Single-cell transcriptomics in plants

Standard and advanced scRNA-seq data analysis workflows

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Wageningen University & Research
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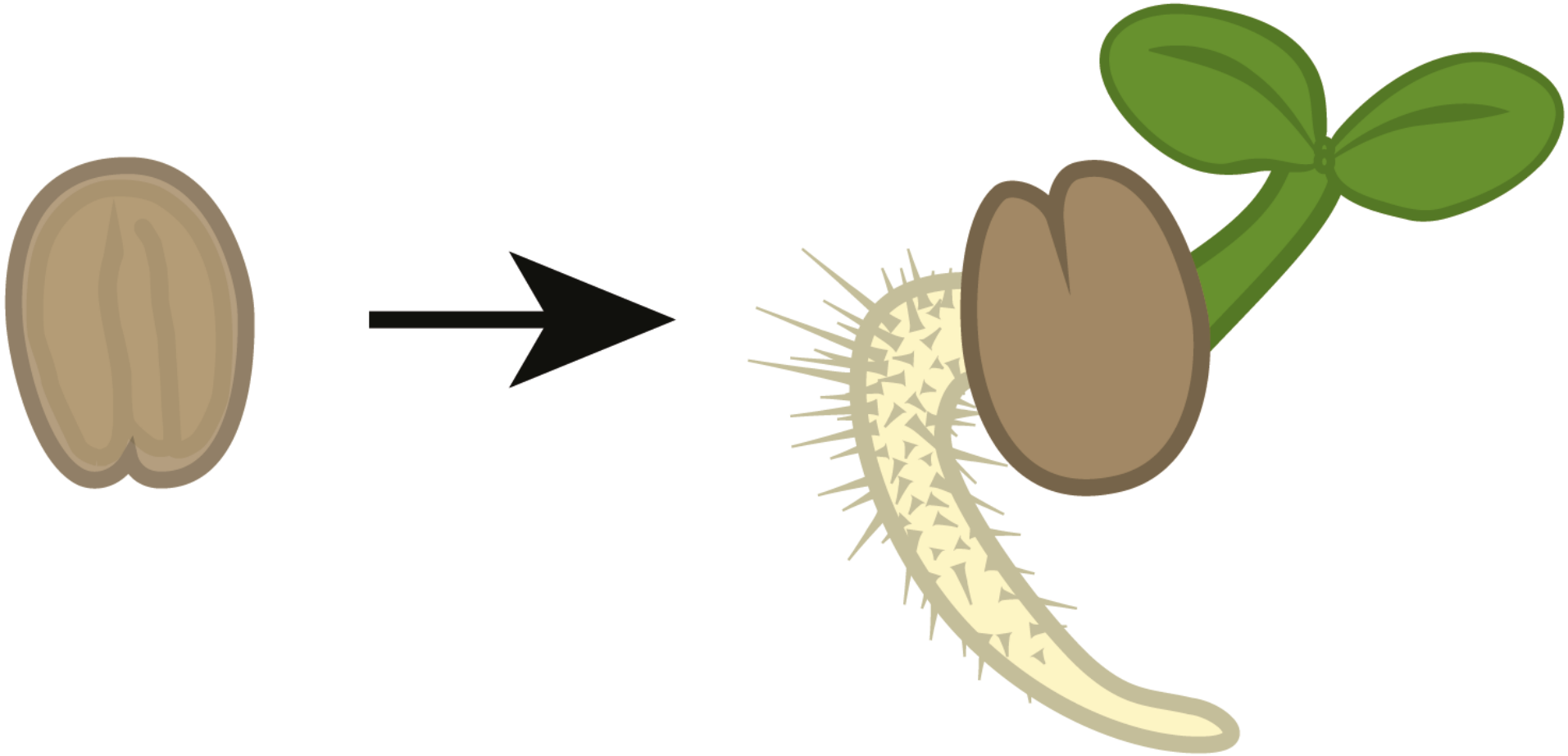
Overview

- Why single cells?
- How-to:
  - Biology → Hardware → Chemistry → Software → *Knowledge?*
- The standard workflow
  - Reads → Counts → Clusters → Markers
  - Dimensionality reduction
- Advanced techniques

Single cell RNA-seq: Why single cells?



