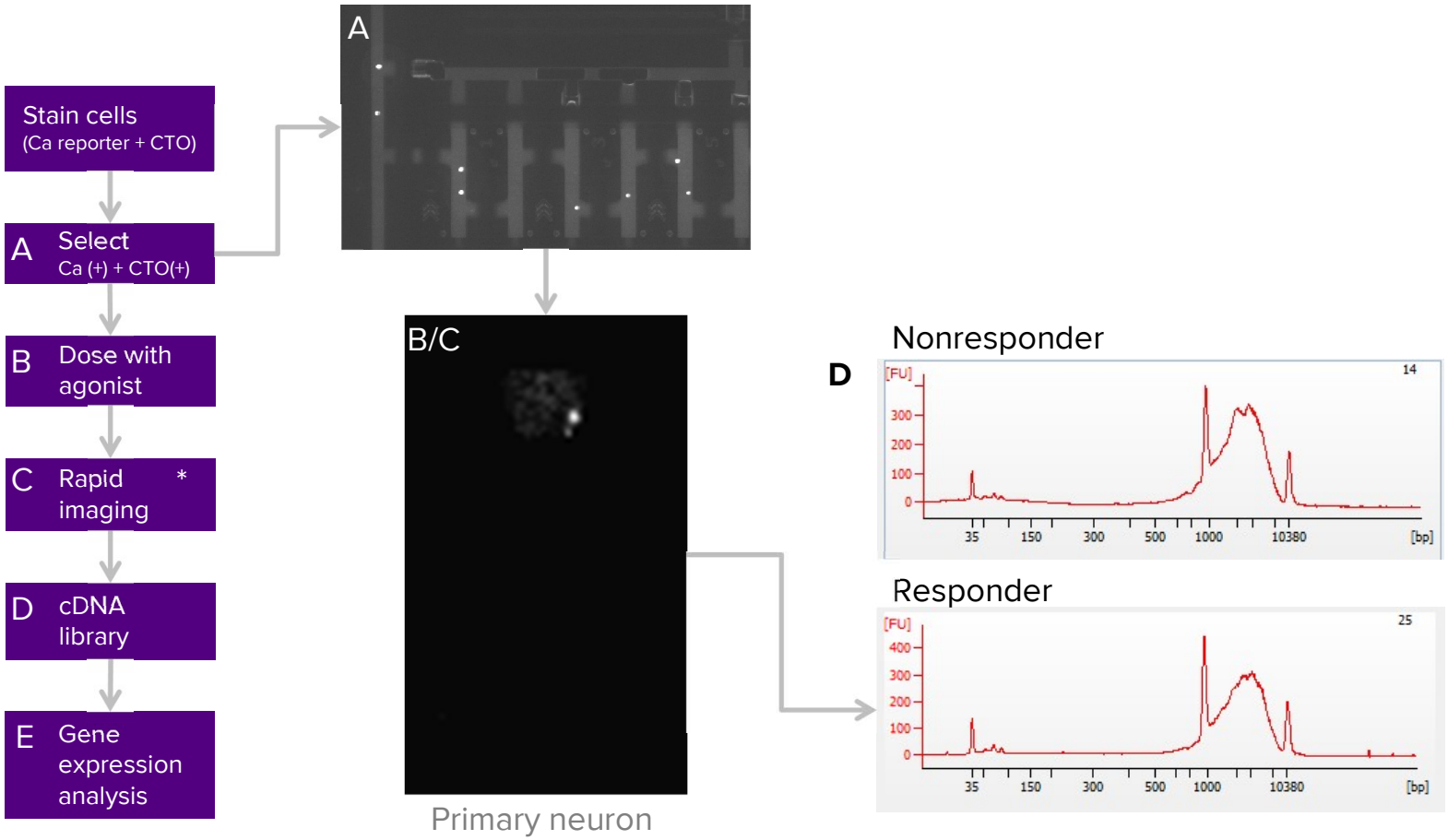
Mayer, S., Chen, J., Velmeshev, D., Mayer, A., Eze, U.C., Bhaduri, A, Cunha, C.E., Jung, D., Arjun, A., Li, E, Alvardo, B., Wang, S., Lovegren, N., Gonzales, M.L., Szpankowski, L., Leyrat, A., West, J.AA., Panagiotakos, G., Alvarez-Buylla, A., Paredes, M.F., Nowakowski, T.J., Pollen., A.A., Kriegstein, A.R

Neuron 102 (2019): 143–158.e7

Polaris enables study of activity responses to agonist application on neural stem cells

