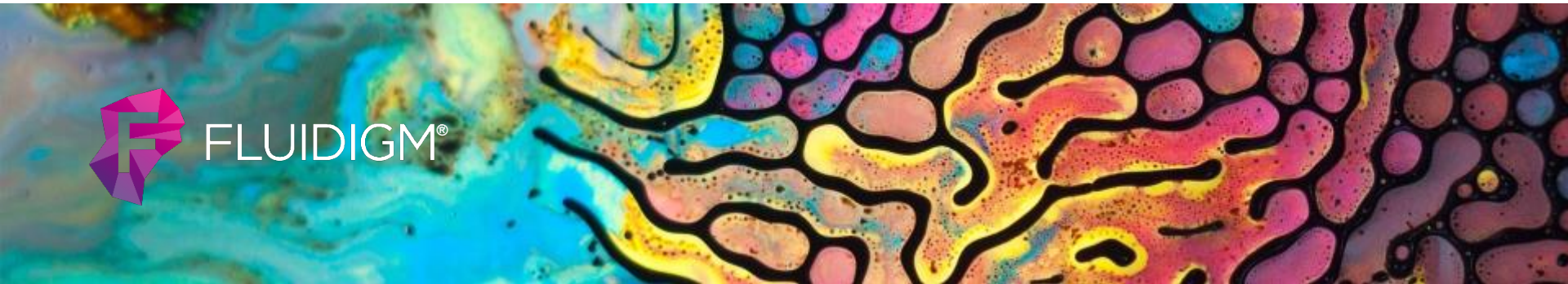
Single-Cell Stranded Total RNA Sequencing



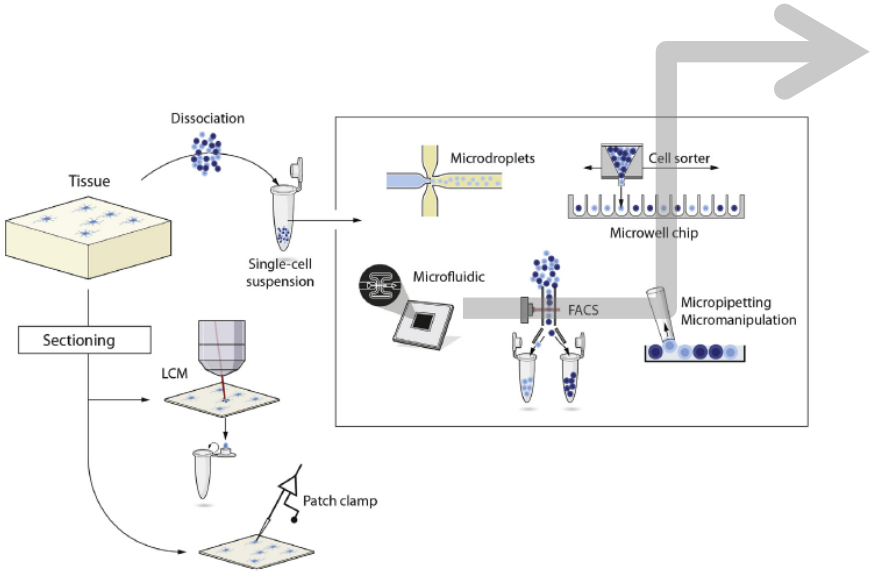
Agenda

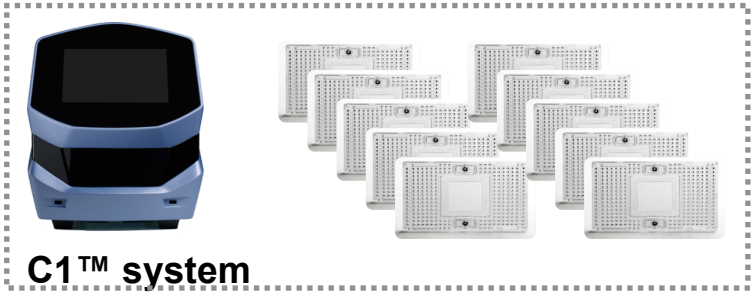
1. Fluidigm technology overview
2. C1 full-length mRNA-seq chemistry complements droplet approaches.
3. C1 supports a flexible array of applications.
4. Total RNA-Seq on C1 enables analysis of nonpoly(A) and noncoding RNA.

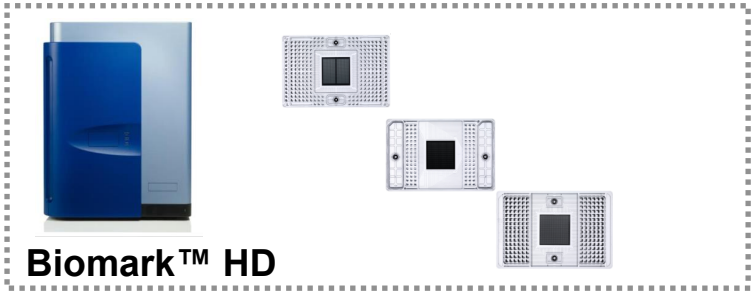
Technology overview

Microfluidics

To capture, verify, perturb, and process individual cells



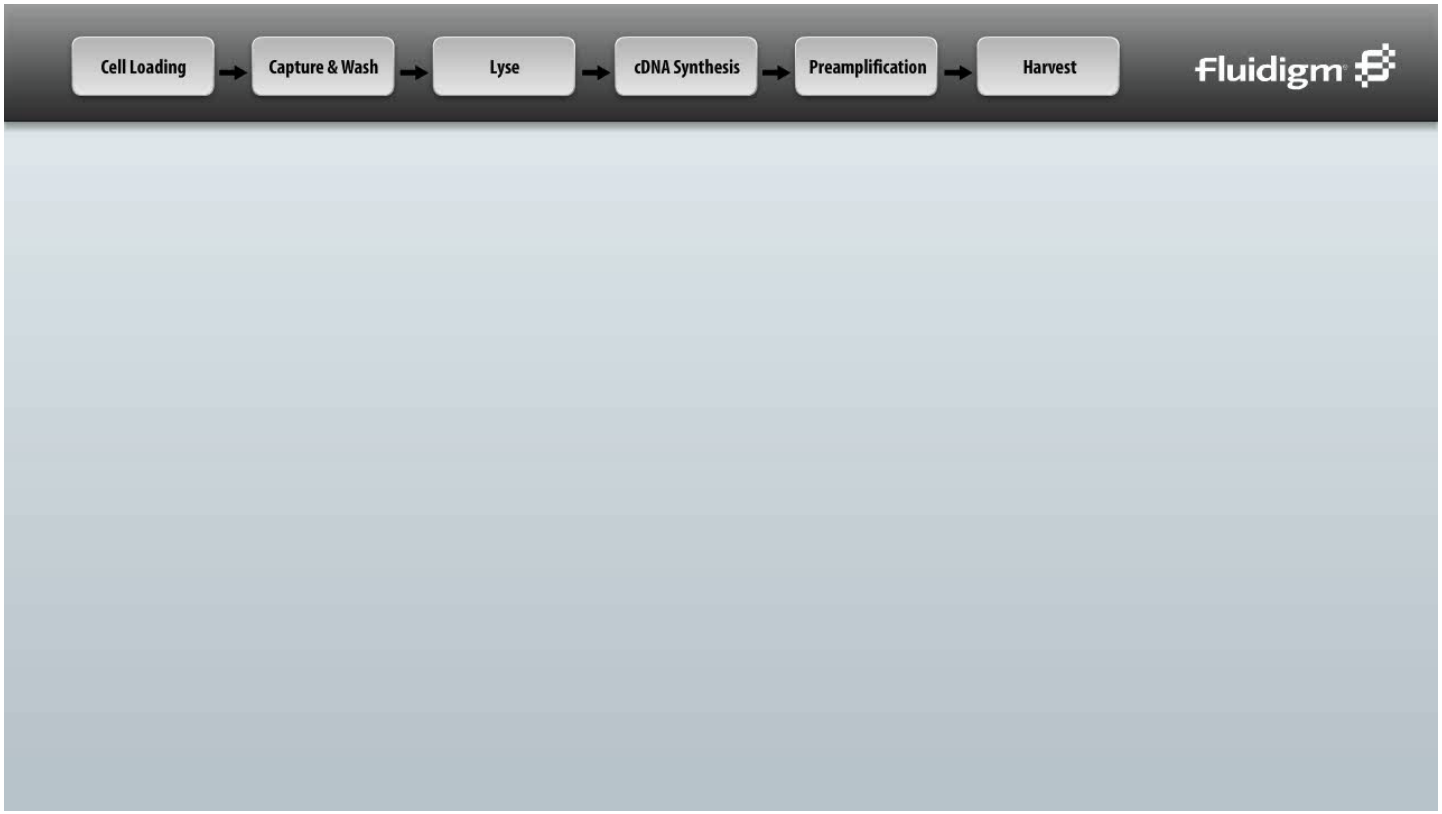




Modified from Hedlund and Deng,
Molecular Aspects of Medicine (2017)

Microfluidics for single-cell genomics

C1 system with integrated fluidic circuits (IFCs)