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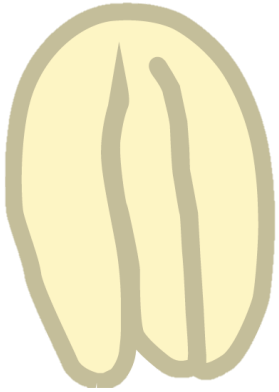
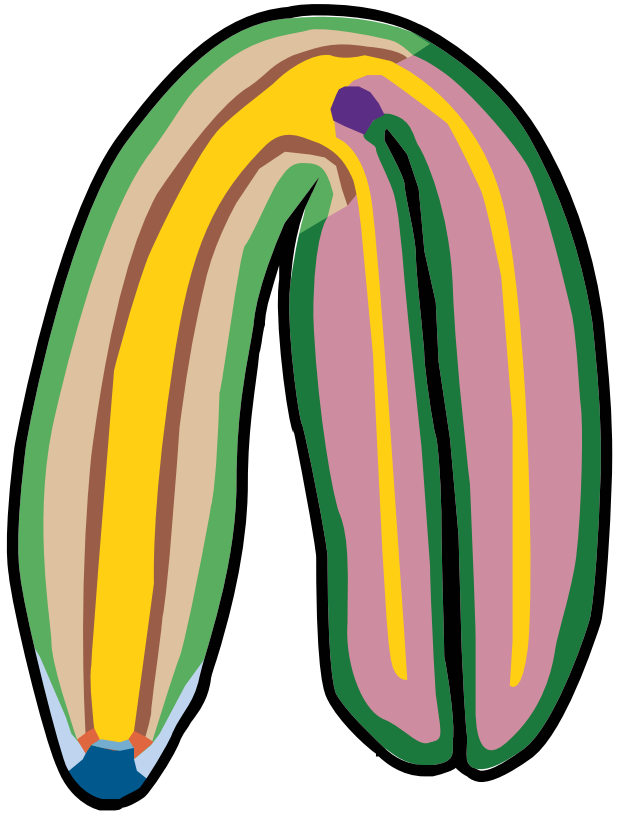


- Vascular
- Ground tissue
- Hypophysis
- Cotyledon
- Shoot meristem
- Cortex
- Endodermis
- Quiescent center
- Columella
- Lateral root cap



