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Single-Cell RNA Sequencing using the Iso-Seq Method

Elizabeth Tseng, Principal Scientist, PacBio

@Magdoll

SINGLE-CELL ISO-SEQ METHOD: WHY?

Cell type specific
mRNA splicing

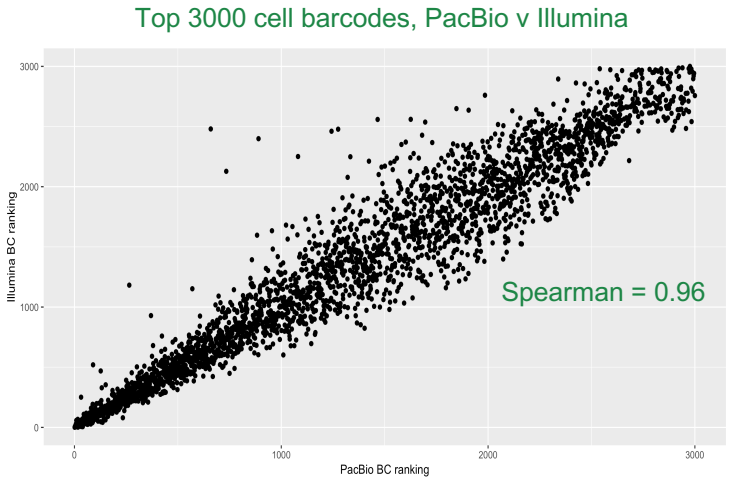
Not captured with 3’-end
short-read scRNA seq

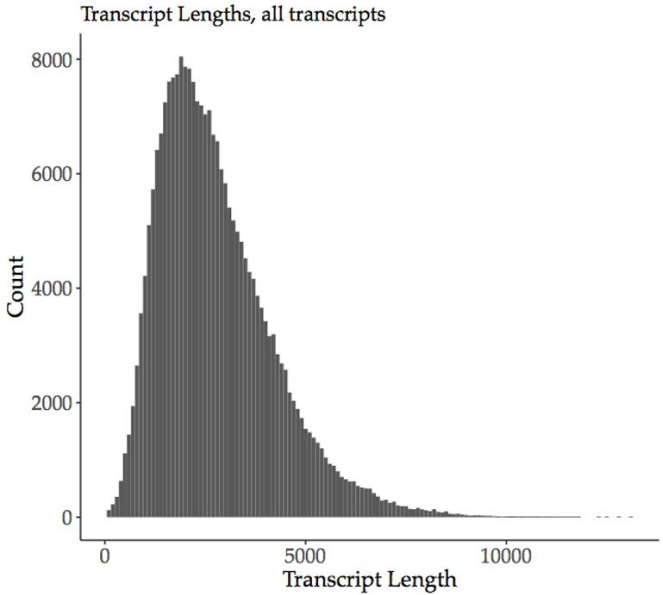
Resolved with single-cell full-length RNA seq

(sclso-Seq)

PACBIO HIFI READS ARE ACCURATE FOR SINGLE-CELL

HiFi reads ~100% concordant with matching Illumina data





COMPATIBLE WITH FULL-LENGTH SINGLE-CELL PLATFORMS

Bulk RNA-seq
on PacBio Systems

Poly-A+ RNA

↓

Full Length
1st Strand cDNA

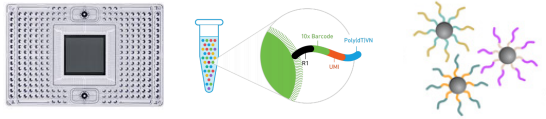
↓

Amplified cDNA

↓

SMRTbell Library

Any single-cell platform that generates full-length cDNA



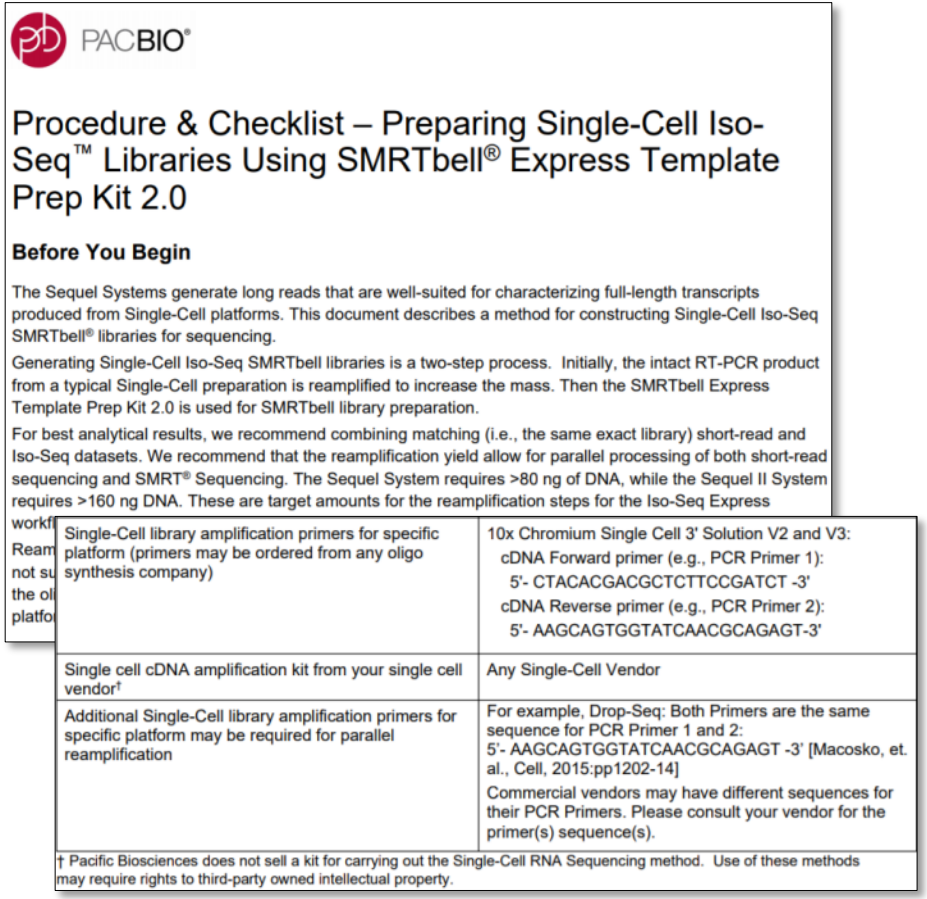
5′ primer	cell BC	UMI	transcript	(A)n	3′ primer

5′ primer	transcript	(A)n	UMI	cell BC	3′ primer

5′ primer	transcript	(A)n	3′ primer

SINGLE-CELL ISO-SEQ WORKFLOW

- Compatible with any full-length single-cell platform
- Detailed workflow guidance
- Standard Iso-Seq library preparation & sequencing workflow
- Can generate matching short read data from same sample
- ~3 million HiFi reads per SMRT Cell 8M
- Flexible barcoding & multiplexing based on desired number of reads per cell



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Procedure & Checklist – Preparing Single-Cell Iso-Seq™ Libraries Using SMRTbell® Express Template Prep Kit 2.0

Before You Begin

The Sequel Systems generate long reads that are well-suited for characterizing full-length transcripts produced from Single-Cell platforms. This document describes a method for constructing Single-Cell Iso-Seq SMRTbell® libraries for sequencing.

Generating Single-Cell Iso-Seq SMRTbell libraries is a two-step process. Initially, the intact RT-PCR product from a typical Single-Cell preparation is reamplified to increase the mass. Then the SMRTbell Express Template Prep Kit 2.0 is used for SMRTbell library preparation.

For best analytical results, we recommend combining matching (i.e., the same exact library) short-read and Iso-Seq datasets. We recommend that the reamplification yield allow for parallel processing of both short-read sequencing and SMRT® Sequencing. The Sequel System requires >80 ng of DNA, while the Sequel II System requires >160 ng DNA. These are target amounts for the reamplification steps for the Iso-Seq Express workf

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Single-Cell library amplification primers for specific platform (primers may be ordered from any oligo synthesis company)	10x Chromium Single Cell 3' Solution V2 and V3: cDNA Forward primer (e.g., PCR Primer 1): 5'- CTACACGACGCTCTTCCGATCT -3' cDNA Reverse primer (e.g., PCR Primer 2): 5'- AAGCAGTGGTATCAACGCAGAGT-3'
Single cell cDNA amplification kit from your single cell vendor†	Any Single-Cell Vendor
Additional Single-Cell library amplification primers for specific platform may be required for parallel reamplification	For example, Drop-Seq: Both Primers are the same sequence for PCR Primer 1 and 2: 5'- AAGCAGTGGTATCAACGCAGAGT -3' [Macosko, et. al., Cell, 2015:pp1202-14] Commercial vendors may have different sequences for their PCR Primers. Please consult your vendor for the primer(s) sequence(s).

† Pacific Biosciences does not sell a kit for carrying out the Single-Cell RNA Sequencing method. Use of these methods may require rights to third-party owned intellectual property.