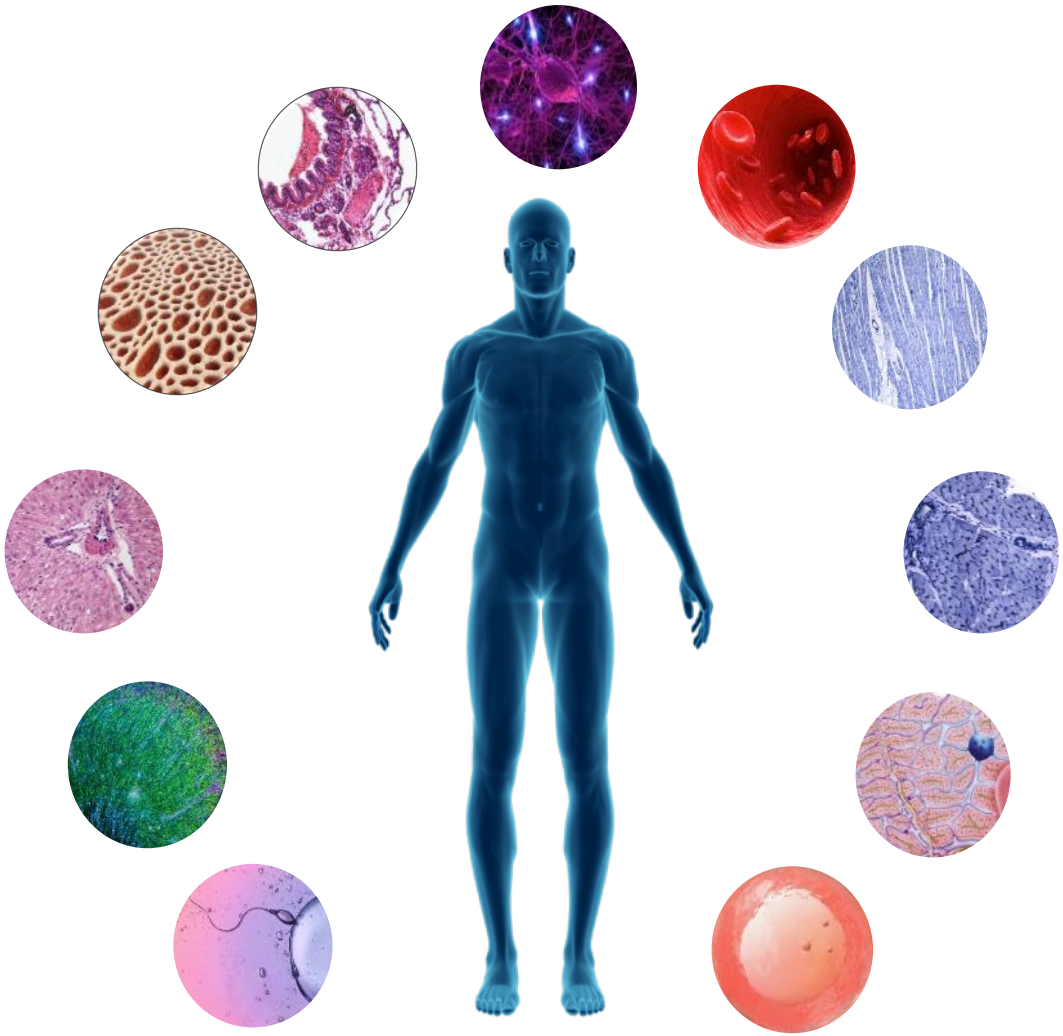
Creating a cell atlas

Single-cell genomic technology is required

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

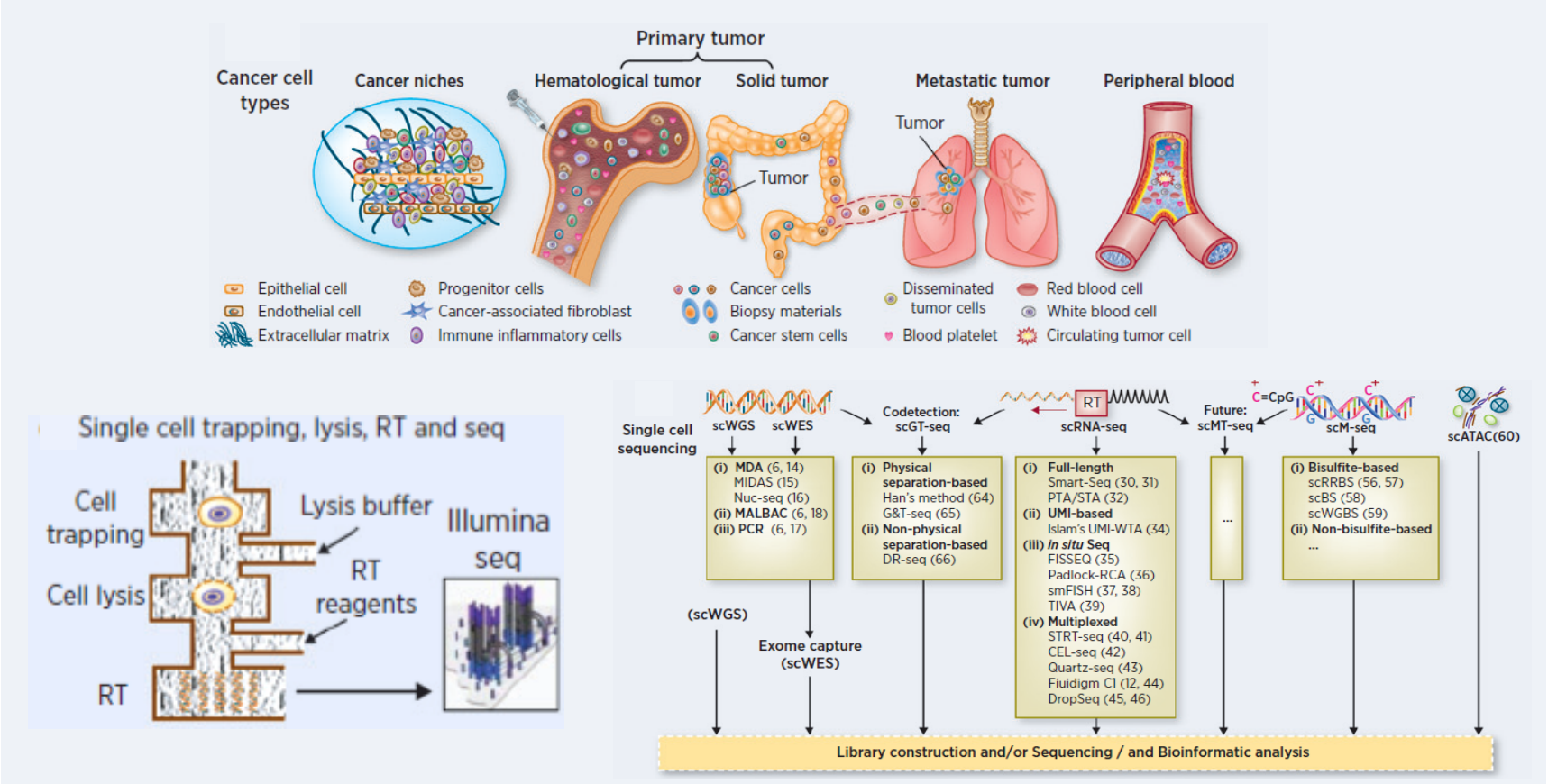
Three cell-based approaches:

1. Classification
2. Characterization
3. Contextual



C1 microfluidics

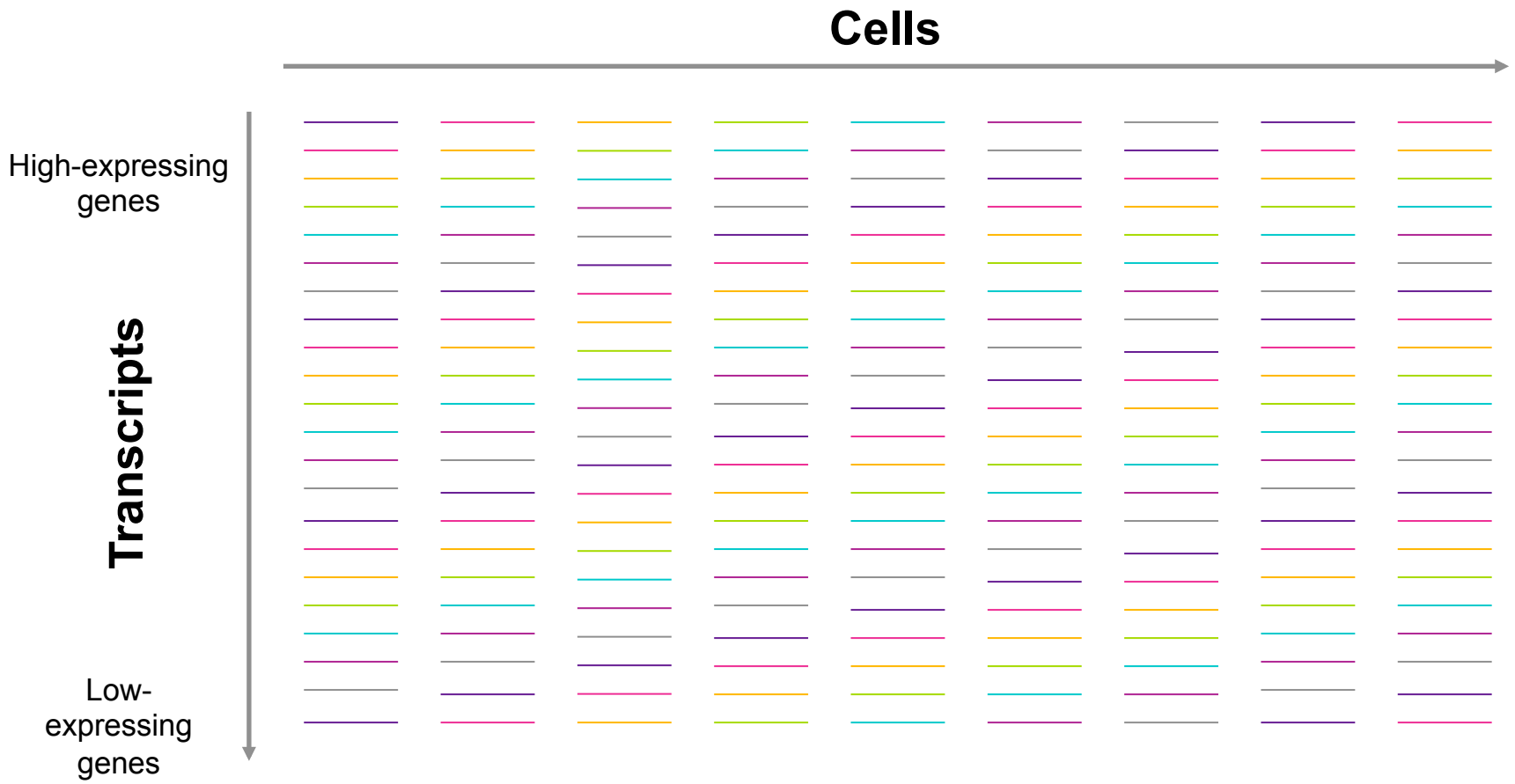
Deep sensitive profiling of cells in multiple modes



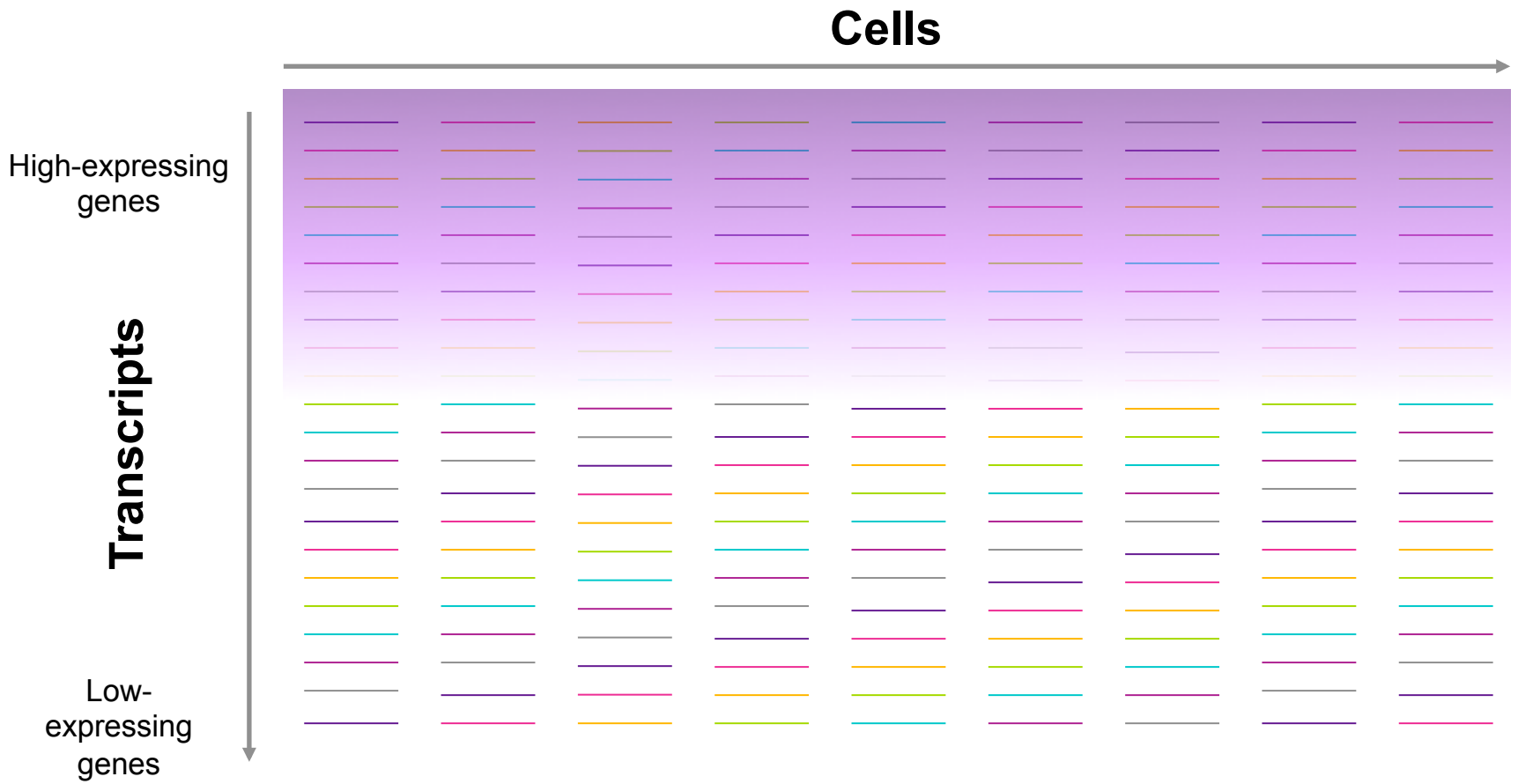
Modified from Zhang et al. *Cancer Research* (2016)