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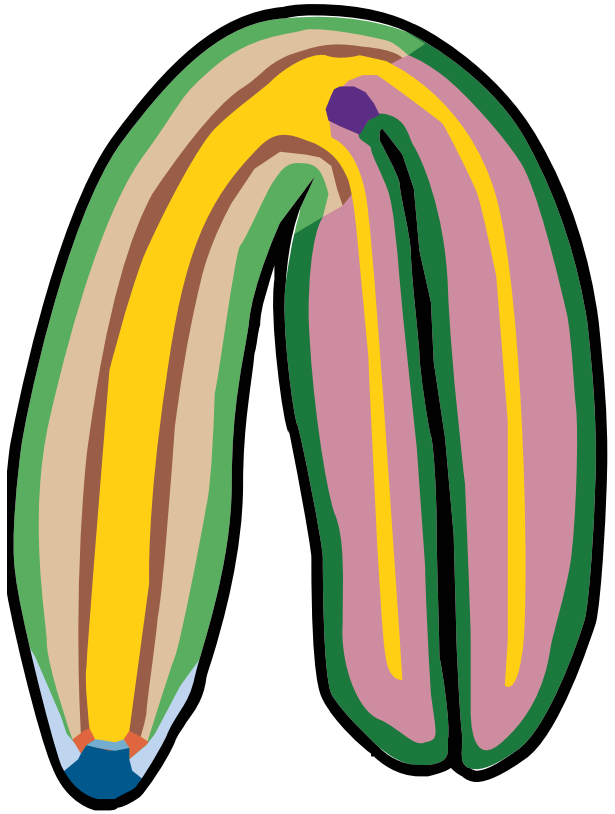
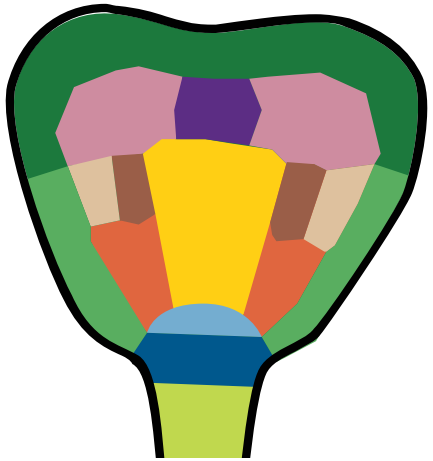
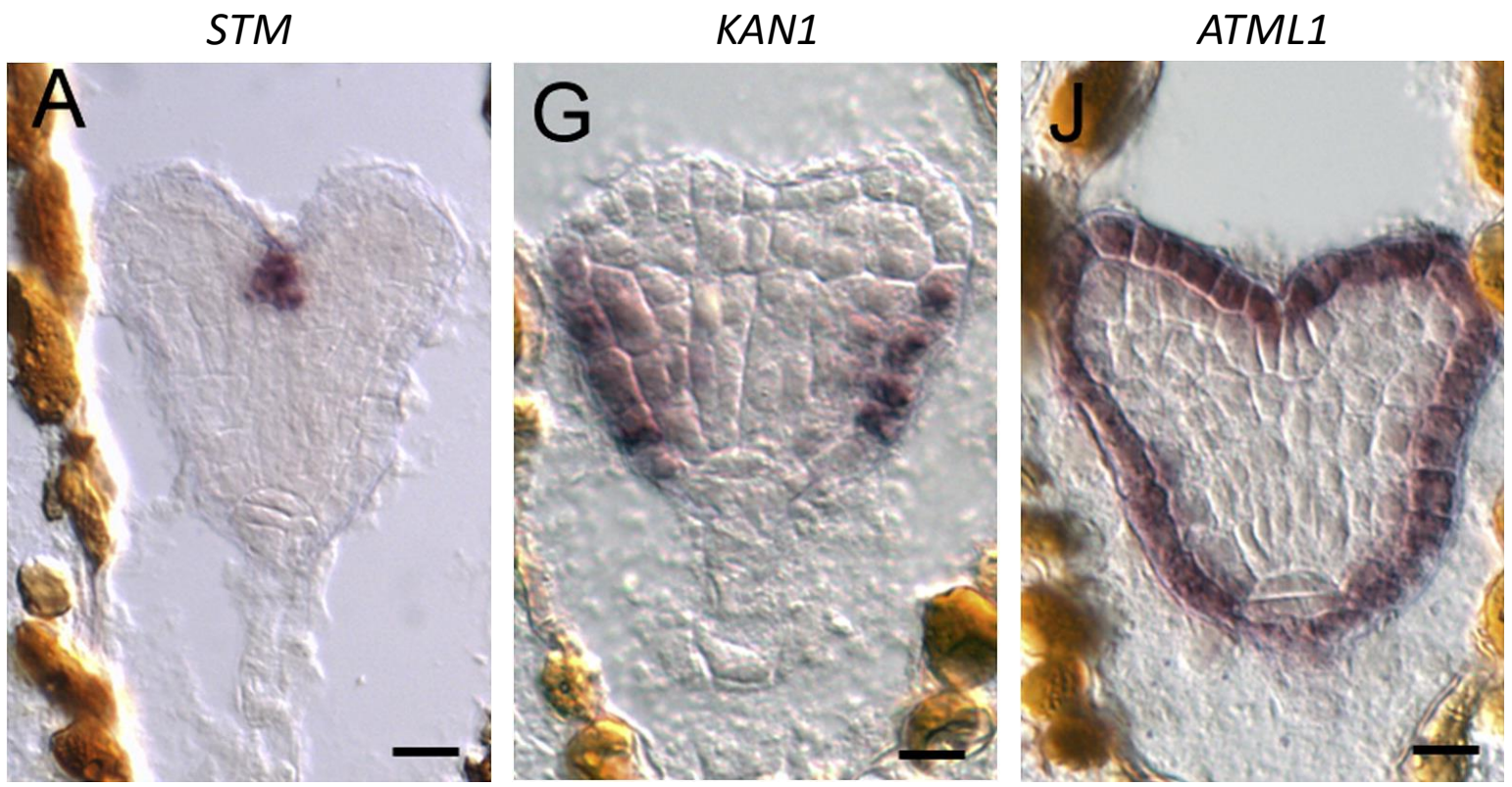