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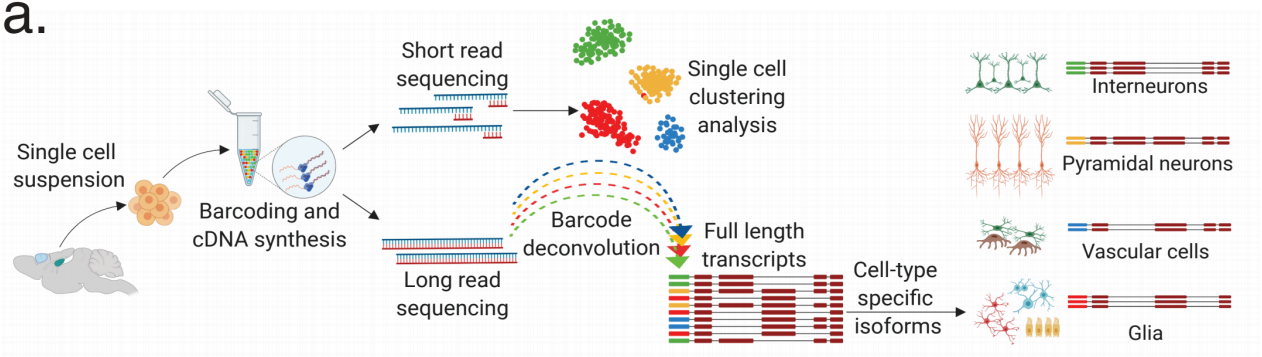
Single-Cell Iso-Seq Research Highlights

SINGLE-CELL ISO-SEQ METHOD ON DIFFERENT PLATFORMS

Single-cell Platform	Publications/Presentations	Research Areas
10x Genomics	Zheng et al. *biorxiv* (2020)	Corneal epithelium
	Tilgner *SMRT Leiden* (2020)	Brain
	Joglekar et al. biorxiv (2020)	Brain
	Mincarelli et al. *biorxiv* (2020)	Immune repertoire
	Russell et al. *J Virology* (2019)	Influenza
Celsee (Bio-Rad)	Underwood *RNA Society* (2020)	Human/mouse cell cycle
Dolomite Bio	Underwood *AGBT* (2019)	Primate organoids
Berkeley Lights	Zost et al. *biorxiv* (2020)	COVID-19 antibodies
Various	Conessa *SMRT Leiden* (2020)	Bioinformatics tools

COMBINING SHORT- AND LONG-READ SINGLE CELL DATA

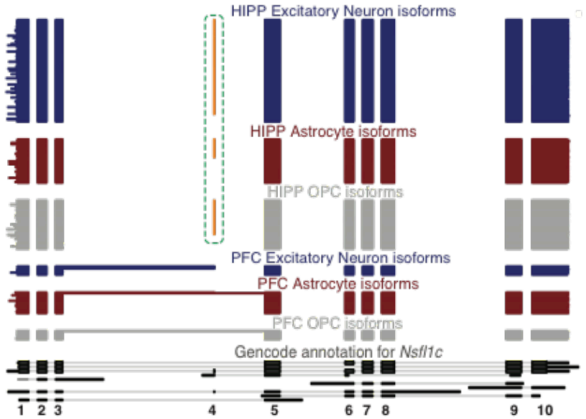
a.



Short read data identifies cell clusters

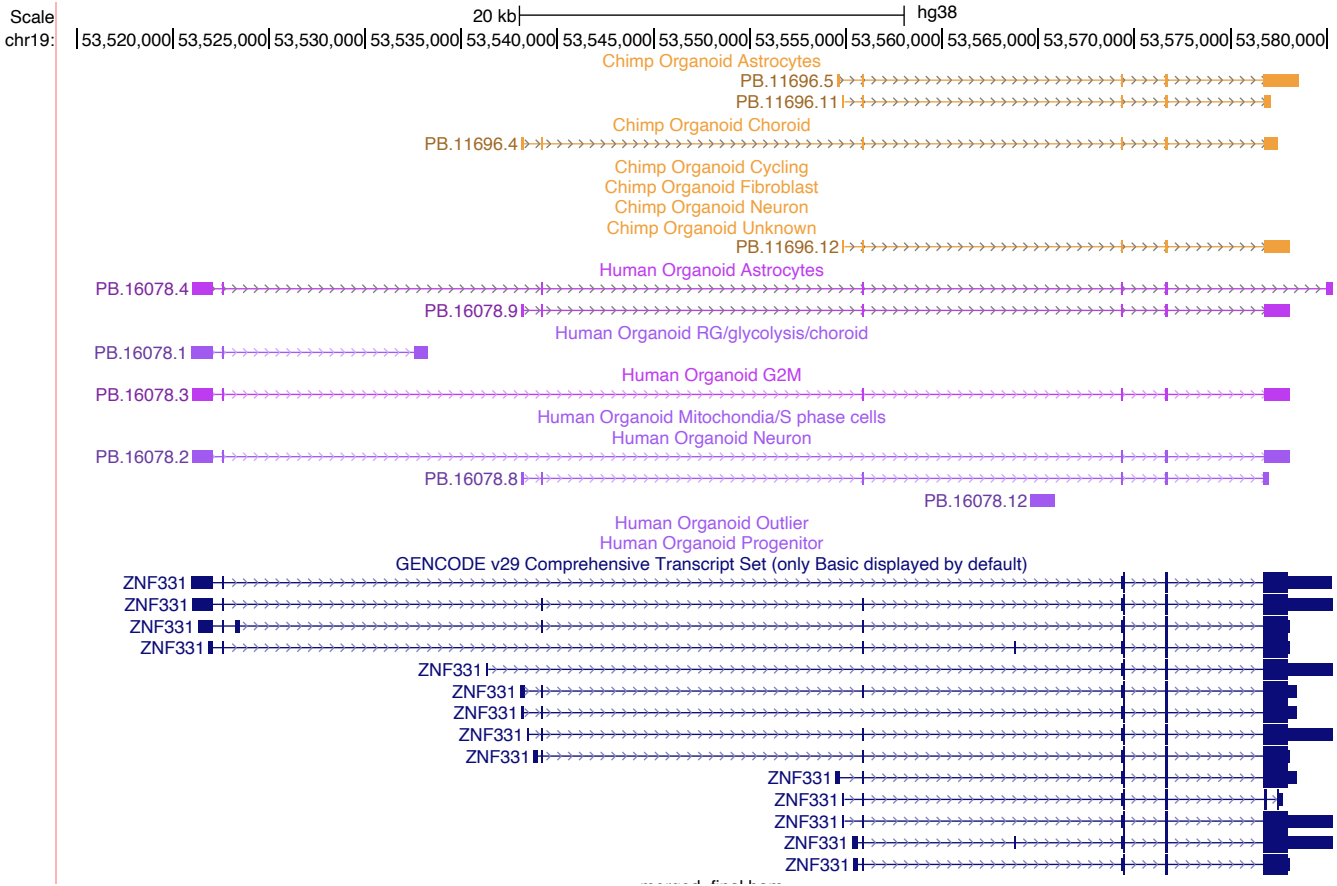
Long read data identifies full-length isoforms

Matching (UMI, BC) links isoforms to cell types



https://www.biorxiv.org/content/10.1101/2020.08.27.268730v1.full.pdf

CHIMP-HUMAN TSS DIFFERENCE IN SINGLE-CELL ORGANOIDS



https://www.youtube.com/watch?v=XKIT0Tw664s

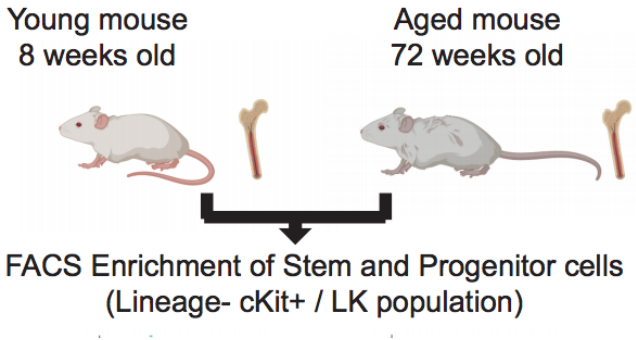
AGED MICE SHOW MORE DIVERSE V(D)J RECOMBINATION

bioRχiv
THE PREPRINT SERVER FOR BIOLOGY

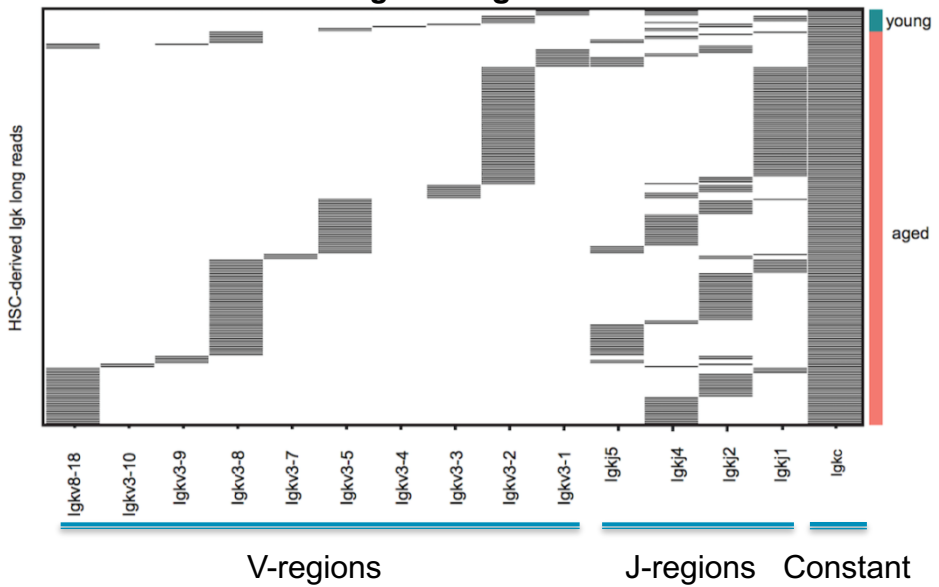
Combined single-cell gene and isoform expression analysis in haematopoietic stem and progenitor cells