C1 complements ultrahigh-throughput methods

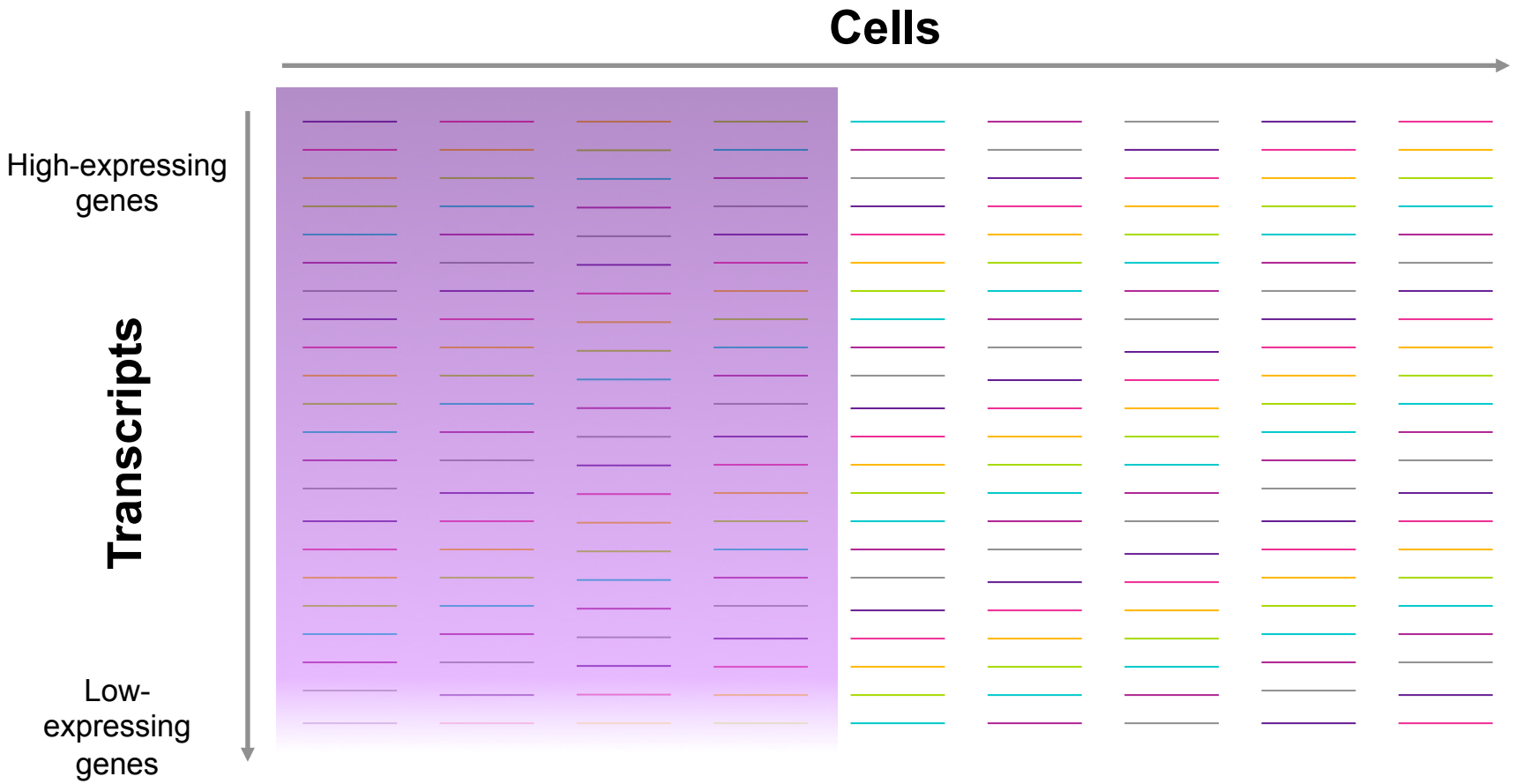
Allocation of reads towards cell number or transcript number



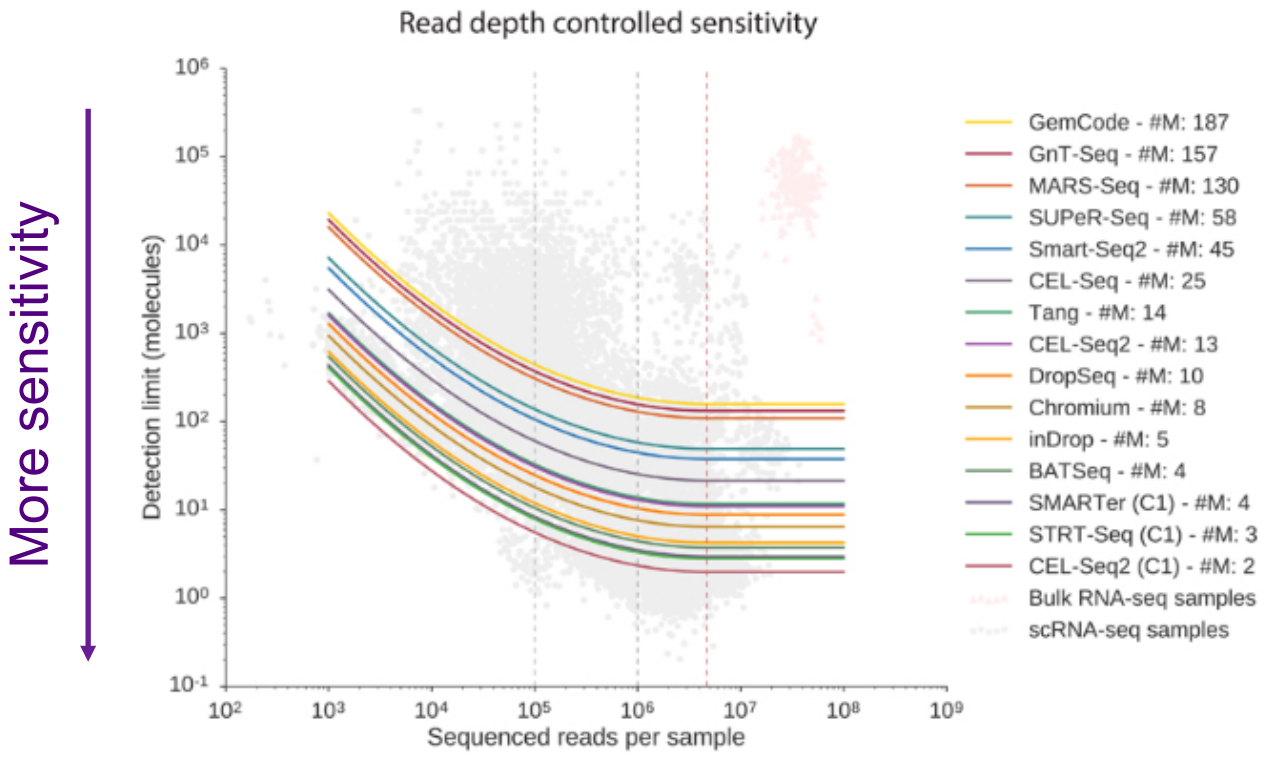
Ultrahigh-throughput methods cover many cells with low-depth sequencing



C1 provides deep full-length mRNA sequencing



C1 methods exhibit the highest sensitivity, even when accounting for read depth



Sequencing other single-cell methods more deeply does not lead to the same gene detection as C1 methods.

Svensson et al., *Nature Methods* (2017)

Uncover molecular pathways with C1

Developmental biology

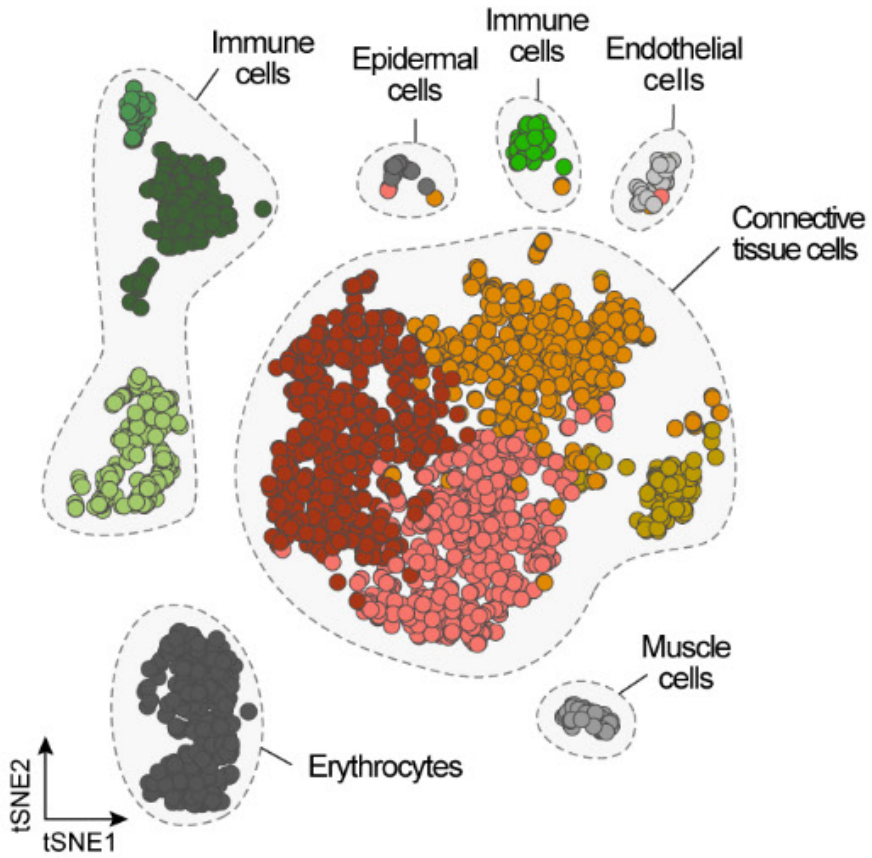
RESEARCH Science

Single-cell analysis uncovers convergence of cell identities during axolotl limb regeneration

Gerber, T., Murawala, P., Knapp, D., Masselink, W., Schuez, M., Hermann, S., Gac-Santel, M., Nowoshilow, S., Kageyama, J., Khattak, S., Currie, J.D., Camp, J.G., Tanaka, E.M., Treutlein, B.