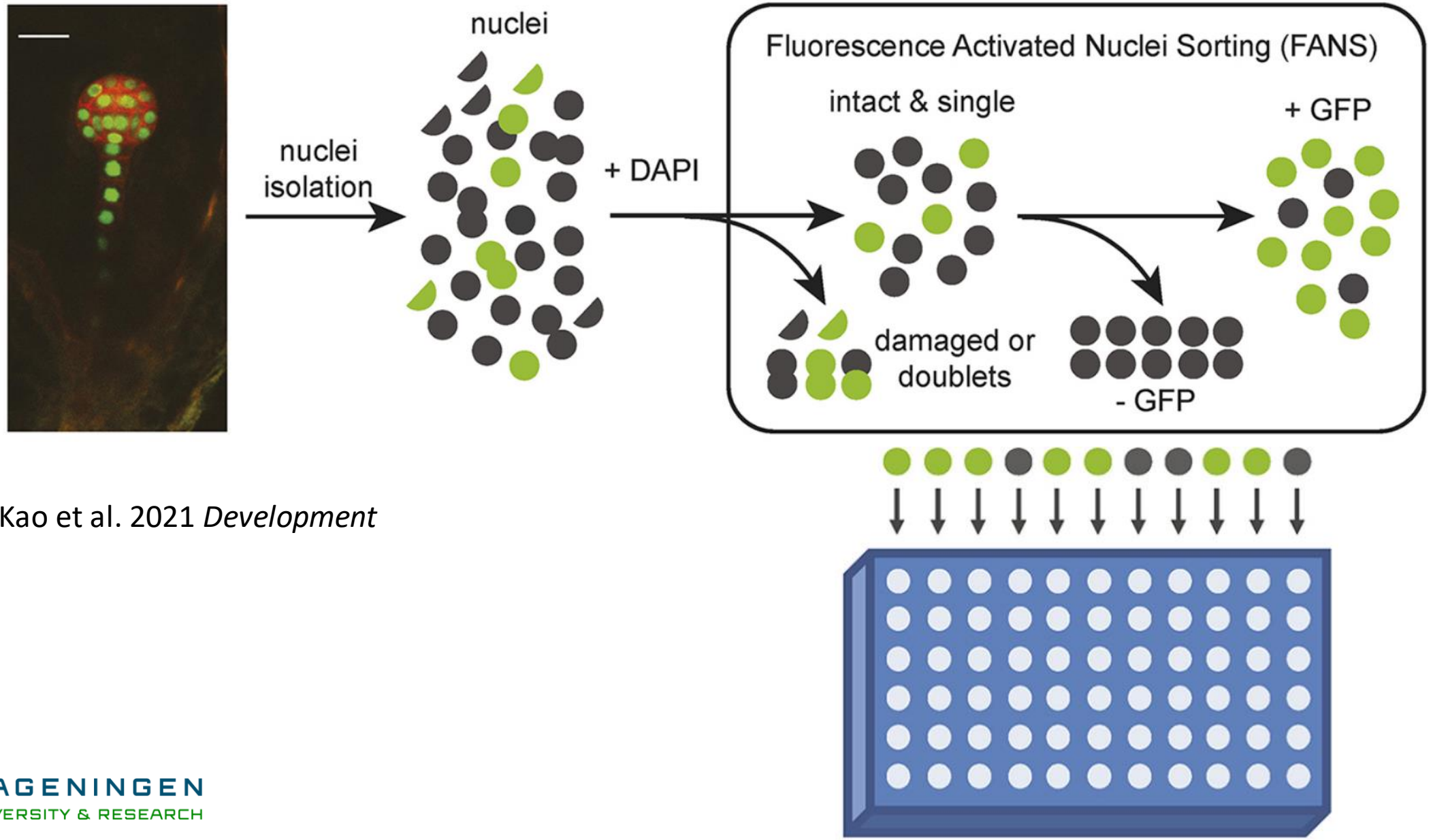
Single cell RNA seq: Why RNA?





Grigg et al. 2009 *Current Biology*

Capturing cells



Kao et al. 2021 *Development*

Capturing the transcriptome

Capturing the transcriptome

	Protocol	Total RNA Input (ng)
Unstranded RNA-seq	TruSeq	≥100
	Ovation	≥0.5
	SMARTer	≥0.01 (single cell)
	Smart-seq2	≥0.01 (single cell)
Strand-specific RNA-seq	Not-so-random (NSR)	≥1,000
	TruSeq Stranded (dUTP)	≥100
	SMART-Seq Stranded	≥10
Barcoded 3′ end sequencing	QuantSeq	≥1
	10X Chromium	≥0.01 (single cell)
	MARS-seq	≥0.01 (single cell)
Poly(A) site sequencing	3P-seq	≥20,000
	TAIL-seq	≥100,000
	mTAIL-seq	≥50
5′ cap sequencing	CAGE	≥50,000
	nanoCAGE	≥50
	STRT-seq	≥0.01 (single cell)
Degradome (5′P) sequencing	PARE	≥50,000
	GMUCT2	≥5,000
	5P-seq	≥5,000
Long-read sequencing	Pacbio Iso-Seq	≥300
	ONT Direct RNA	~25,000 (≥500ng polyA+)