Laura Mincarelli, Vladimir Uzun, Stuart A. Rushworth, Wilfried Haerty, Iain C. Macaulay

doi: https://doi.org/10.1101/2020.04.06.027474



***Igk* – long read**



CONCATENATION OF SINGLE-CELL TRANSCRIPTS

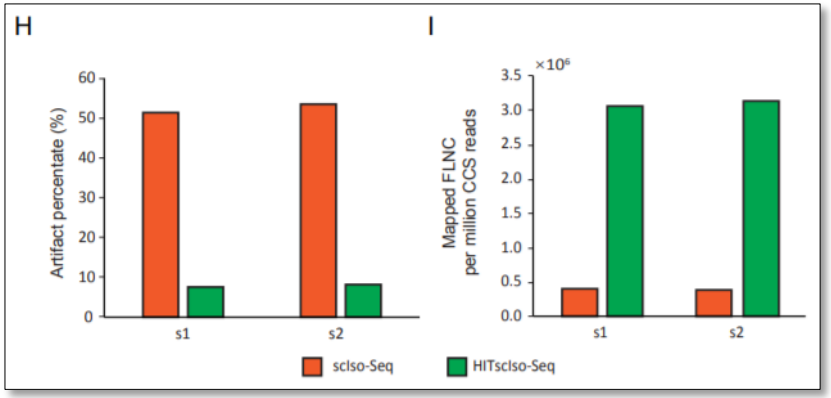
New Results

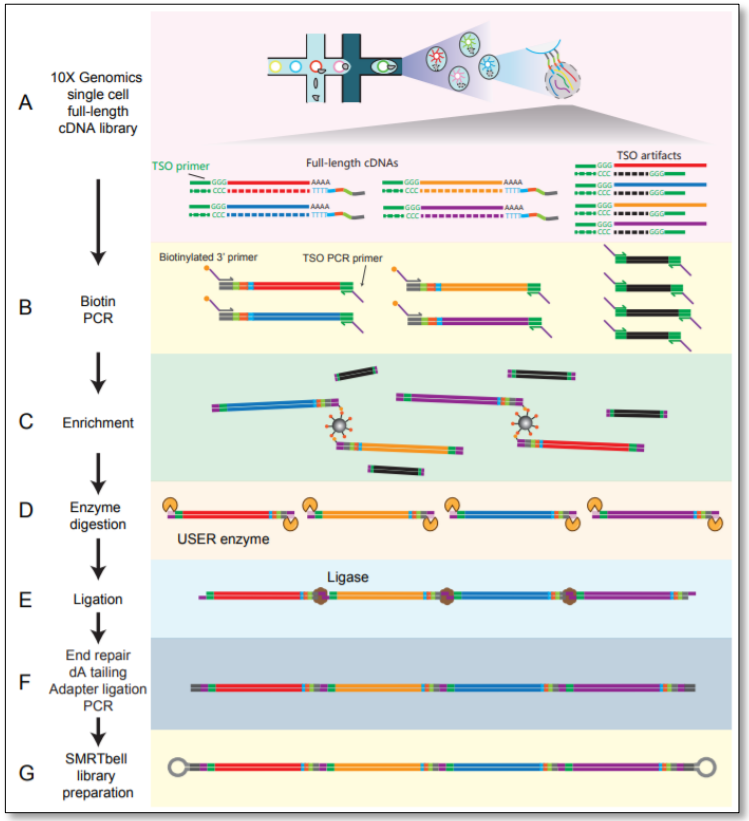
Comment on this paper

HIT-scISOseq: High-throughput and High-accuracy Single-cell Full-length Isoform Sequencing for Corneal Epithelium

Ying-Feng Zheng, Zhi-Chao Chen, Zhuo-Xing Shi, Kun-Hua Hu, Jia-Yong Zhong, Chun-Xiao Wang, Wen Shi, Ying Chen, Shang-Qian Xie, Feng Luo, Xiao-Chen Bo, Chong Tang, Yi-Zhi Liu, Chuan-Le Xiao

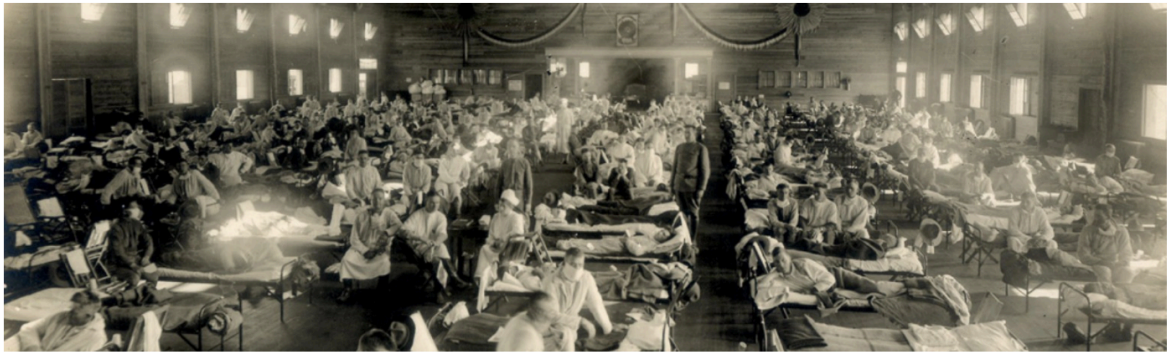
doi: https://doi.org/10.1101/2020.07.27.222349



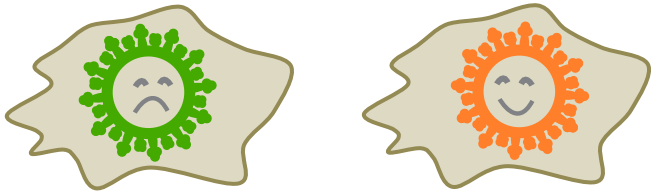


Zheng et al. (2020) bioRxiv 2020.07.27.222349

https://medium.com/@magdoll

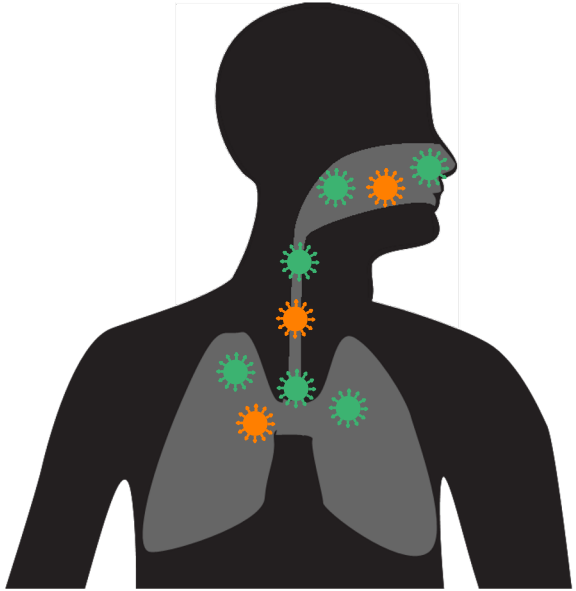


A Tale of Mutants: Sequencing the Full-Length Influenza Virus at the Single Cell Level



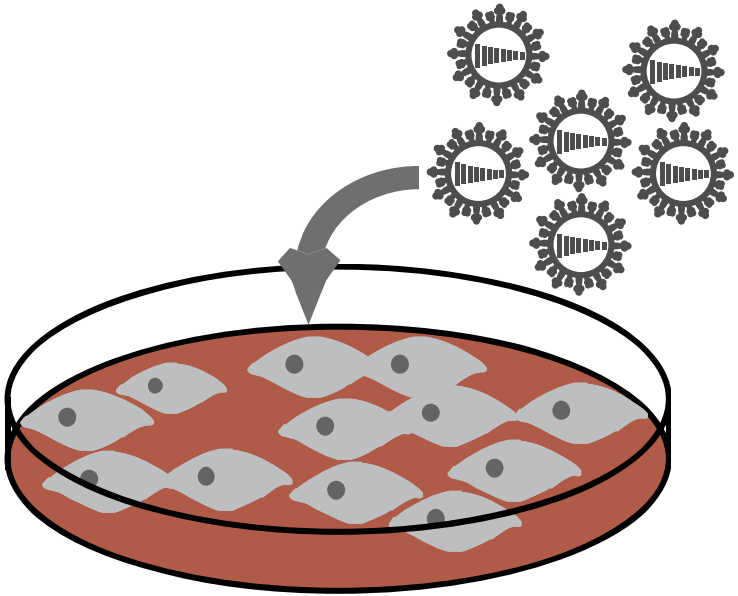
(slides hereafter courtesy of Jesse Bloom)