Science (2018): 10.1126

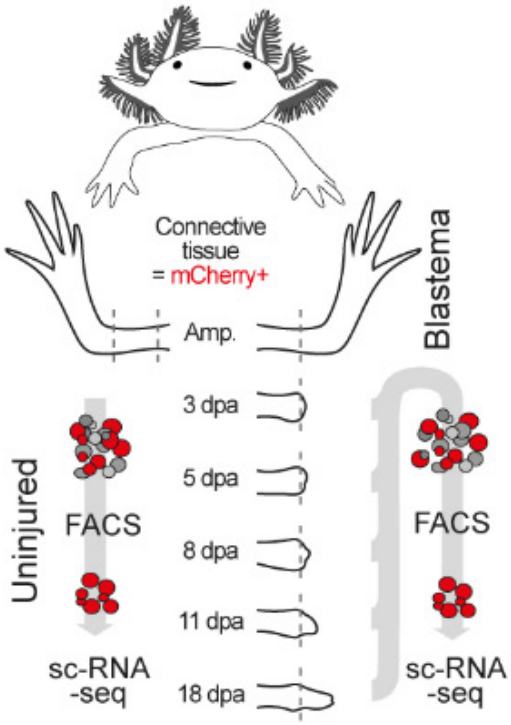
Understand cellular diversity with ultrahigh-throughput methods



10x Genomics® Chromium™ was used to sample the cellular diversity in the uninjured adult limb.

Gerber et al. (2018)

Deeper profiling with C1 to uncover molecular pathways



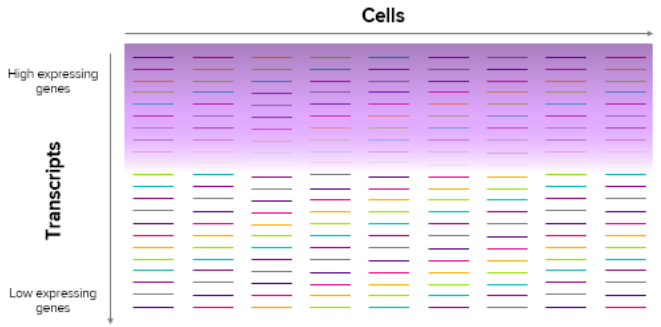
(3 days post amputation, 108 cells; 5 dpa, 167 cells; 8 dpa, 121 cells; 11 dpa, 163 cells; 18 dpa, 135 cells)

Fluidigm C1 was used to obtain high transcriptome coverage of mCherry+ cells and understand the molecular pathways involved in connective tissue regeneration.

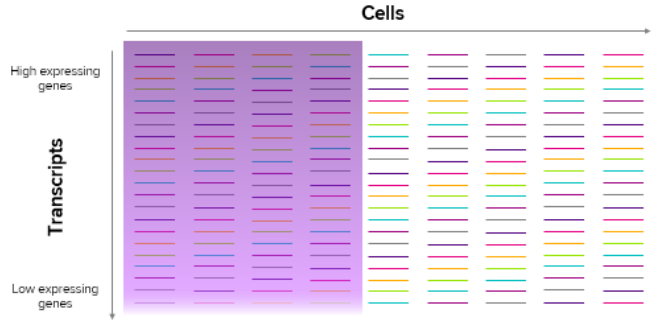
Gerber et al. (2018)

Comprehensive single-cell transcriptome analysis

Classify with ultrahigh-throughput methods such as Drop-seq or 10x Chromium with low-depth mRNA sequencing analysis to identify and atlas single cells.



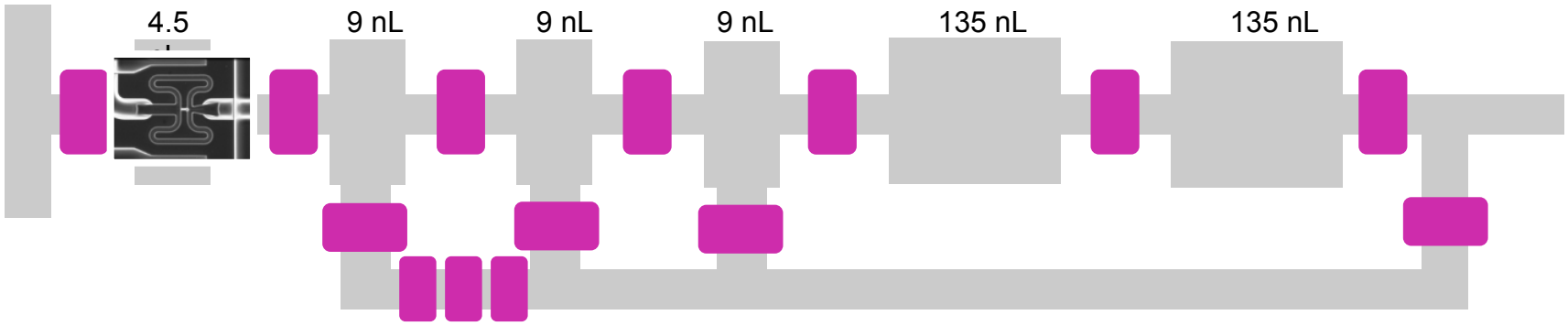
Characterize with deep profiling using C1 to verify the shallow-based findings of ultrahigh-throughput
methods and uncover molecular pathways.



C1 applications including Total RNA-Seq

Architecture of the C1 IFC

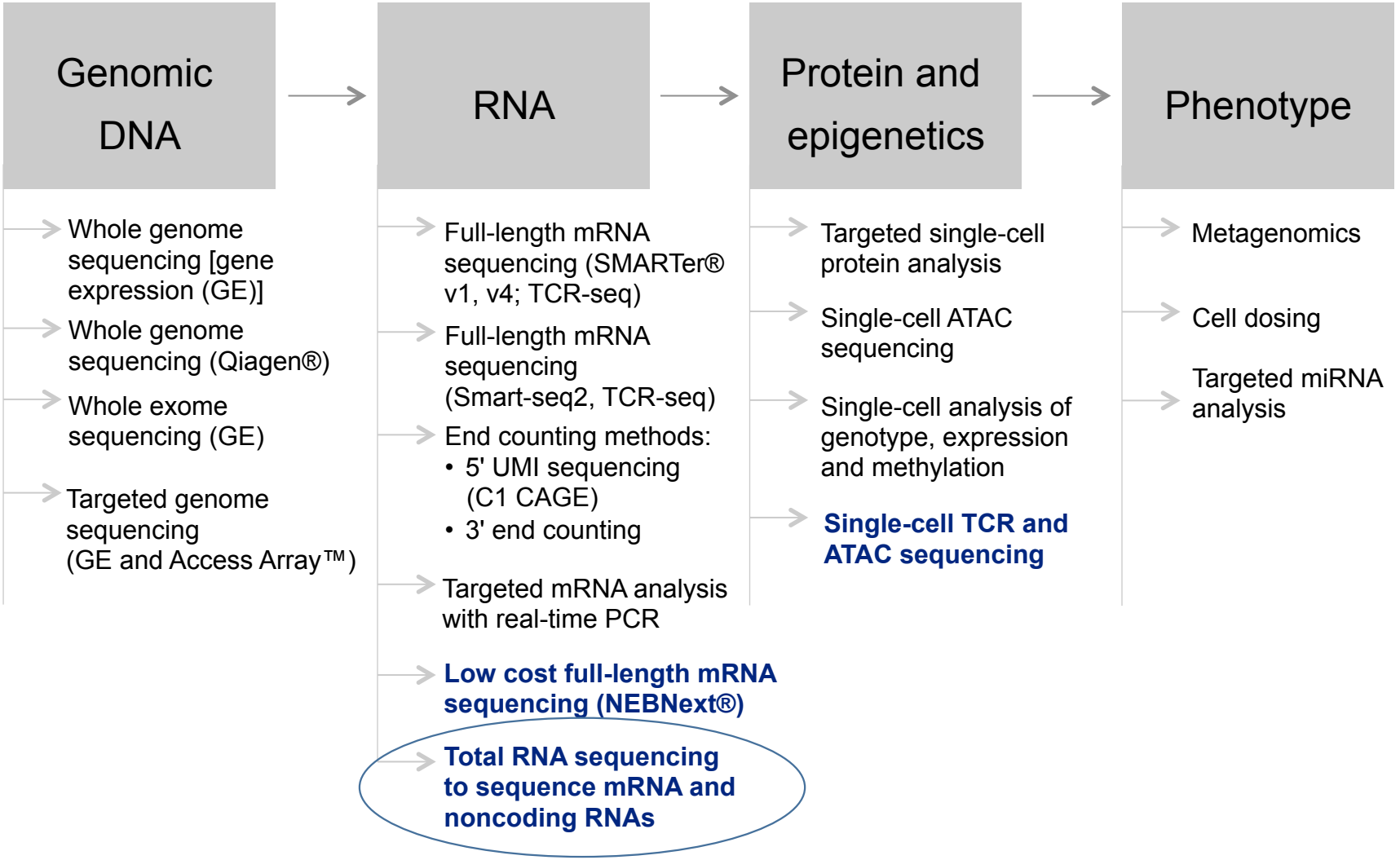
Allows chemistry flexibility



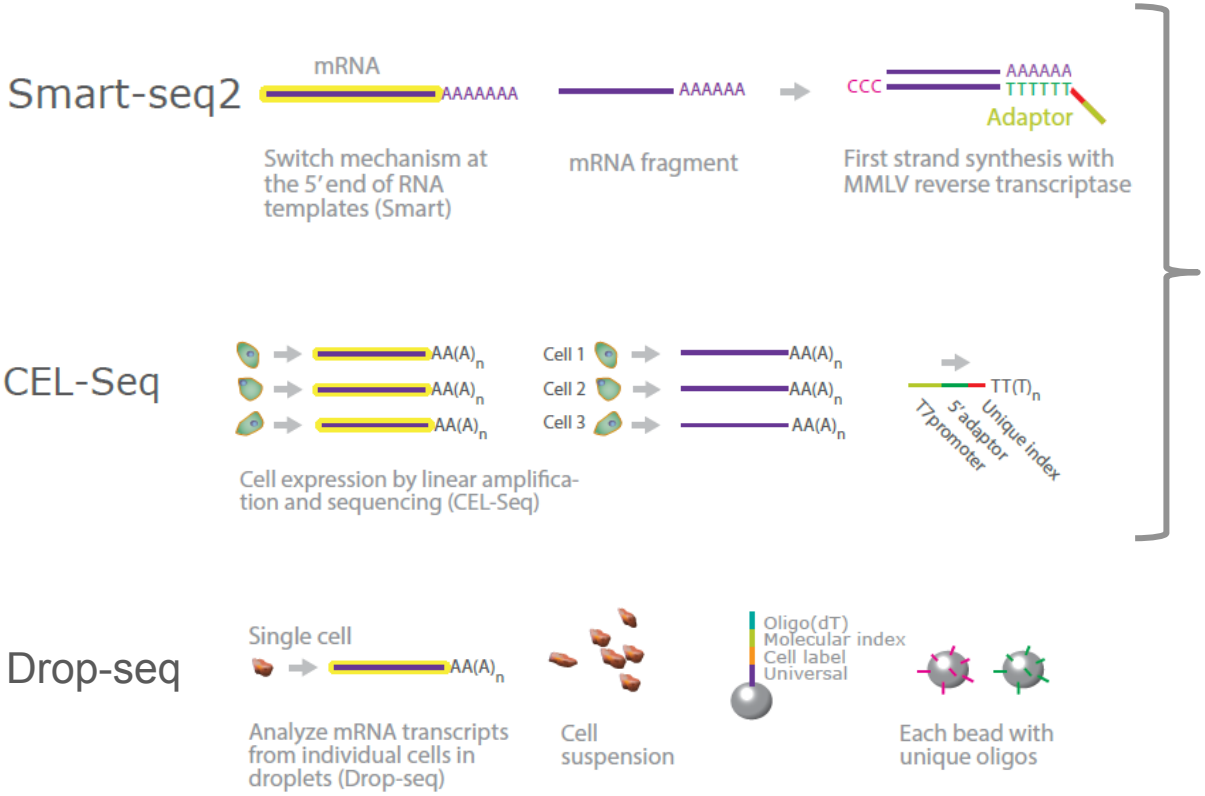
The architecture of the C1 IFC enables **sensitive** gene detection, **full-length** transcript coverage and **novel** chemistry development.