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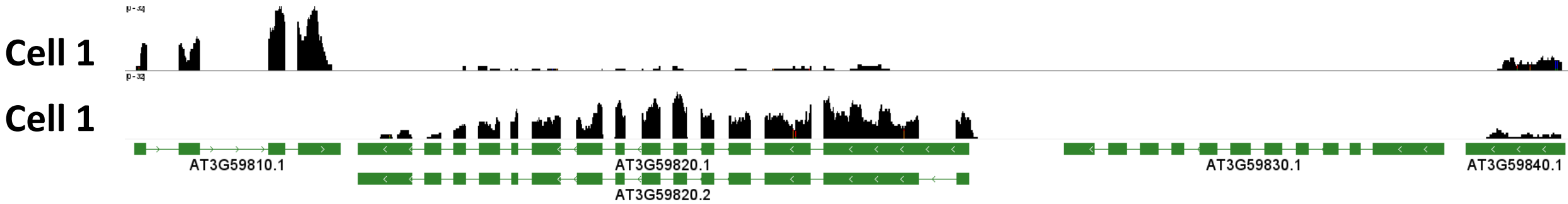
RNA x Cells... Now what?

- Align sequenced reads with transcripts
  - Genome alignments (STAR, HiSat)
  - Pseudoalignment (Kallisto, Salmon)



	Cell 1	Cell 2
AT3G59810	110	0
AT3G59820	20	304
AT3G59830	0	0
AT3G59840	22	13

More information in Practical 1!

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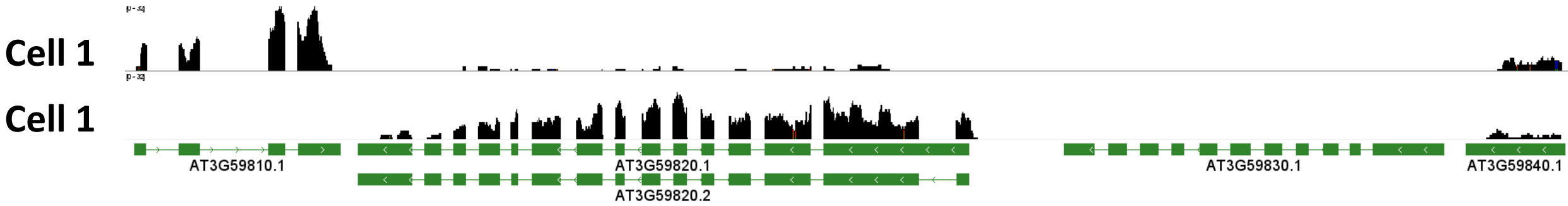

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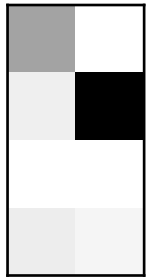