Human influenza infections are initiated by just a few virions

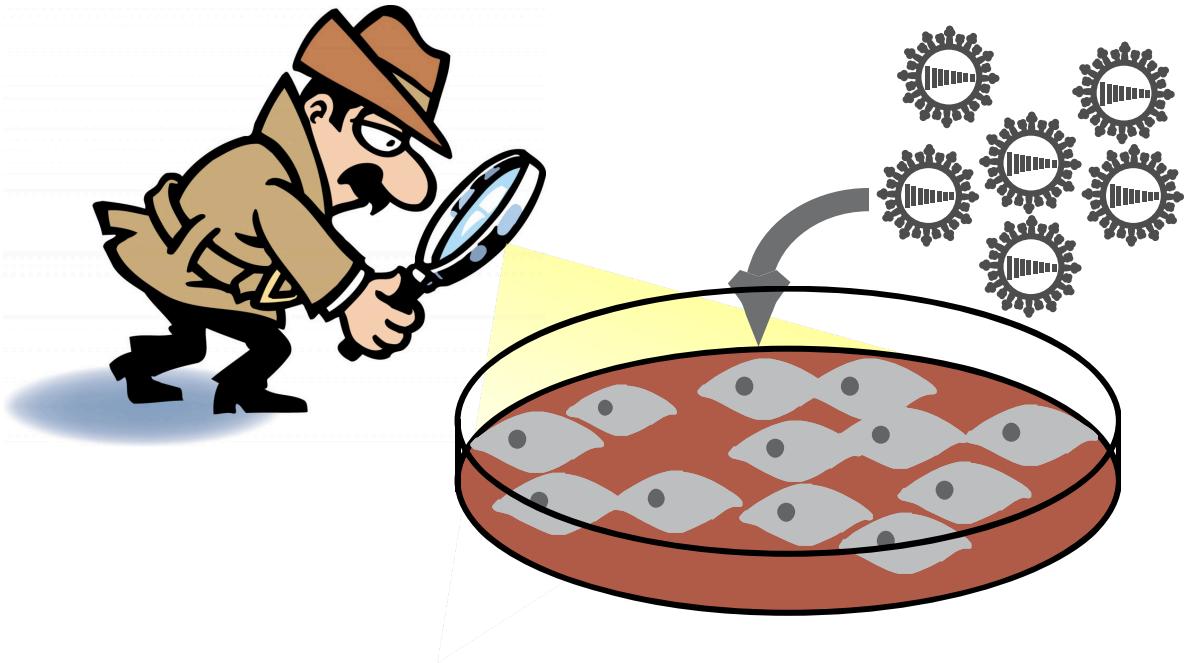


McCrone, John T., Robert J. Woods, Emily T. Martin, Ryan E. Malosh, Arnold S. Monto, and Adam S. Lauring. 2018. “Stochastic Processes Constrain the within and between Host Evolution of Influenza Virus.” *eLife* 7 (May). https://doi.org/10.7554/eLife.35962.
Xue, Katherine S., and Jesse D. Bloom. 2020. “Linking Influenza Virus Evolution within and between Human Hosts.” *Virus Evolution* 6 (1): veaa010.

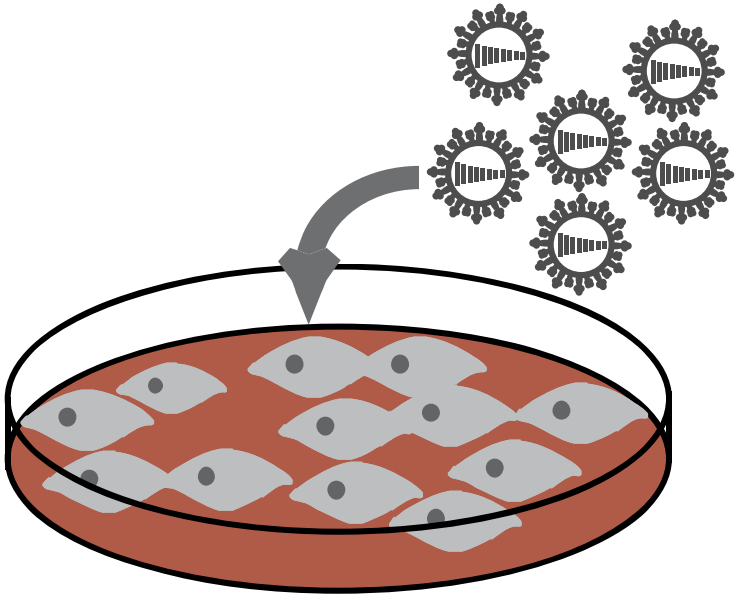
We take many cells, and infect them with many virions



We usually **average** over this entire process to study infection



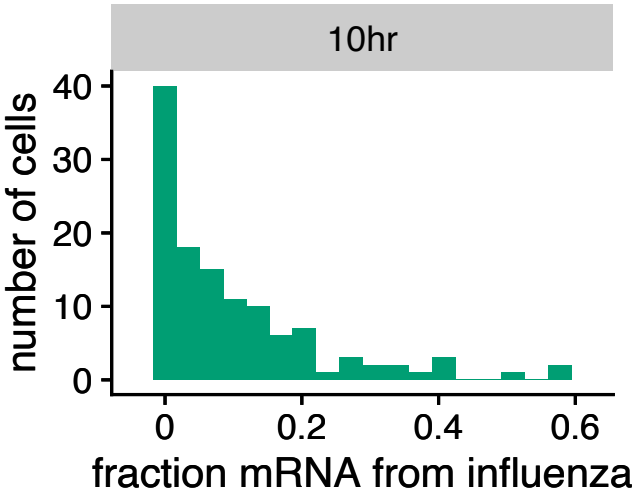
We infect A549 cells at low multiplicity of infection (MOI)



This yields a large **cell-gene matrix** that we can analyze computationally

	Cellular gene 1	**Cellular gene 2**	**…**	**Cellular gene 19,960**	**Cellular gene 19,961**	**Viral gene 1**	**…**	**Viral gene 8**
Cell 1	23	1	…	120	0	0	…	0
Cell 2	17	0	…	88	3	967	…	588
…	…	…	…	…	…	…	…	…
Cell 9,999	35	2	…	159	1	32	…	16
Cell 10,000	22	0	…	102	0	0	…	0

Extreme variation across single cells

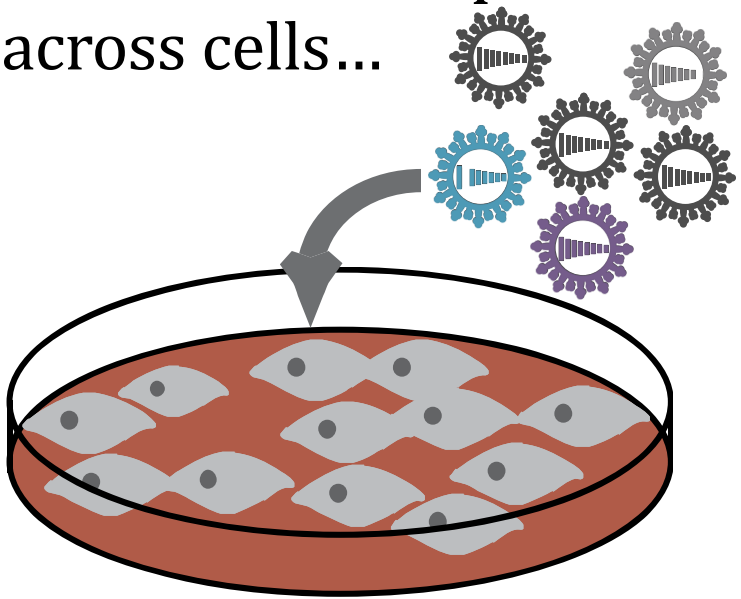


Russell, Alistair B., Cole Trapnell, and Jesse D. Bloom. 2018. “Extreme Heterogeneity of Influenza Virus Infection in Single Cells.” *eLife* 7 (February). https://doi.org/10.7554/eLife.32303.