C1 offers prospects with more

30 ready-to-use applications via Script Hub™



Current scRNA-seq methods target only polyadenylated RNAs with oligo-dT-based capture



Adapted version of these methods available on C1

Images taken from https://www.illumina.com/science/sequencing-method-explorer.html

Types of nonpoly(A) RNA

mRNA

- Replication-dependent histone mRNAs lack poly(A) tail.

Noncoding RNA (ncRNA)

- ncRNA is not synonymous with nonpoly(A) RNA.
- Some ncRNA (for example, certain lincRNAs) have poly(A) tail and can be detected by SMART-Seq® chemistry on C1*.

*Liu et al. *Genome Biology* (2016). “Single-cell analysis of long non-coding RNAs in the developing human neocortex.” (Used SMART-Seq chemistry on C1)

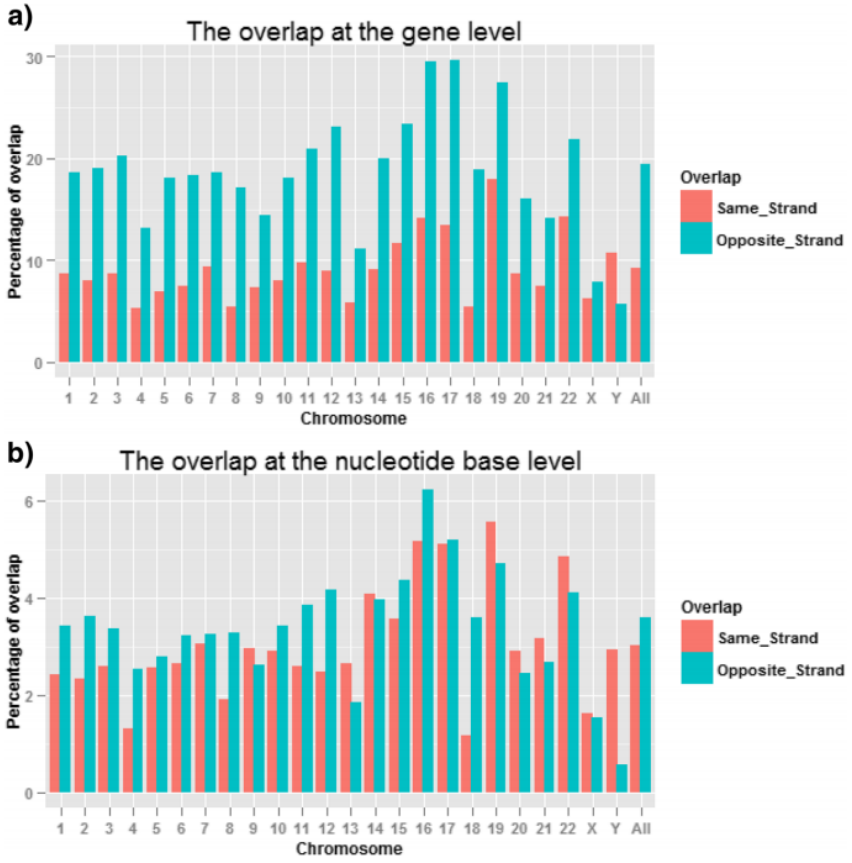
ncRNA biotypes

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 libraries improve accuracy

- Around 19% (11,000) of genes overlap with one or more genes on the opposite strand.
- Those regions translate to about 3% of overlapping nucleotides.
- Without strand information, reads are either wrongly assigned (false positive) or discarded (false negative).



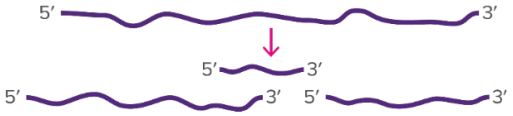
Zhao et al. *BMC Genomics* (2015). "Comparison of stranded and non-stranded RNA-seq transcriptome profiling and investigation of gene overlap."

Adapting Stranded Total RNA Seq for C1

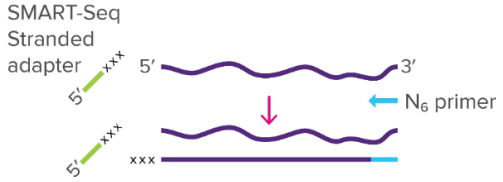
Stranded Total RNA-Seq workflow

Automated steps on C1

1. RNA fragmentation

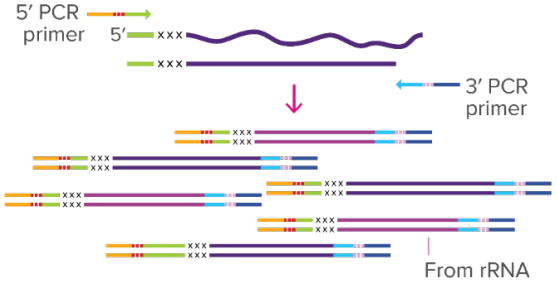


2. First-strand synthesis and tailing by RT



3. Template switching and extension by RT

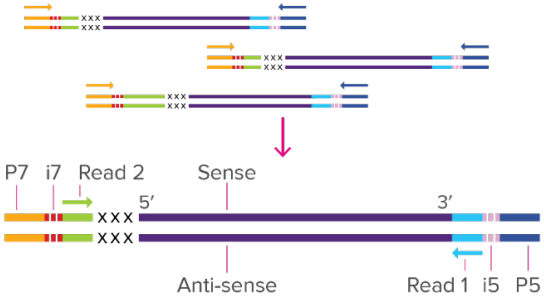
4. **PCR 1:** Addition of Illumina® adapters with barcodes



Single-tube library prep after C1

1. Cleavage of ribosomal cDNA

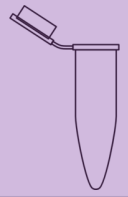
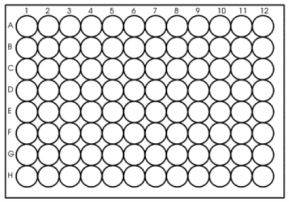
2. **PCR 2:** Enrichment of uncleaved fragments



Adaption of SMART-Seq Stranded Kit (Takara Bio)

Benefits

Workflow and cost

	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 
Third-party kit cost	• Chemistry: $4,320 (15 IFCs) • Cost per IFC: $688 • Total cost per IFC: $688 • Library prep: $0 • Cost per cell: $7.16	• Chemistry: $2,688 (10 IFCs) • Cost per IFC: $1,075.00 • Library prep: $550 • Total cost per IFC: $1,625.00 • Cost per cell: $16.92

C1 Total RNA Seq provides an easier and much cheaper workflow without compromising biological data.

Total RNA-Seq performance

Development objectives

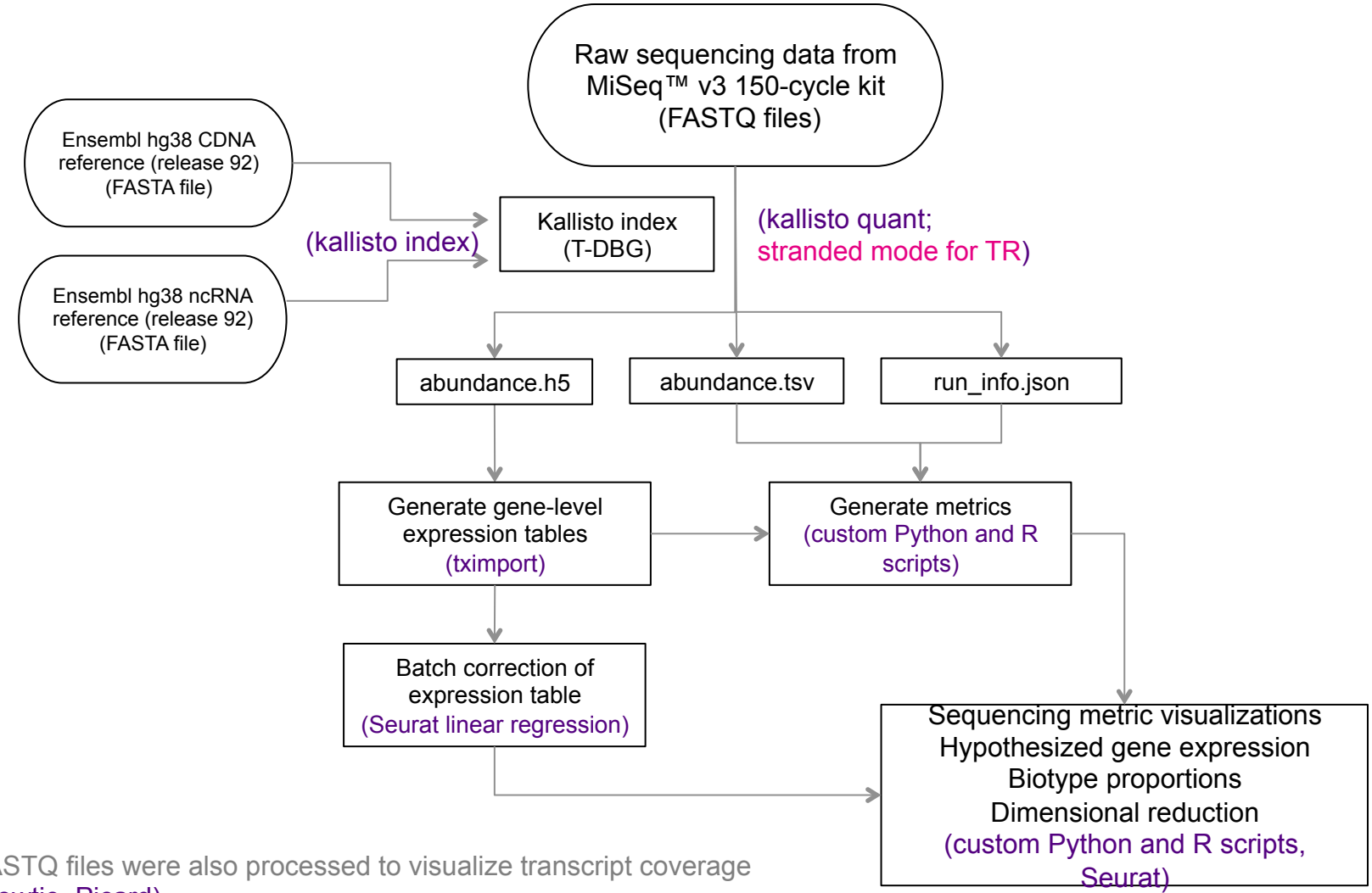
- Assess the performance of C1 Total RNA-Seq application on multiple cell types.
- Evaluate the efficiency of Total RNA Seq in comparison to a poly(A)-based method.
  - Experiments were performed side by side with the SMART-Seq v4 Ultra® Low Input RNA Kit.
- Employed an analysis pipeline for stranded single-cell total RNA-seq data.