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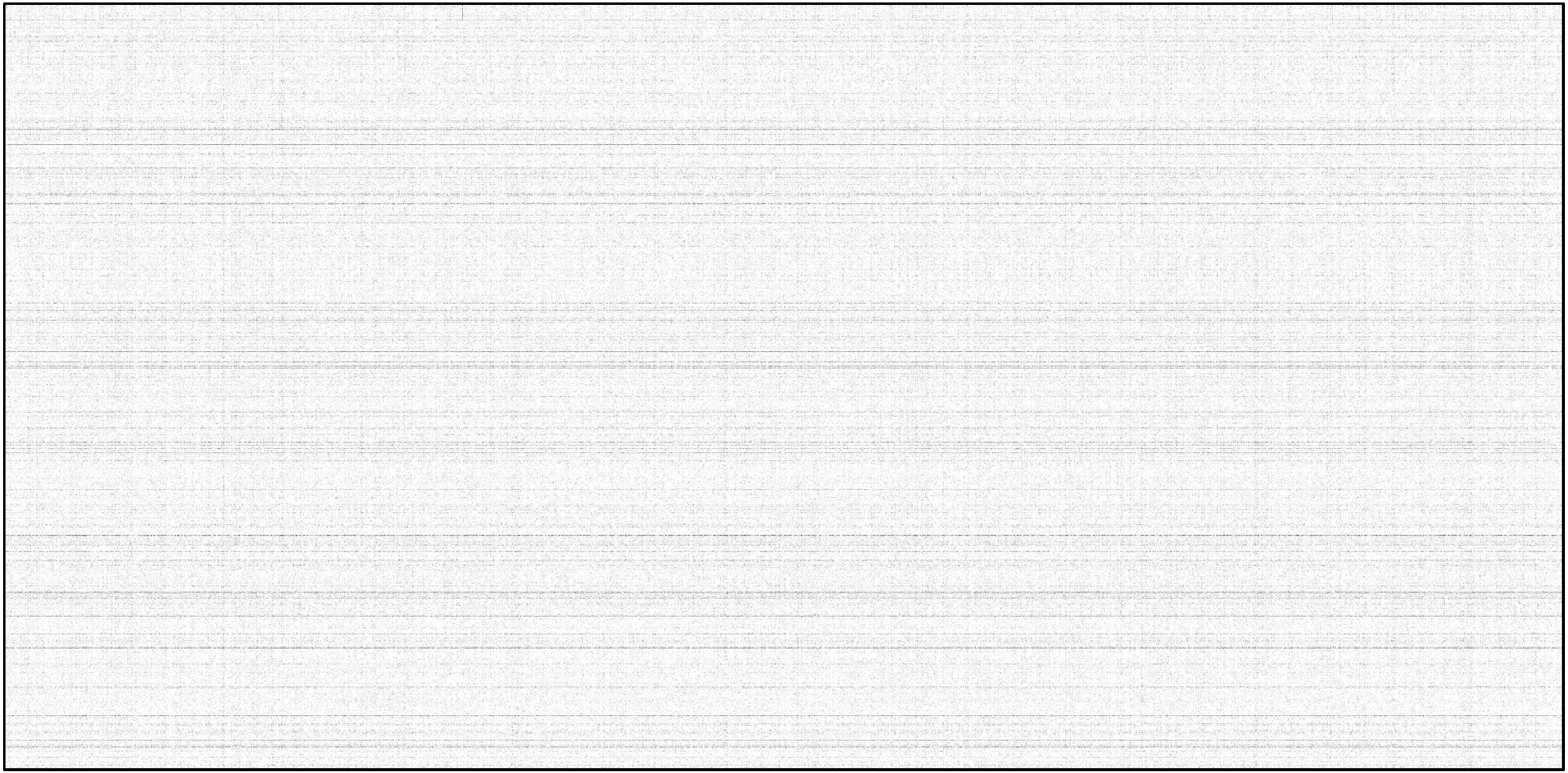
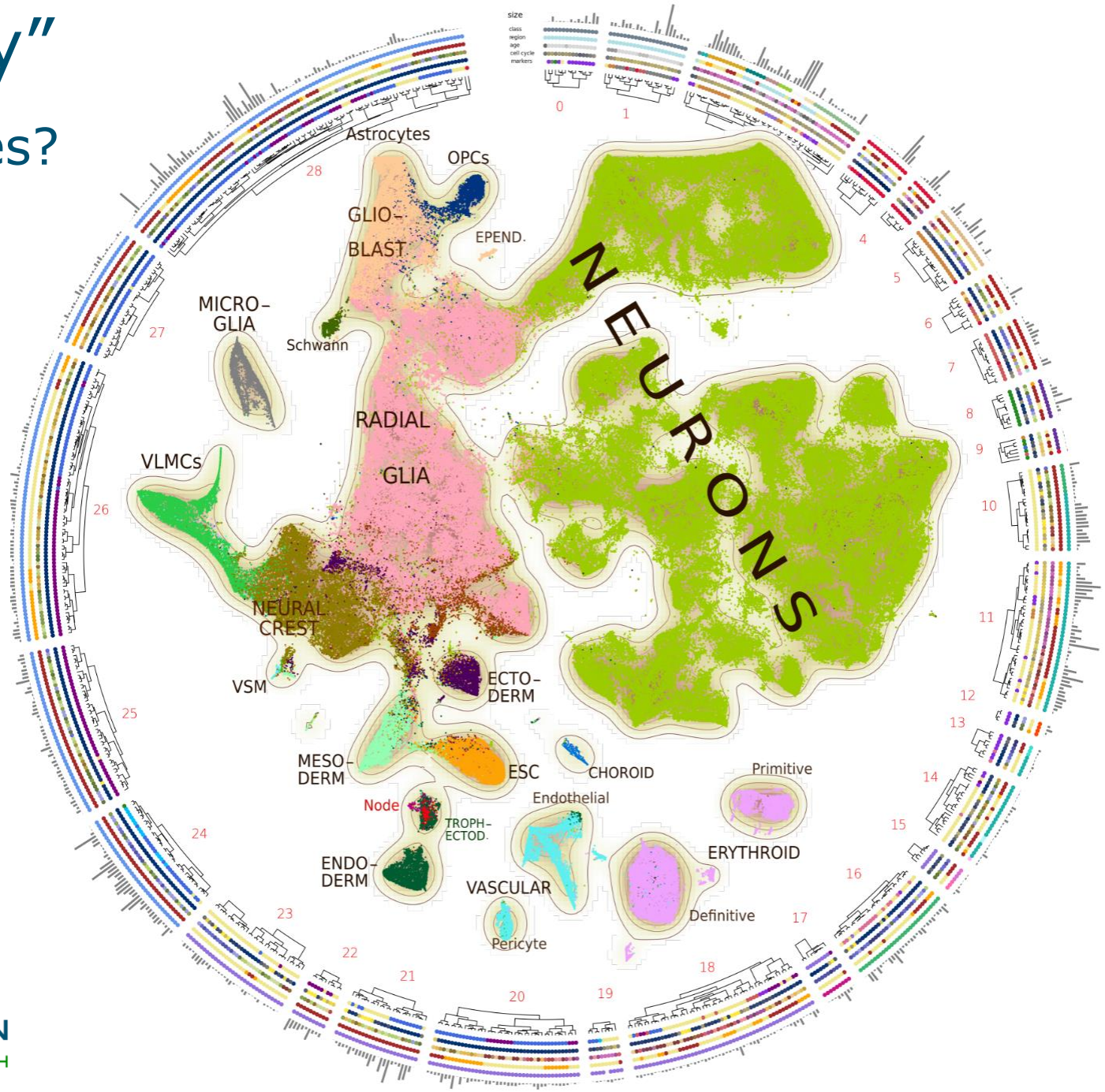