I said that we used a stock of "wildtype" virus

Actually, ***all*** viral stocks contain mutants

Maybe mutations in the virions explain variation across cells…



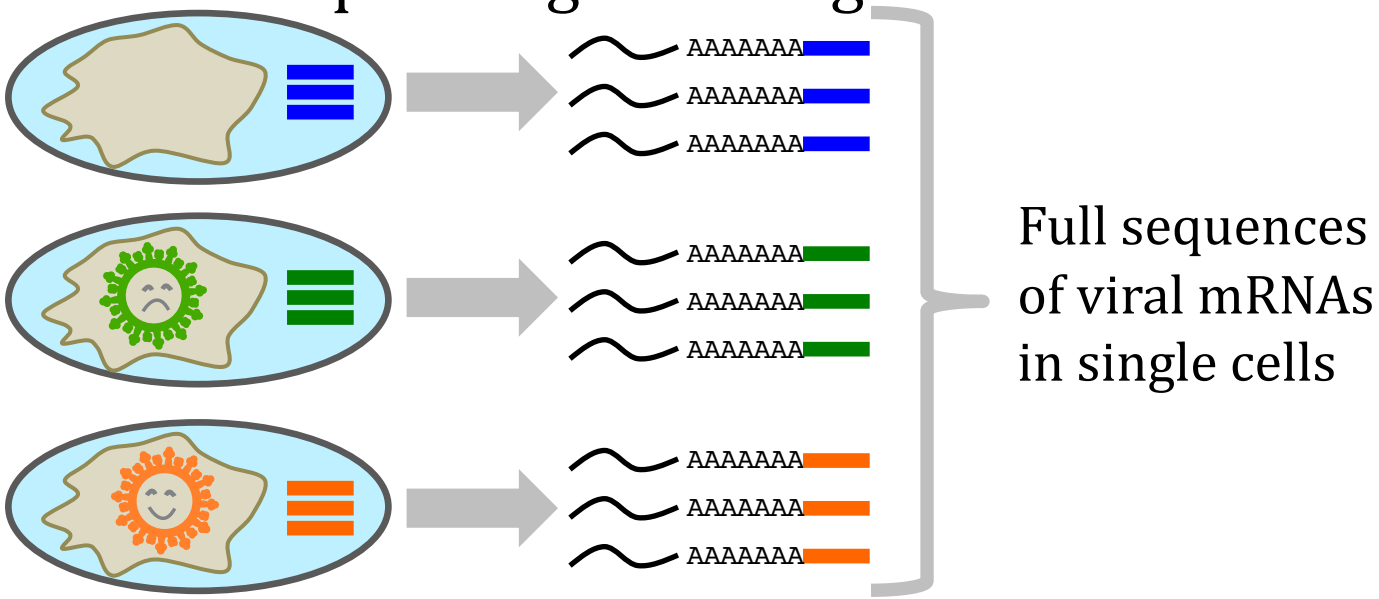
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Single-cell transcriptomics counts mRNAs, it doesn't tell us if they have mutations

	Cellular gene 1	**Cellular gene 2**	**…**	**Cellular gene 19,960**	**Cellular gene 19,961**	**Viral gene 1**	**…**	**Viral gene 8**
Cell 1	23	1	…	120	0	0	…	0
Cell 2	17	0	…	88	3	967	…	588
…	…	…	…	…	…	…	…	…
Cell 9,999	35	2	…	159	1	32	…	16
Cell 10,000	22	0	…	102	0	0	…	0

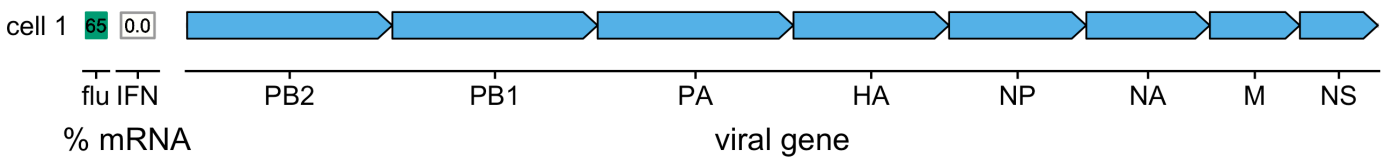
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

We start with the same process as for single-cell transcriptomics
But we also perform full-length PacBio sequencing on viral genes



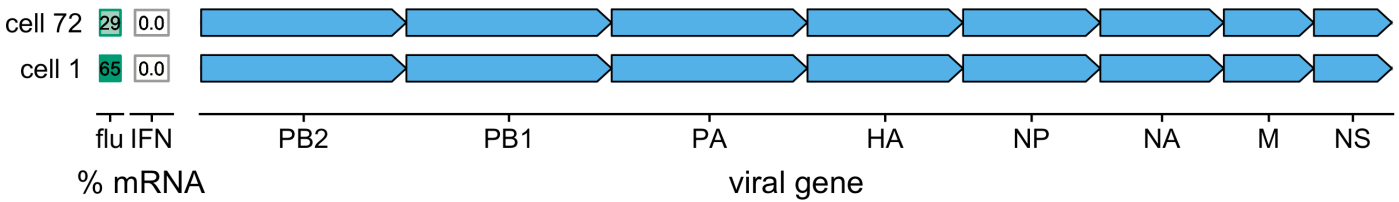
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Cells infected by wildtype virions often produce lots of viral mRNA



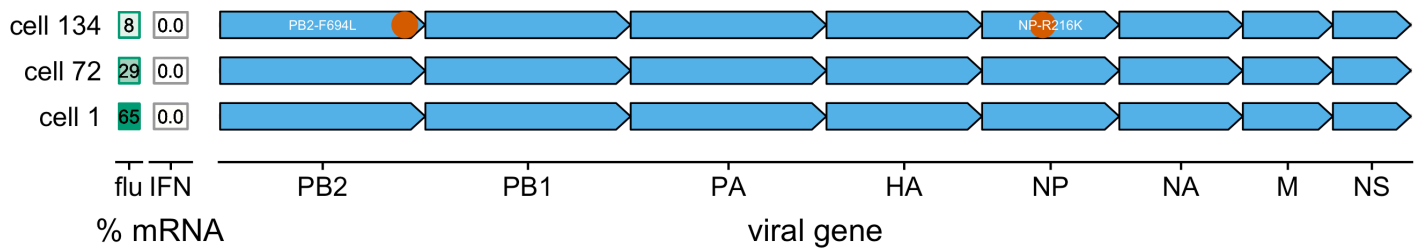
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Although some wildtype virions produce less viral mRNA than others



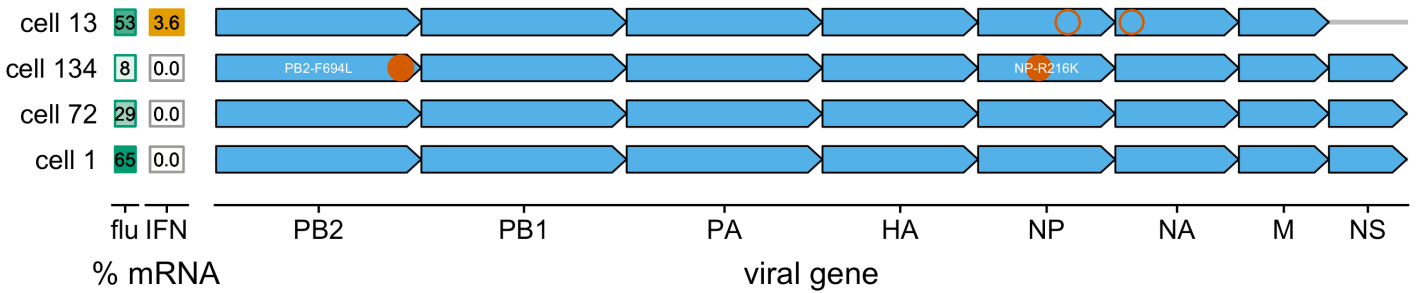
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Virions with mutations sometimes produce little viral mRNA



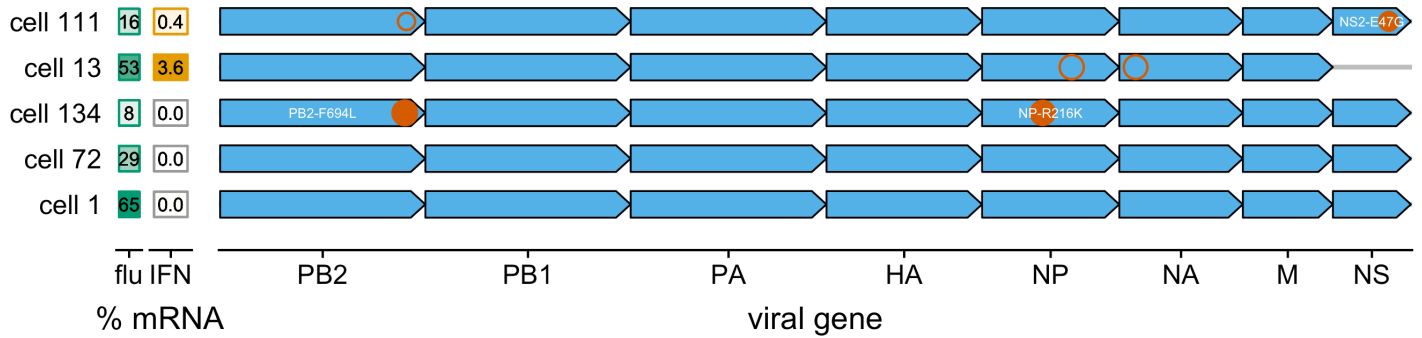
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Virions with defects sometimes produce IFN: fails to express NS