Experimental design

	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 TR 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.

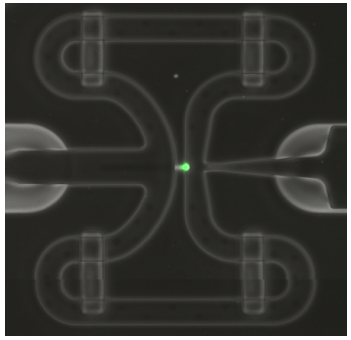
Bioinformatic analysis workflow



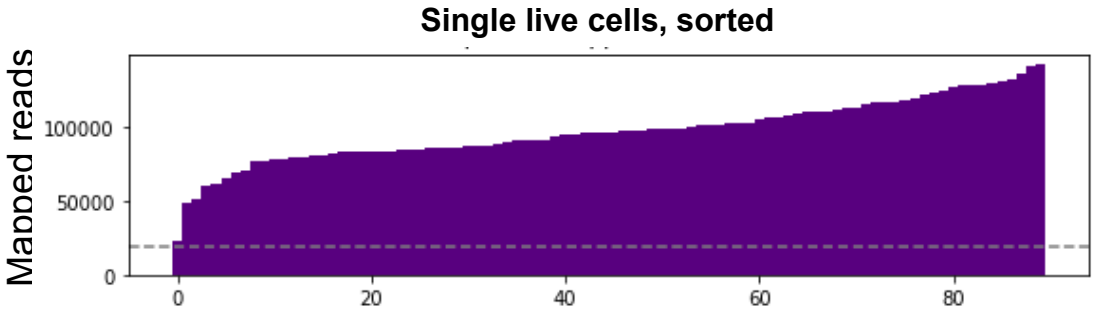
FASTQ files were also processed to visualize transcript coverage (Bowtie, Picard)

Processing live cells

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



Single live cell



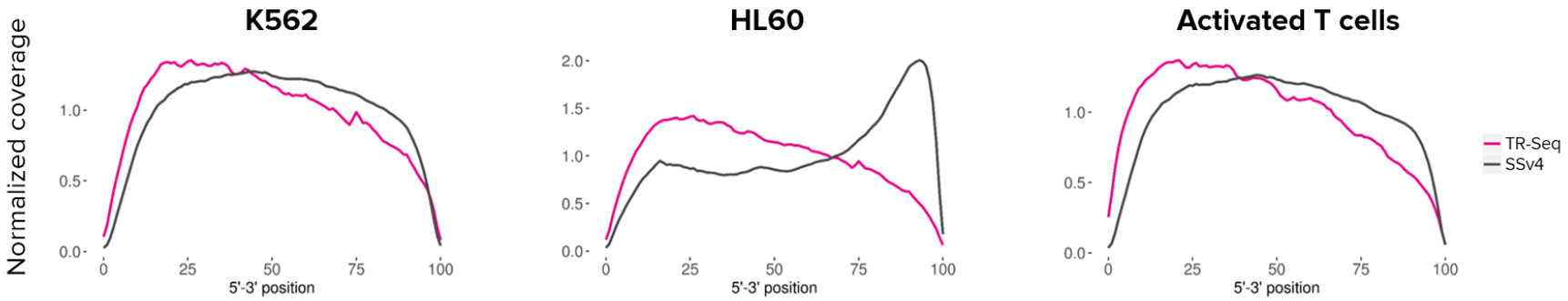
One IFC shown as an example. Dashed line indicates 20,000 mapped reads.

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

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.

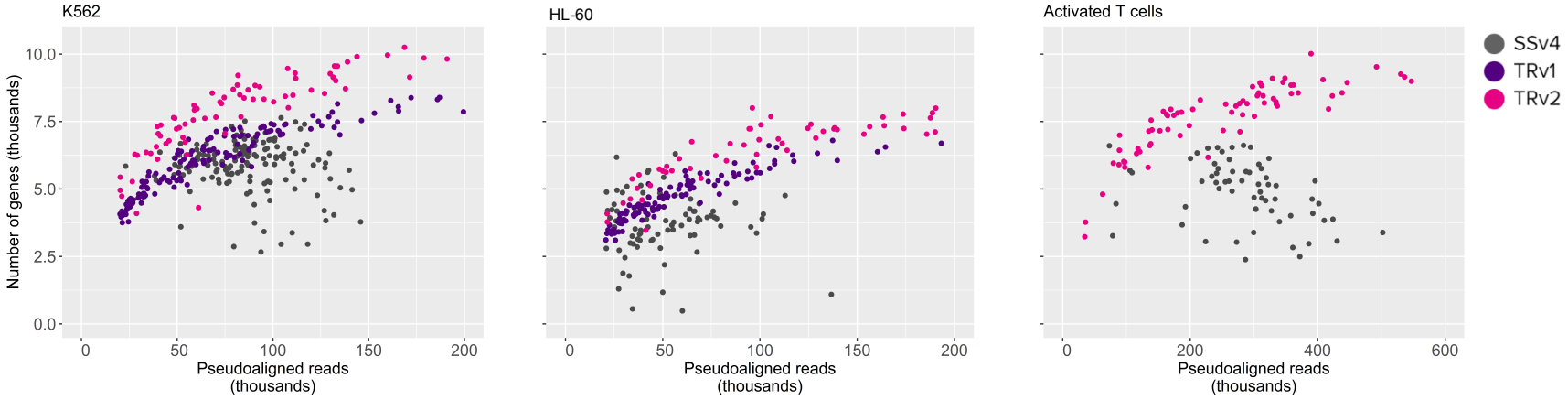
Total RNA-Seq shows similar or better transcript coverage than SMART-Seq v4



Total RNA Seq provides better coverage across transcripts in cell types that show high 3’ bias in SMART-Seq v4 chemistry.

Total RNA-Seq

Shows higher gene detection with increasing mapped reads

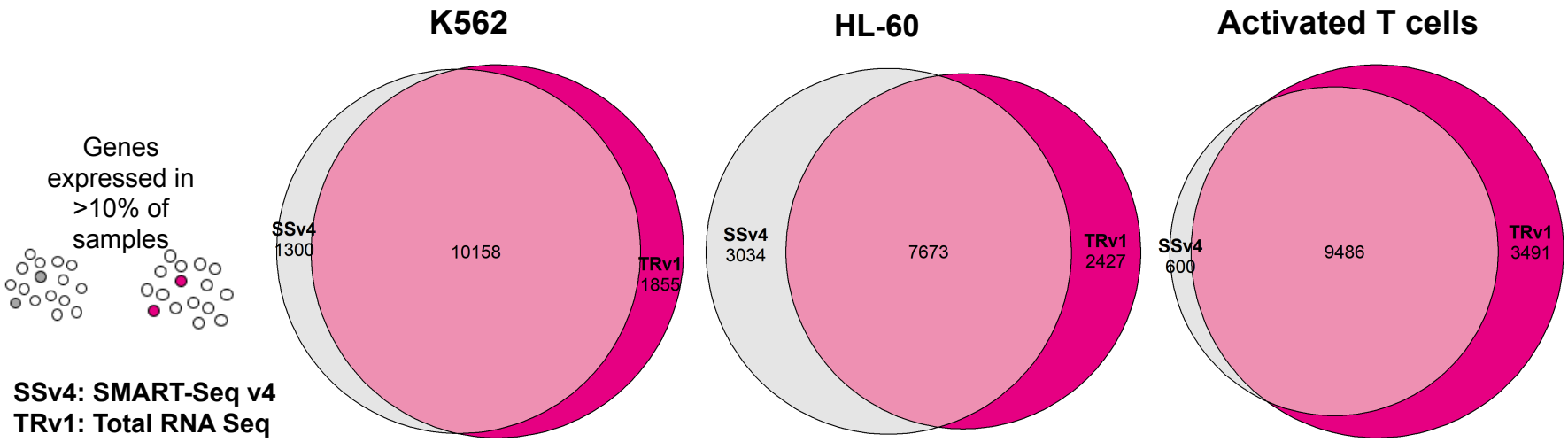


Gene counted if TPM >1

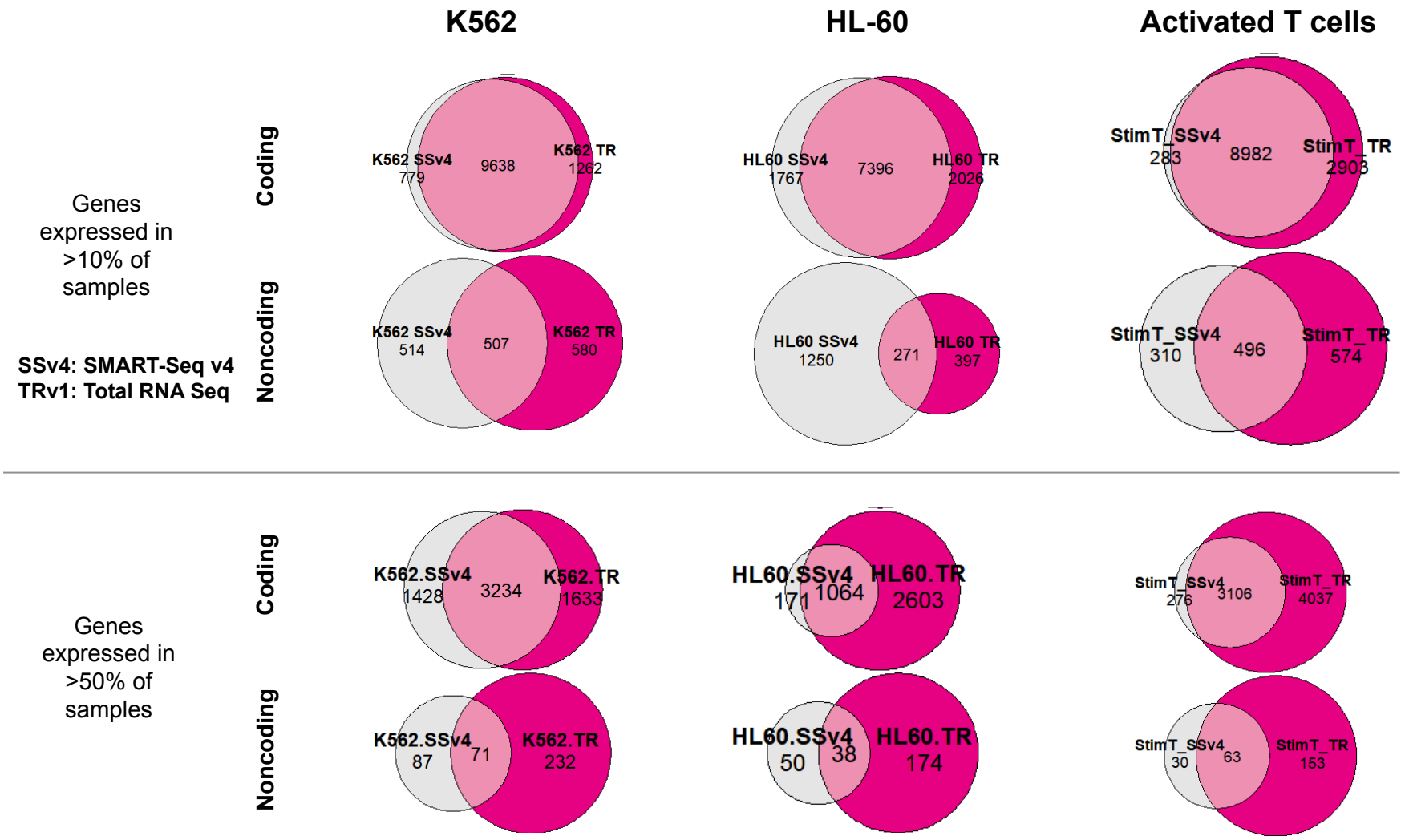
Total RNA Seq demonstrates a deeper cell characterization methodology aiding full single-cell transcriptome analysis.