“Similarity”

- Marker genes?
  - *NANOG*



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La Manno et al. 2021 *Nature*

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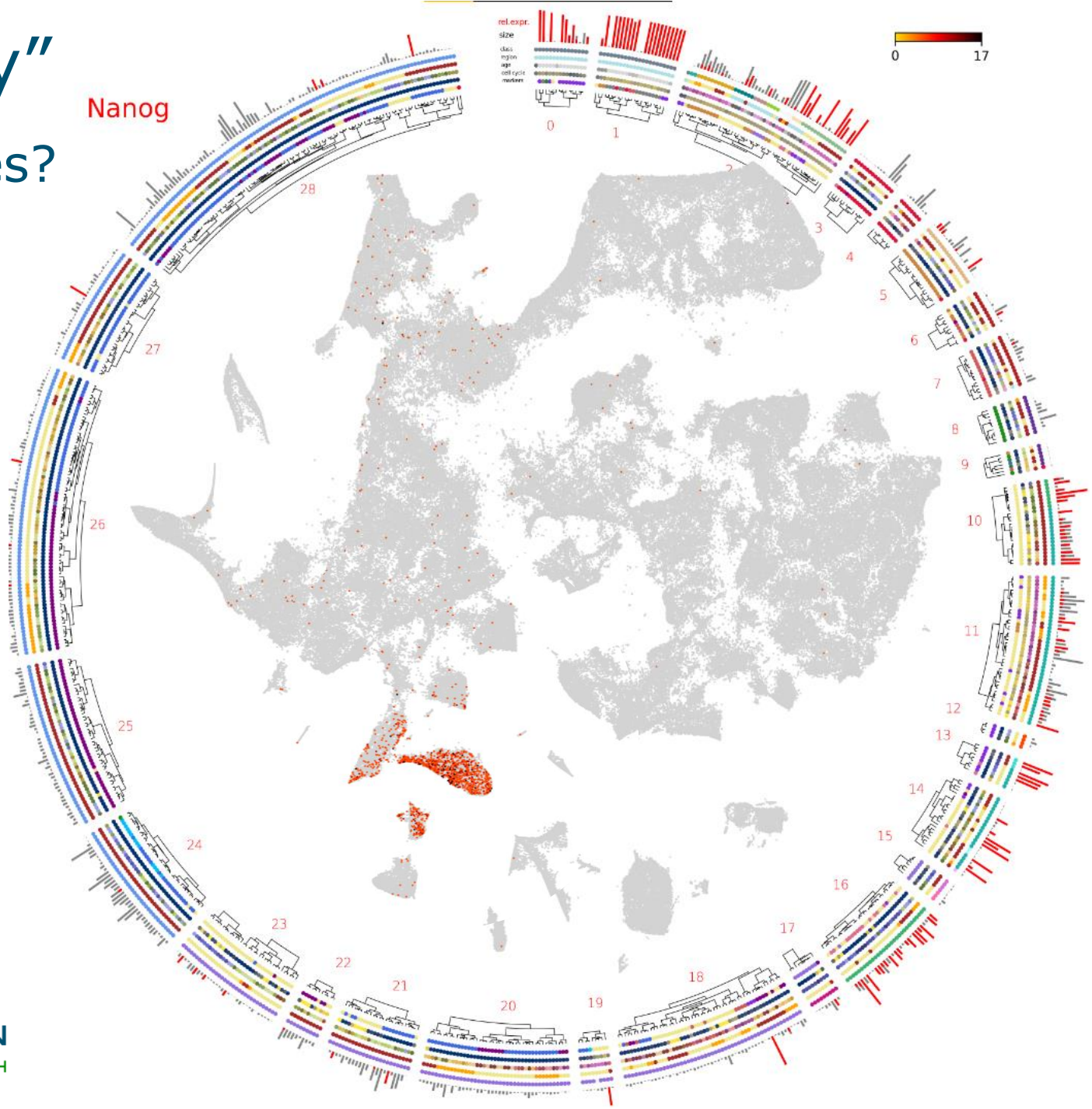