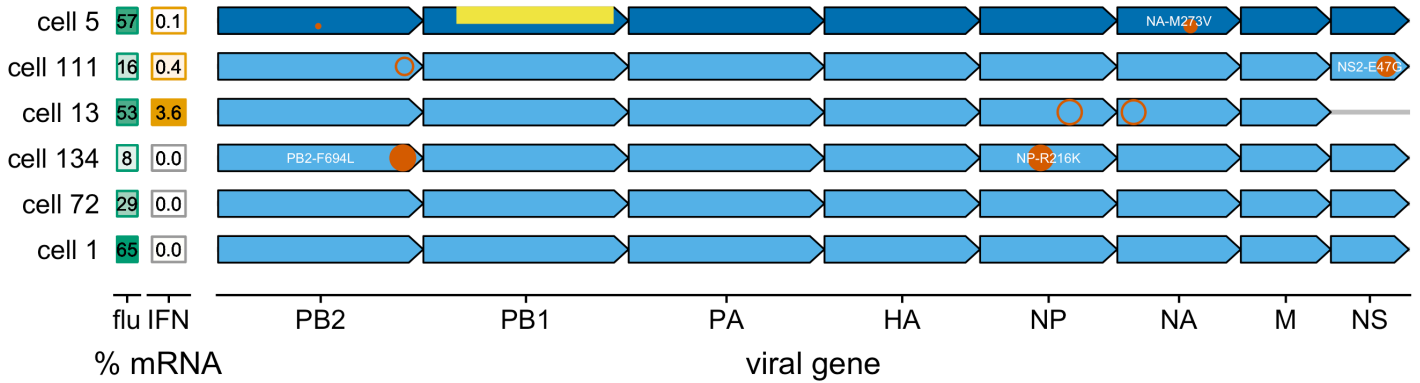
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Virions with defects sometimes produce IFN: point mutation in NS



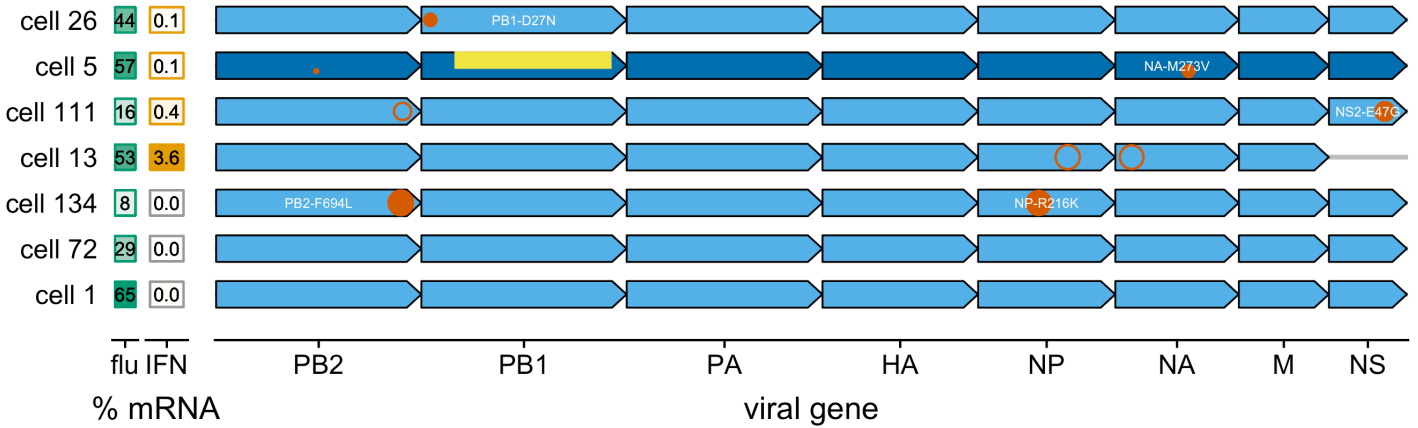
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Virions with defects sometimes produce IFN: internal deletion in PB1



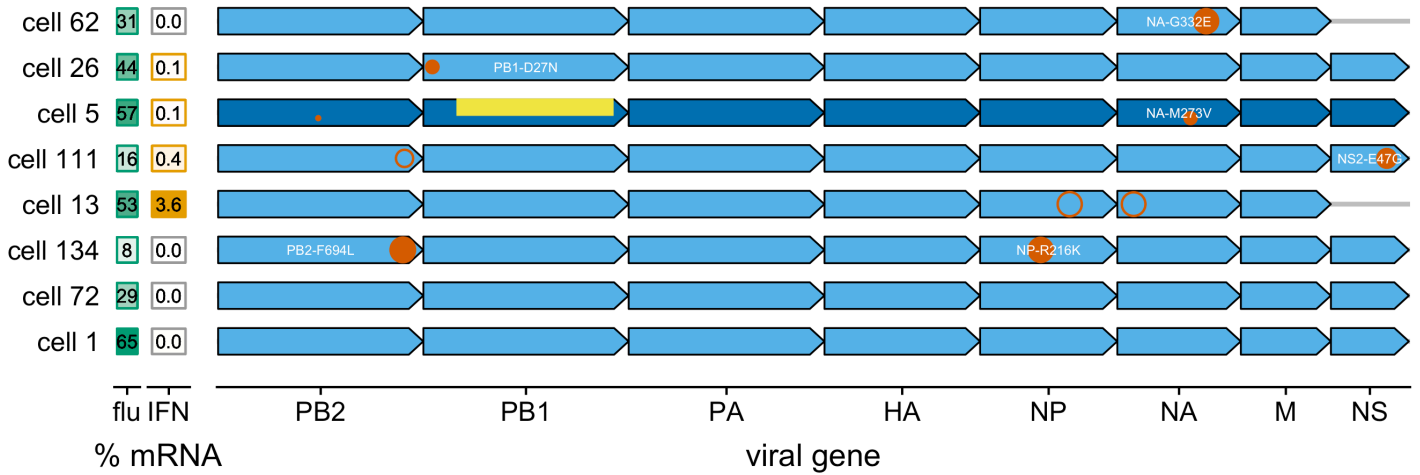
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Virions with defects sometimes produce IFN: point mutation in PB1



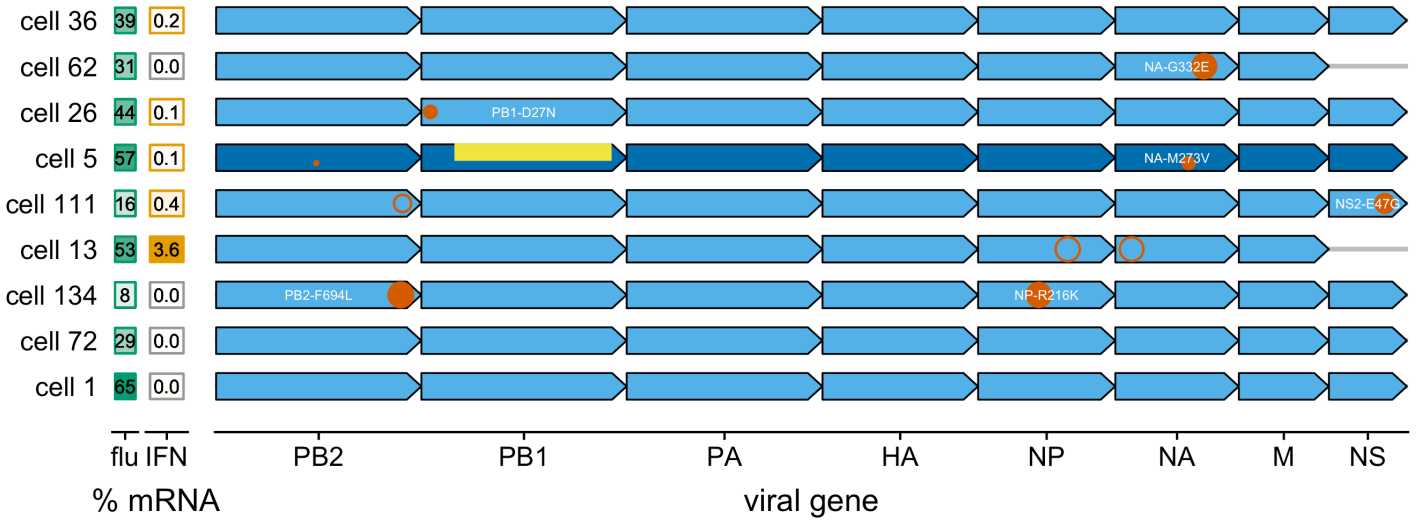
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