Total RNA-Seq

Detects most of the same genes that SMART-Seq v4 detects

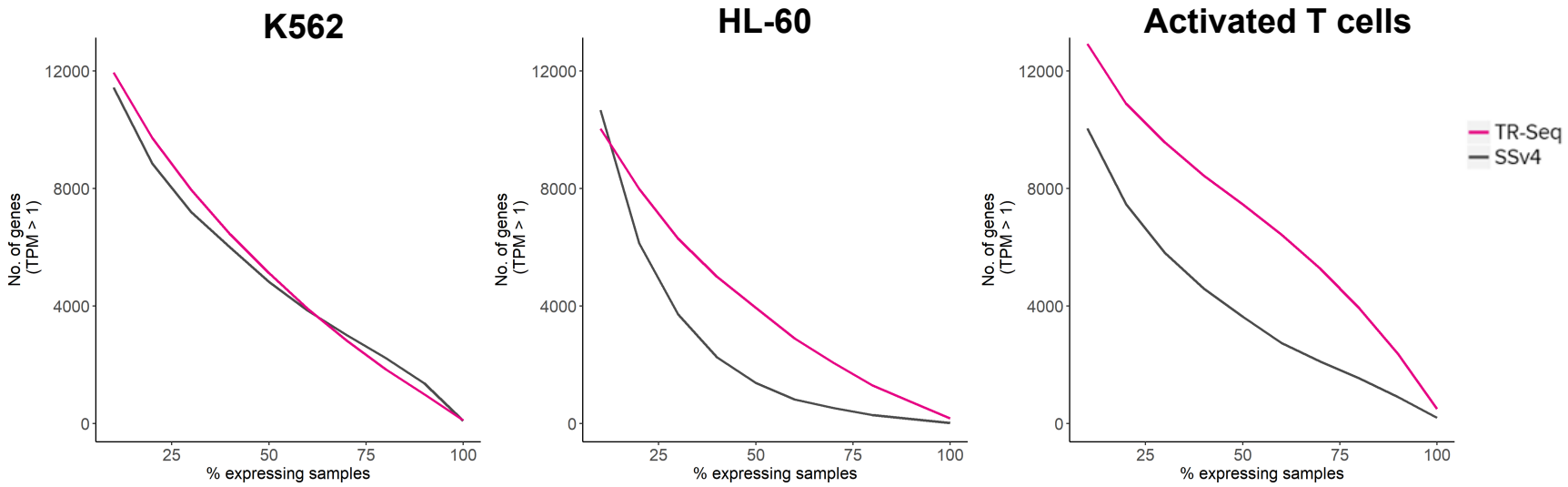


Method comparison in detection of coding and noncoding genes



Total RNA-Seq

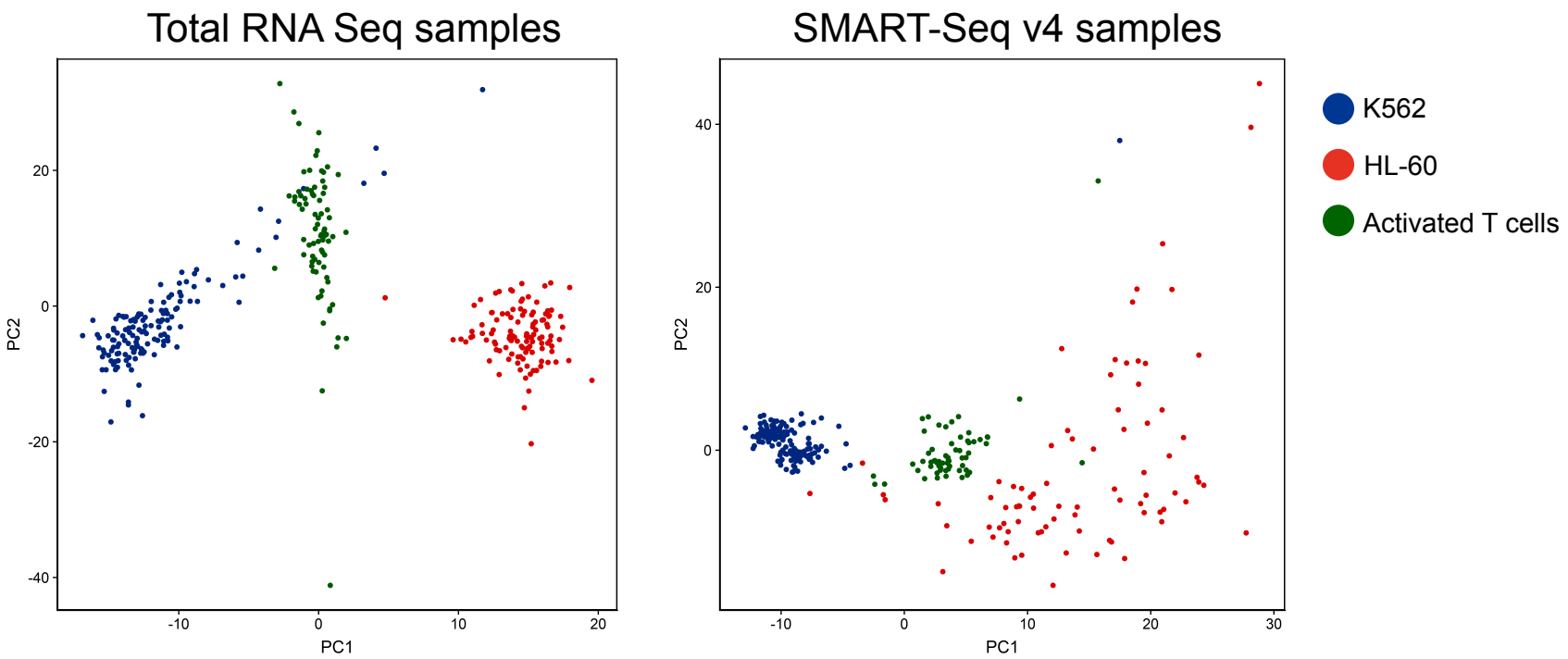
Shows gene expression similar to or more consistent than SSv4



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

Total RNA-Seq

Shows greater resolution when visualized by PCA

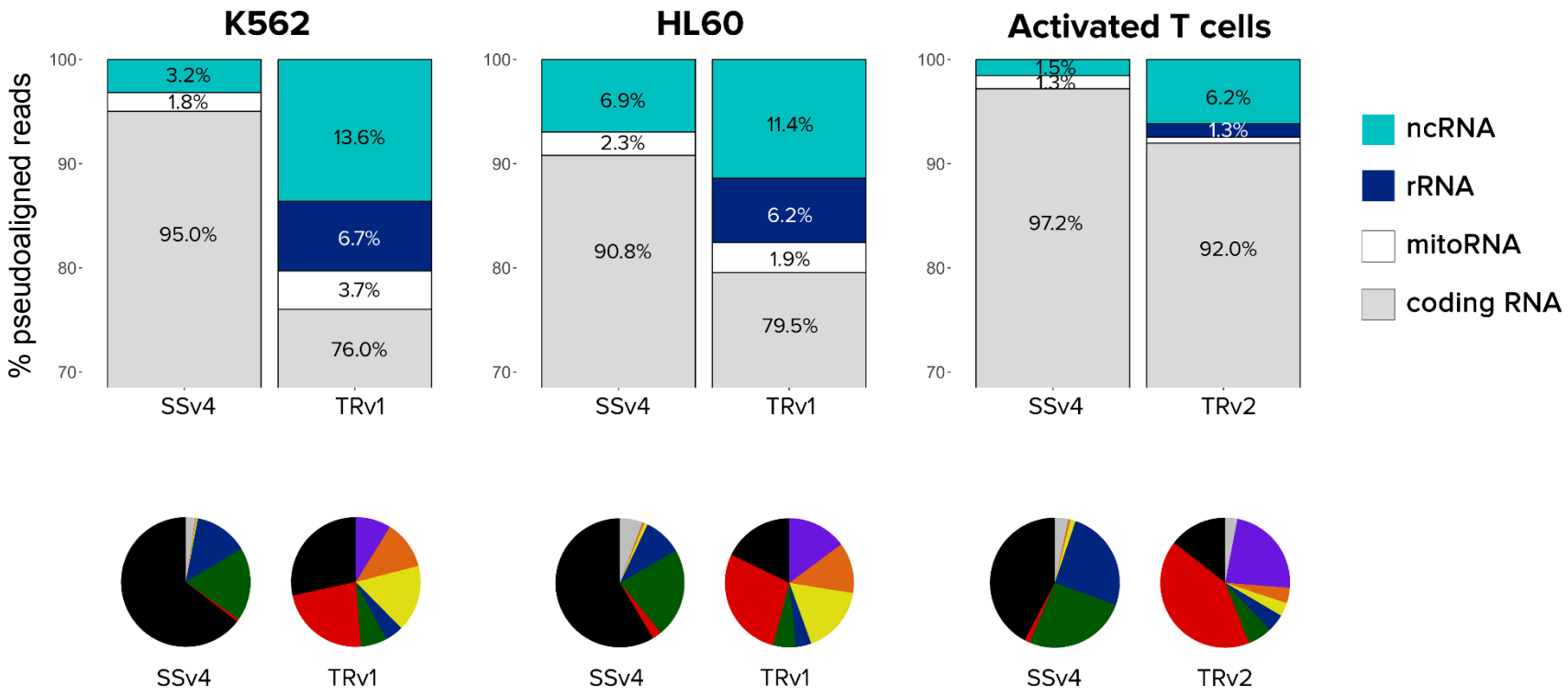


Cells that show more variability with SMART-Seq v4 exhibit tighter grouping with Total RNA-Seq chemistry.