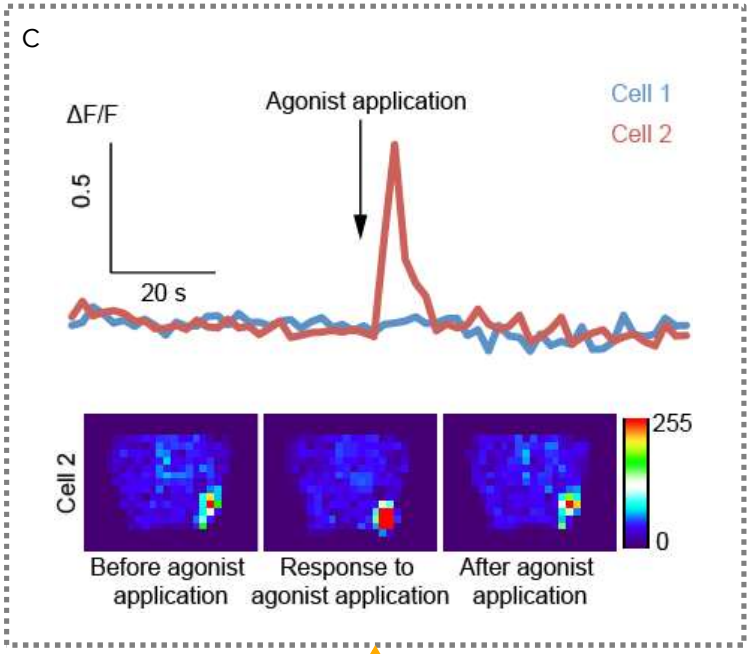
- Embryonic neural stem cells give rise to functioning neurons.
- Development of neural stem cells depends in part on secreted molecules.
- Polaris single-cell isolation allows evaluation of **agonist-specific neural responses**, without confounding extracellular signals.
- Polaris enables study of **neural activity and differential gene expression** following mRNA Seq chemistry.

Polaris dosing applied short-acting agonist on primary neural cells



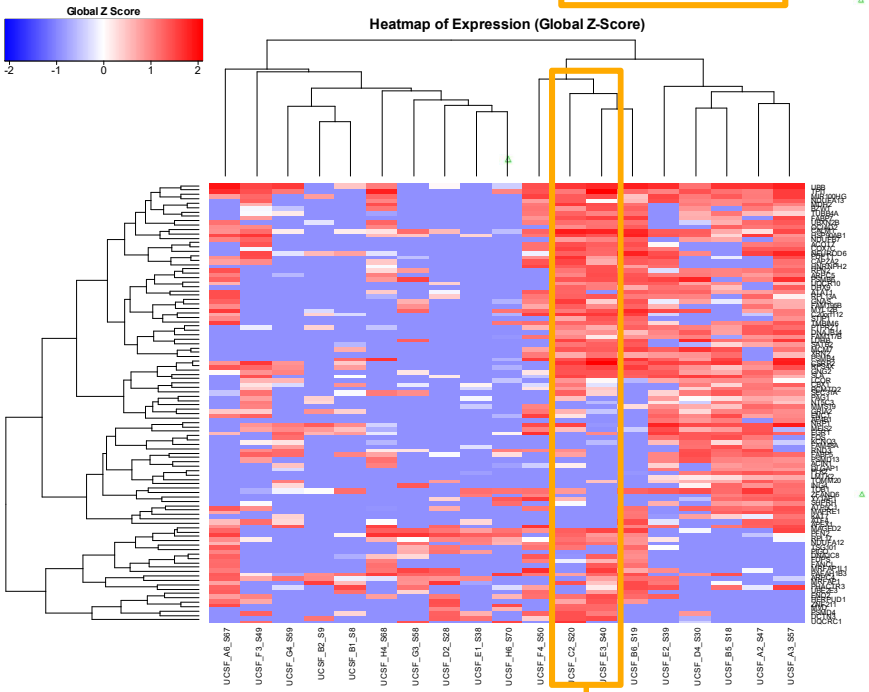
Short-acting agonist dosing of primary neural cells

Differential expression is coordinated blinking cell



Top 100 genes

Responders



Use gene expression data to improve selection of responders based on novel biomarkers.

Summary

Single-cell functional analysis with Polaris

Cell selection:

- Low cell input numbers
- Size-independent selection of desired cells based on fluorescent markers
- Gentle path to capture chamber (great for delicate cells)

Short-term culture (24 hr):

- Suspension or adherent culture on extracellular matrix of choice
- Control of microenvironment

Dosing: Use addressable fluidics to deliver dosing reagents.

***In situ* assays:** Determine the functional response of single cells.

Single-cell genomics: Generate mRNA-seq libraries.

Thank you.

For Research Use Only. Not for use in diagnostic procedures.