But even virions lacking NS do not always induce IFN



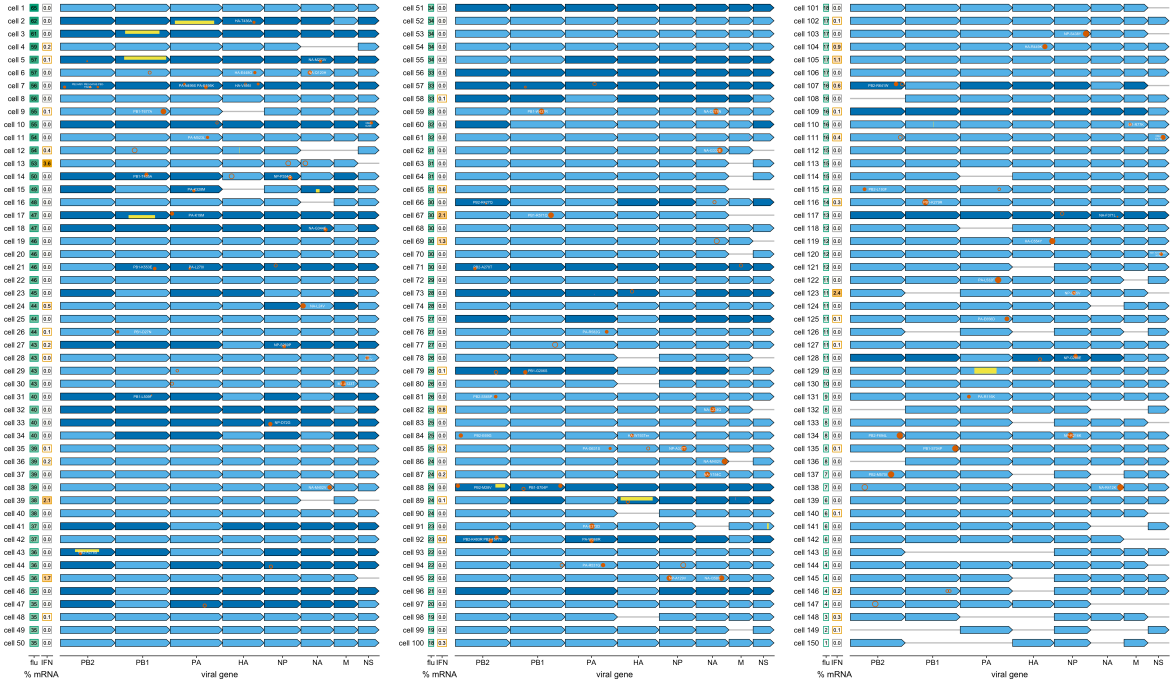
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

And sometimes wildtype virions induce IFN



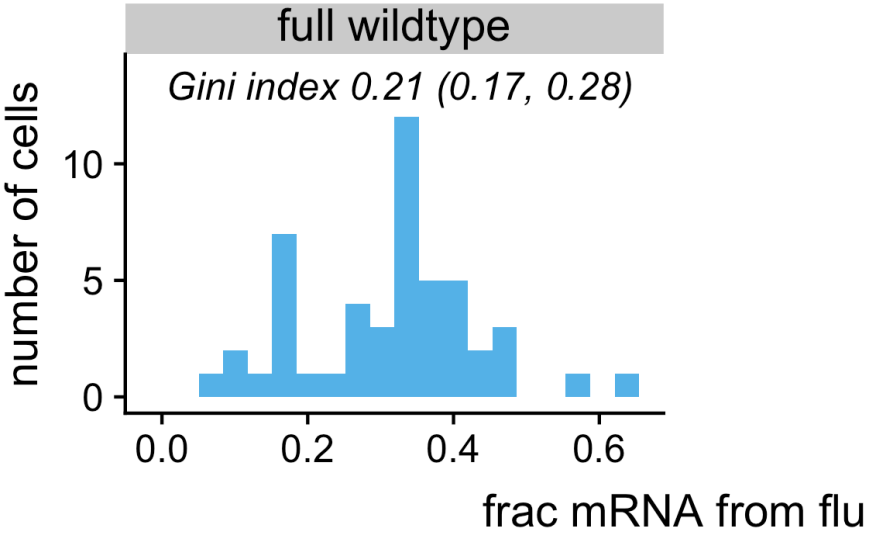
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

150 cells infected, only 49 by wildtype virions



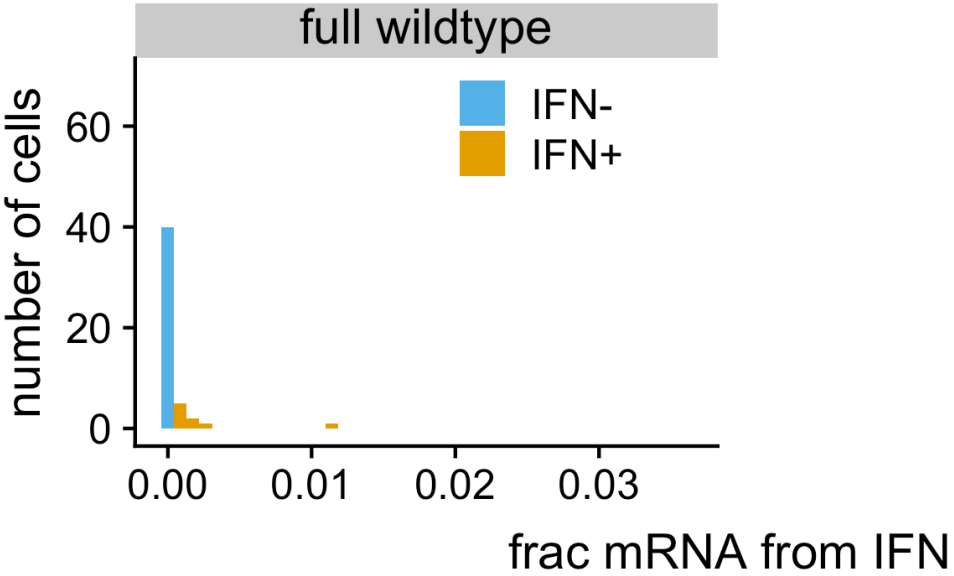
Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Infections by wildtype virions are less heterogeneous than ones by mutant ones



Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

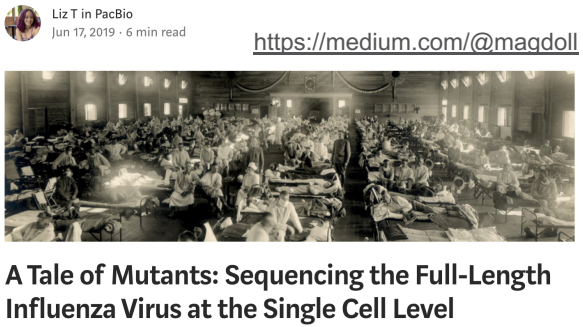
Infections by mutant virions induce more IFN

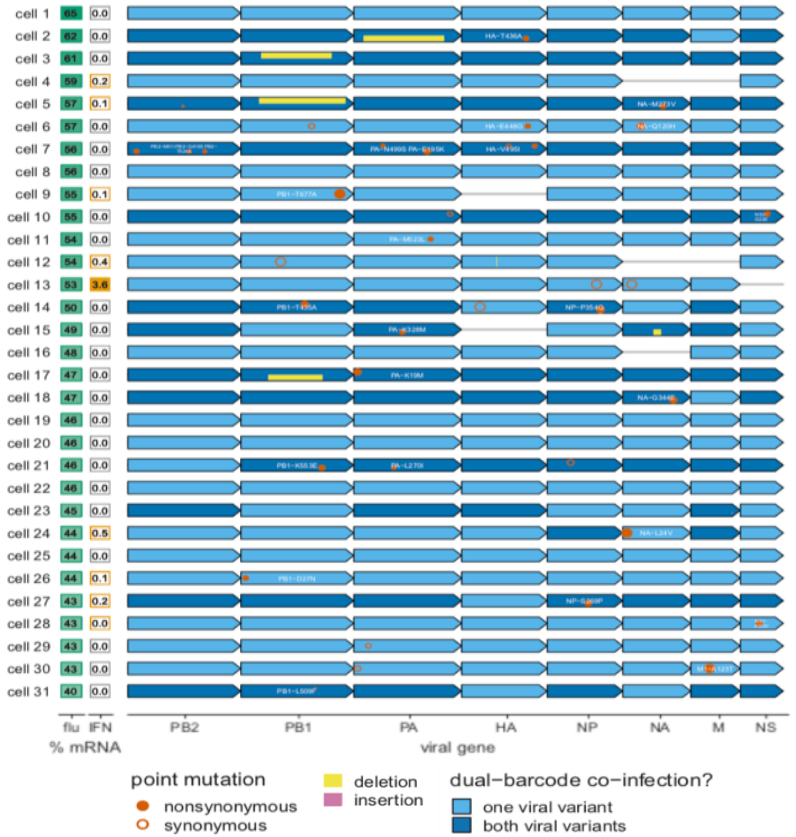


Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

SINGLE-CELL VIRAL SEQUENCING

- Single-cell sequencing of H1N1 influenza
- Full-length viral transcripts reveal cell-to-cell variation on mutational landscape
- 10hr post-infection, only 49 of 150 infected cells remain wild type
- Mutations linked to differences in viral load and the innate immune response





Russell, AB, et. al. (2019). Single-cell virus sequencing of influenza infections that trigger innate immunity. *Journal of Virology*. doi: 10.1128/JVI.00500-19.