PCA generated with Seurat

Total RNA-Seq

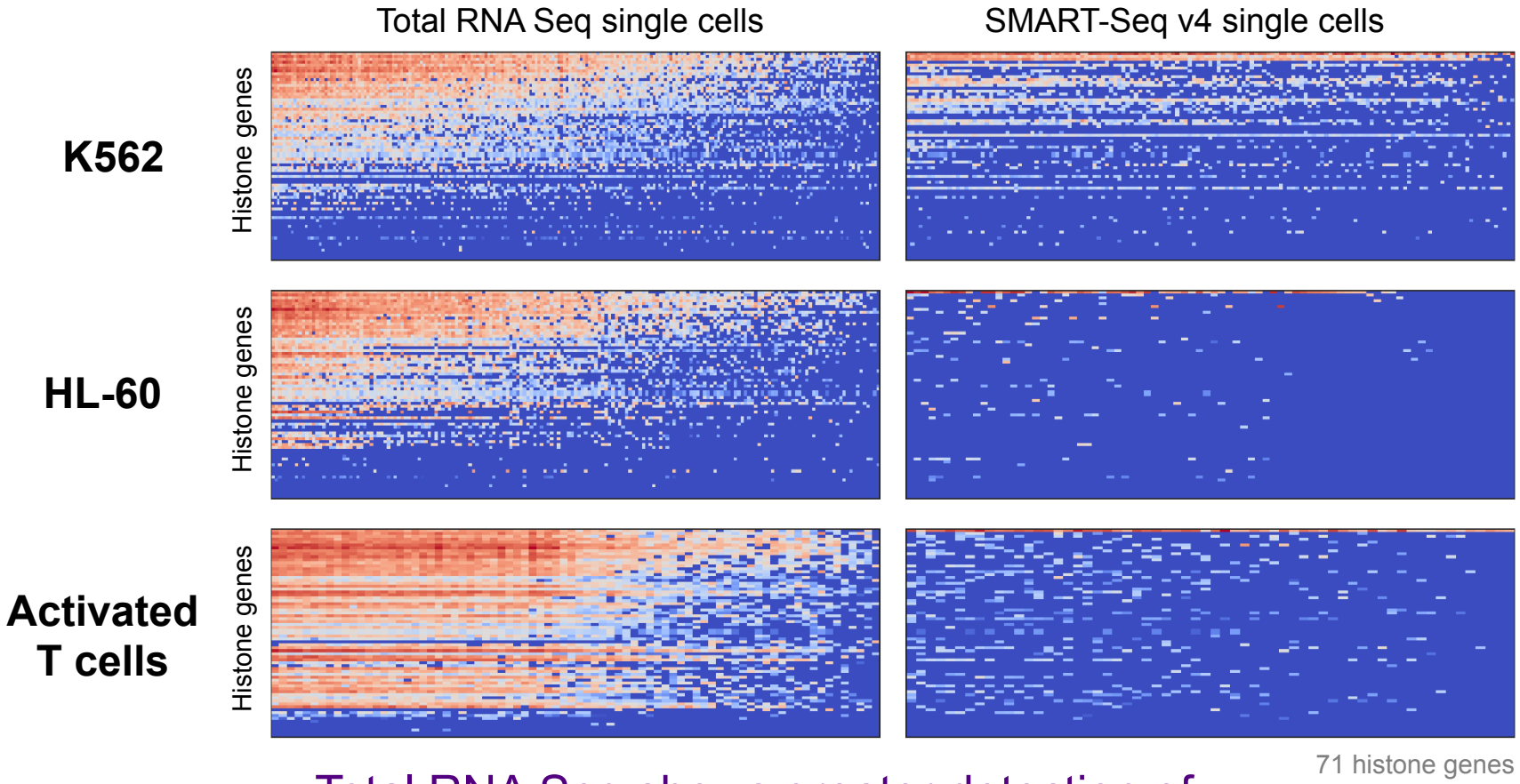
Exhibits a greater detection of noncoding RNA



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

Total RNA Seq

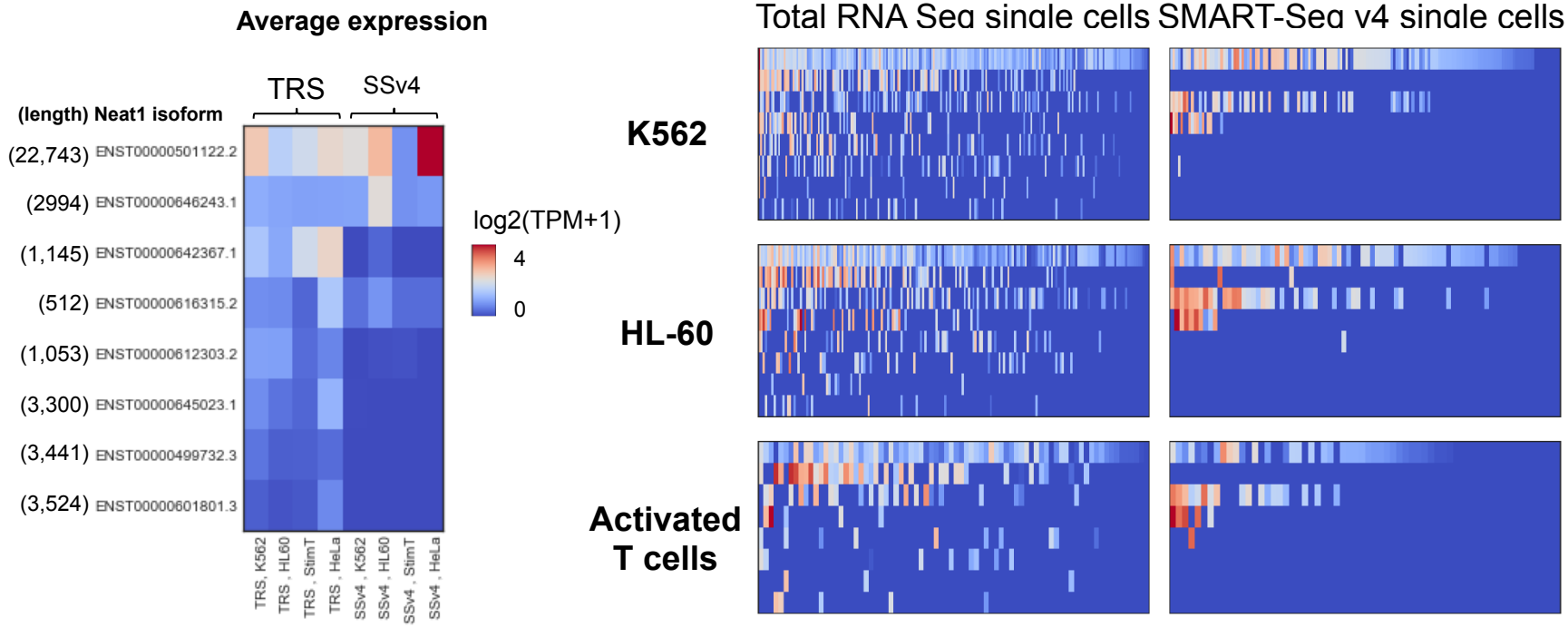
Detects more non poly(A) histone genes than SMART-Seq v4



Total RNA Seq shows greater detection of histone genes, a nonpoly(A) mRNA.

Total RNA Seq

Detects more nonpoly(A), Neat1 isoforms than SMART-Seq v4



Neat1 = nuclear-enriched abundant transcript 1

Total RNA Seq shows greater detection in the isoforms of a nonpoly(A), noncoding RNA.

Conclusion

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

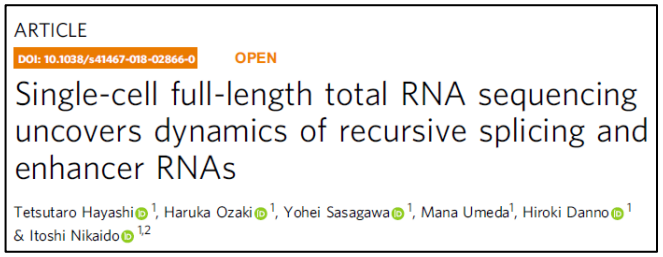
Method	Read Depth	Transcript Coverage	Poly(A) Transcript Isoforms	Nonpoly(A) Transcript Isoforms
Droplet-based methods	Low	3′ only	No	No
C1 high-throughput	Medium	3′ only	No	No
C1 96 (SMART-Seq v4)	High	Full-length	Yes	No
C1 96 Total RNA Seq	High	Full-length	Yes	Yes

Methods for ncRNA and nonpoly(A) RNA produced with C1

Hayashi et al.

Nature Communications

February 2018



ARTICLE

DOI: 10.1038/s41467-018-02866-0 OPEN

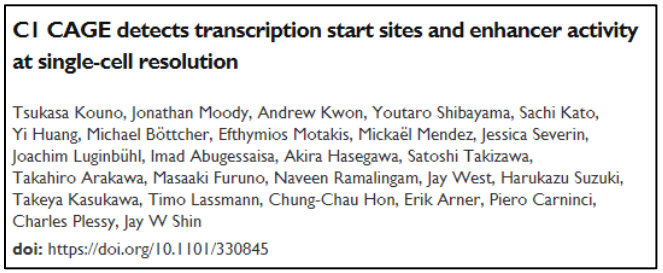
Single-cell full-length total RNA sequencing uncovers dynamics of recursive splicing and enhancer RNAs

Tetsutaro Hayashi[1], Haruka Ozaki[1], Yohei Sasagawa[1], Mana Umeda[1], Hiroki Danno[1] & Itoshi Nikaido[1,2]

Kouno et al.

BioRxiv, May 2018

Launched on Script Hub May 2016



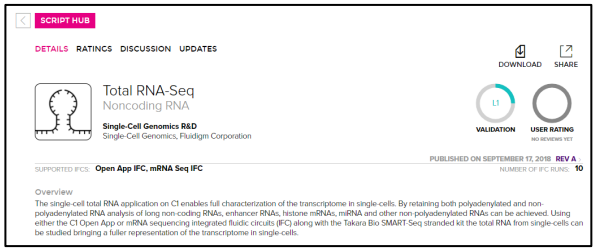
C1 CAGE detects transcription start sites and enhancer activity at single-cell resolution

Tsukasa Kouno, Jonathan Moody, Andrew Kwon, Youtaro Shibayama, Sachi Kato, Yi Huang, Michael Böttcher, Efthymios Motakis, Mickaël Mendez, Jessica Severin, Joachim Luginbühl, Imad Abugessaisa, Akira Hasegawa, Satoshi Takizawa, Takahiro Arakawa, Masaaki Furuno, Naveen Ramalingam, Jay West, Harukazu Suzuki, Takeya Kasukawa, Timo Lassmann, Chung-Chau Hon, Erik Arner, Piero Carninci, Charles Plessy, Jay W Shin

doi: https://doi.org/10.1101/330845

Fluidigm Total RNA Seq

Launched on Script Hub September 2018



SCRIPT HUB

DETAILS RATINGS DISCUSSION UPDATES

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Total RNA-Seq
Noncoding RNA

Single-Cell Genomics R&D
Single-Cell Genomics, Fluidigm Corporation

VALIDATION USER RATING

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SUPPORTED IFCS: Open App IFC, mRNA Seq IFC

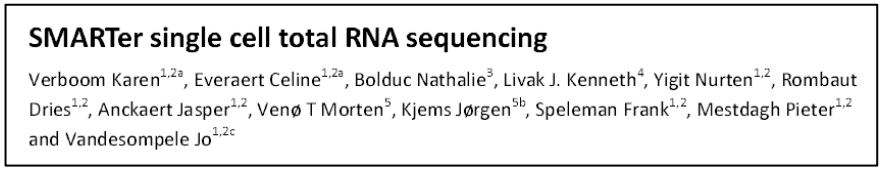
NUMBER OF IFC RUNS: 10

Overview

The single-cell total RNA application on C1 enables full characterization of the transcriptome in single-cells. By retaining both polyadenylated and non-polyadenylated RNA analysis of long non-coding RNAs, enhancer RNAs, histone mRNAs, miRNA and other non-polyadenylated RNAs can be achieved. Using either the C1 Open App or mRNA sequencing integrated fluidic circuits (IFC) along with the Takara Bio SMART-Seq stranded kit the total RNA from single-cells can be studied bringing a fuller representation of the transcriptome in single-cells.

Verboom et al.

BioRxiv, September 2018



SMARTer single cell total RNA sequencing

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Total RNA Seq

Summary

- C1 IFC architecture enables myriad applications including Total RNA Seq.
- 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 indexing PCR and single-tube post-C1 prep
- Maintains full-length 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 non-coding RNA features

Thank you.

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