Single-Cell Iso-Seq Bioinformatics

SINGLE-CELL ISO-SEQ BIOINFORMATICS WORKFLOW

HiFi reads

SINGLE-CELL ISO-SEQ BIOINFORMATICS WORKFLOW

HiFi reads

Remove cDNA primers

Extract UMI and BC

SINGLE-CELL ISO-SEQ BIOINFORMATICS WORKFLOW

HiFi reads

Remove cDNA primers

Extract UMI and BC

Cluster by (UMI,BC)

Classify Transcripts

Remove Artifacts

id	length	UMI	BC	count
molecule/34465	833	ATGGTATA	GAACGTGGTCGA	1
molecule/34597	807	GTACGATG	CAGGATTAAGAG	3
molecule/34598	807	ACCTCCTG	GACCAGACTGGA	1
molecule/34638	807	AAAGTCAC	TATATGGTAGGT	2

https://github.com/PacificBiosciences/IsoSeq/blob/master/isoseq-deduplication.md

SINGLE-CELL ISO-SEQ BIOINFORMATICS WORKFLOW

HiFi reads

Remove cDNA primers

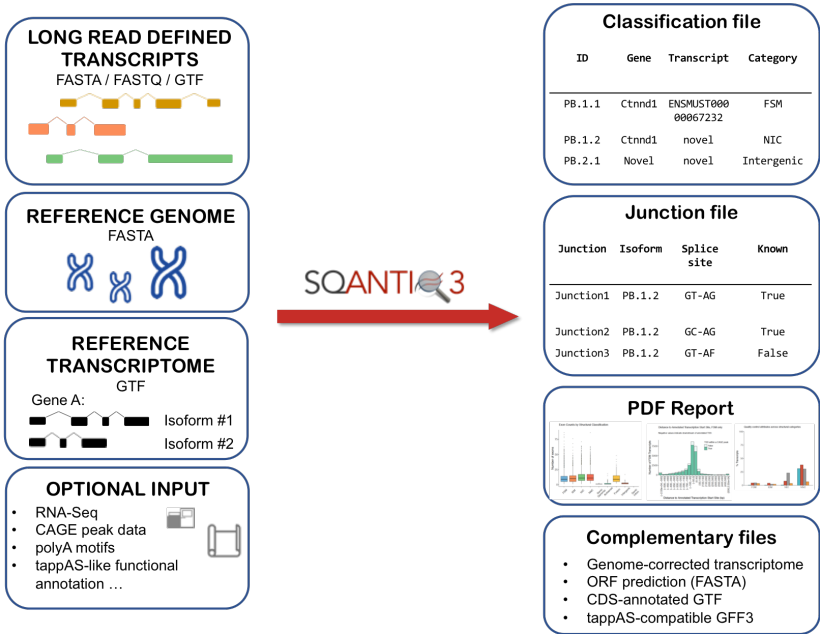
Extract UMI and BC

Cluster by UMI

Classify Transcripts

Remove Artifacts

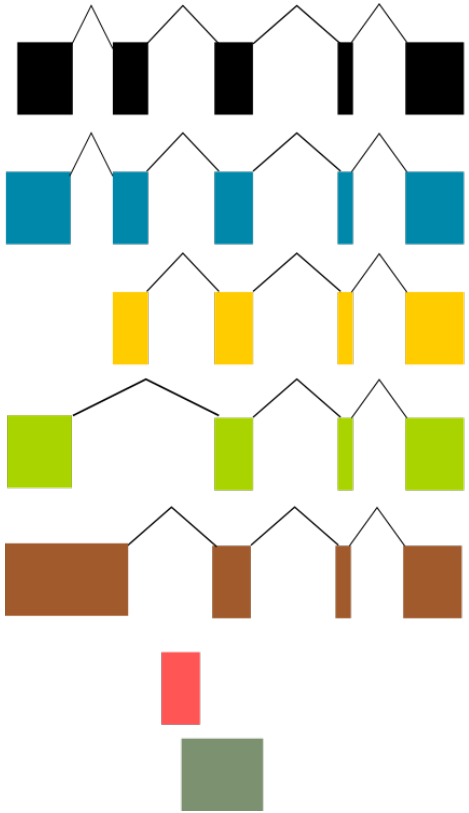
SQANTI3: QUALITY CONTROL OF TRANSCRIPTOMES



https://github.com/ConesaLab/SQANTI3

https://github.com/Magdoll/cDNA_Cupcake/wiki/Iso-Seq-Single-Cell-Analysis:-Recommended-Analysis-Guidelines

TRANSCRIPT CLASSIFICATION BY SQANTI



Reference

FSM Full Splice Match, matches reference perfectly

ISM Incomplete Splice Matches, matches reference partially

NIC Novel In Catalog, novel isoform using known junctions

NNC Novel Not in Catalog, novel isoforms using novel junctions

Genic Intron Within intron

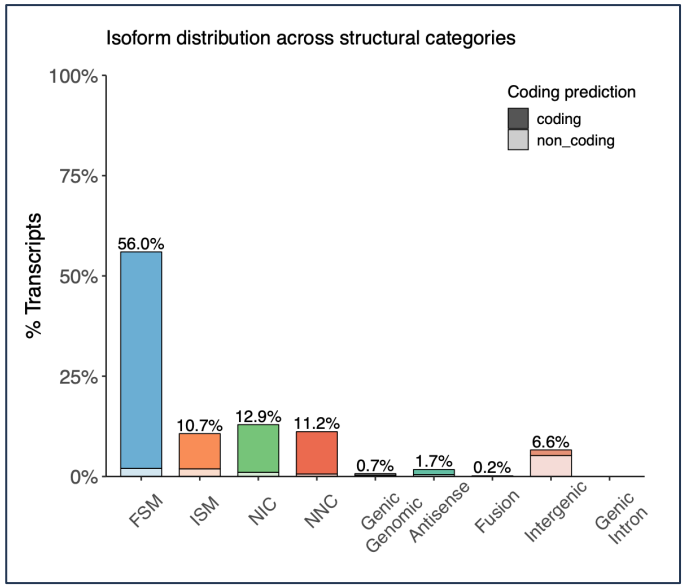
Genic Genomic Overlap with introns and exons

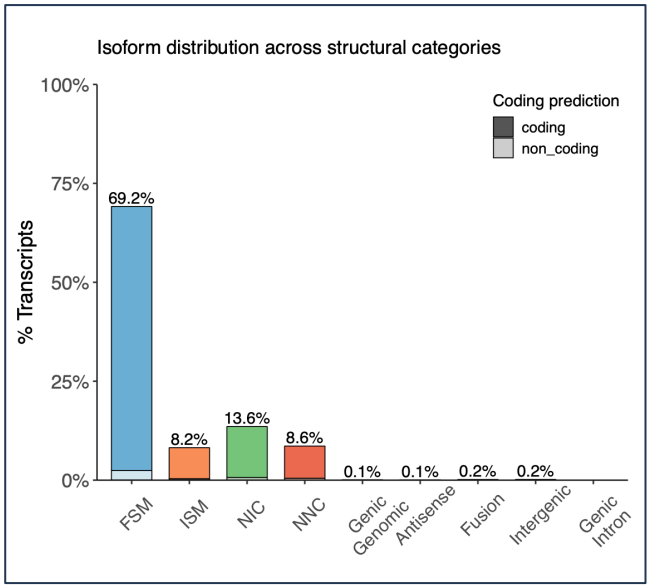
Tardaguila, M. *et al.* SQANTI: extensive characterization of long read transcript sequences for quality control in full-length transcriptome identification and quantification. 1–31 (2017). doi:10.1101/118083

SQANTI3: BEFORE AND AFTER FILTERING

After SQANTI3 filtering, percentage of FSMs increase

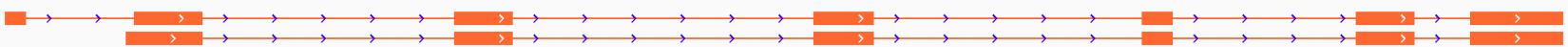
Most filtered transcripts are genic, genomic, or intergenic





SINGLE-CELL ISO-SEQ BIOINFORMATICS WORKFLOW

id	length	transcript	gene	category	UMI	BC
molecule/34465	833	ENST00000392221.5	ENSG00000114942.14	full-splice_match	ATGGTATA	GAACGTGGTCGA
molecule/34597	807	ENST00000392222.7	ENSG00000114942.14	full-splice_match	GTACGATG	CAGGATTAAGAG
molecule/34598	807	ENST00000392222.7	ENSG00000114942.14	full-splice_match	ACCTCCTG	GACCAGACTGGA
molecule/34638	807	ENST00000392222.7	ENSG00000114942.14	full-splice_match	AAAGTCAC	TATATGGTAGGT



Cell:	1	2	...	N
Transcript 1	10	1		5
Transcript 2	8	6		0
.	.	.		.
.	.	.		.
Transcript M	3	0		12

https://github.com/Magdoll/cDNA_Cupcake/wiki/Iso-Seq-Single-Cell-Analysis:-Recommended-Analysis-Guidelines

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