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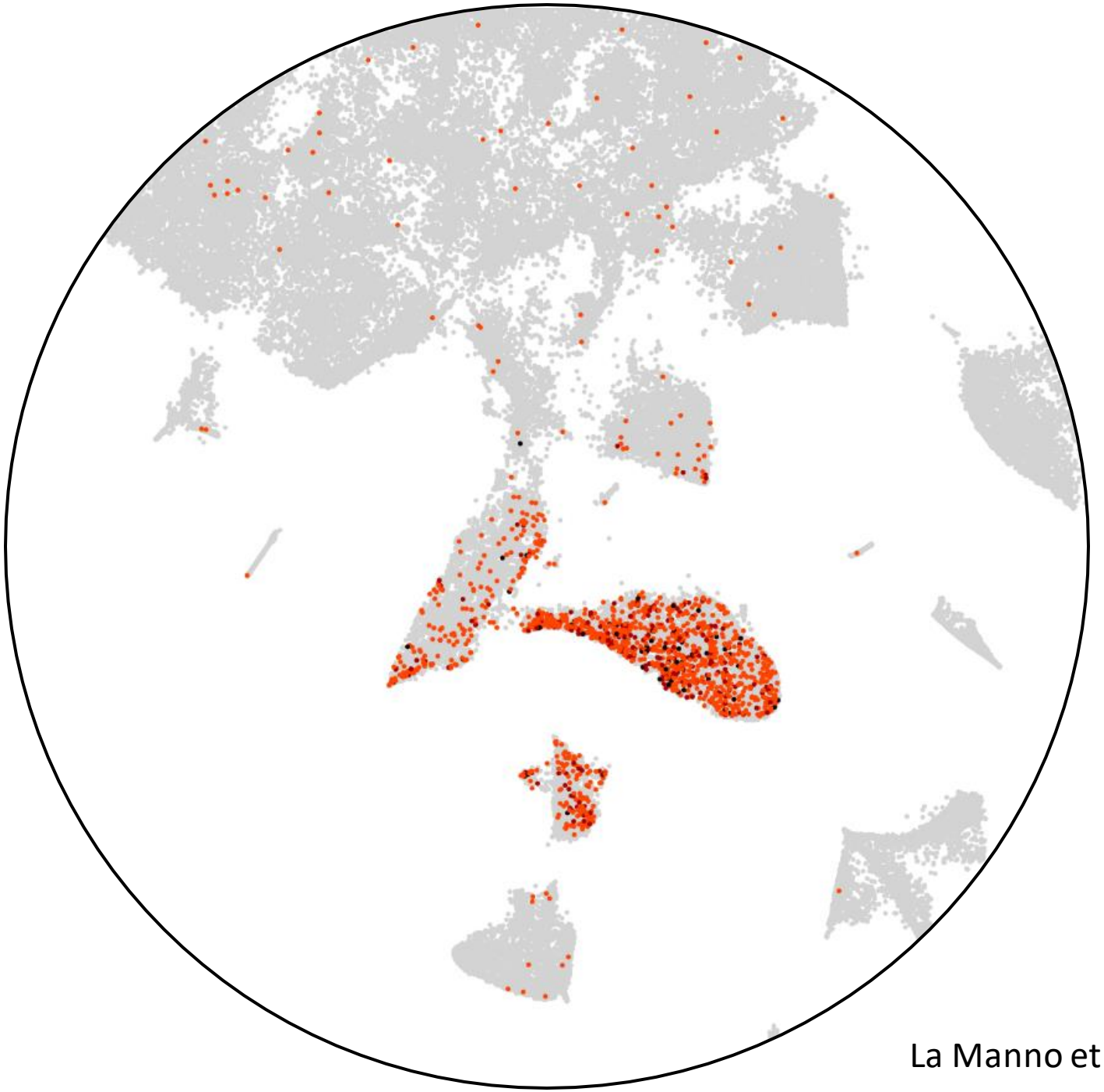
La Manno et al. 2021 *Nature*

“Similarity”

- Marker genes?
  - *NANOG*

⚠ Not all ESCs have *NANOG*

⚠ Not all *NANOG*+ cells are ESCs



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La Manno et al. 2021 *Nature*

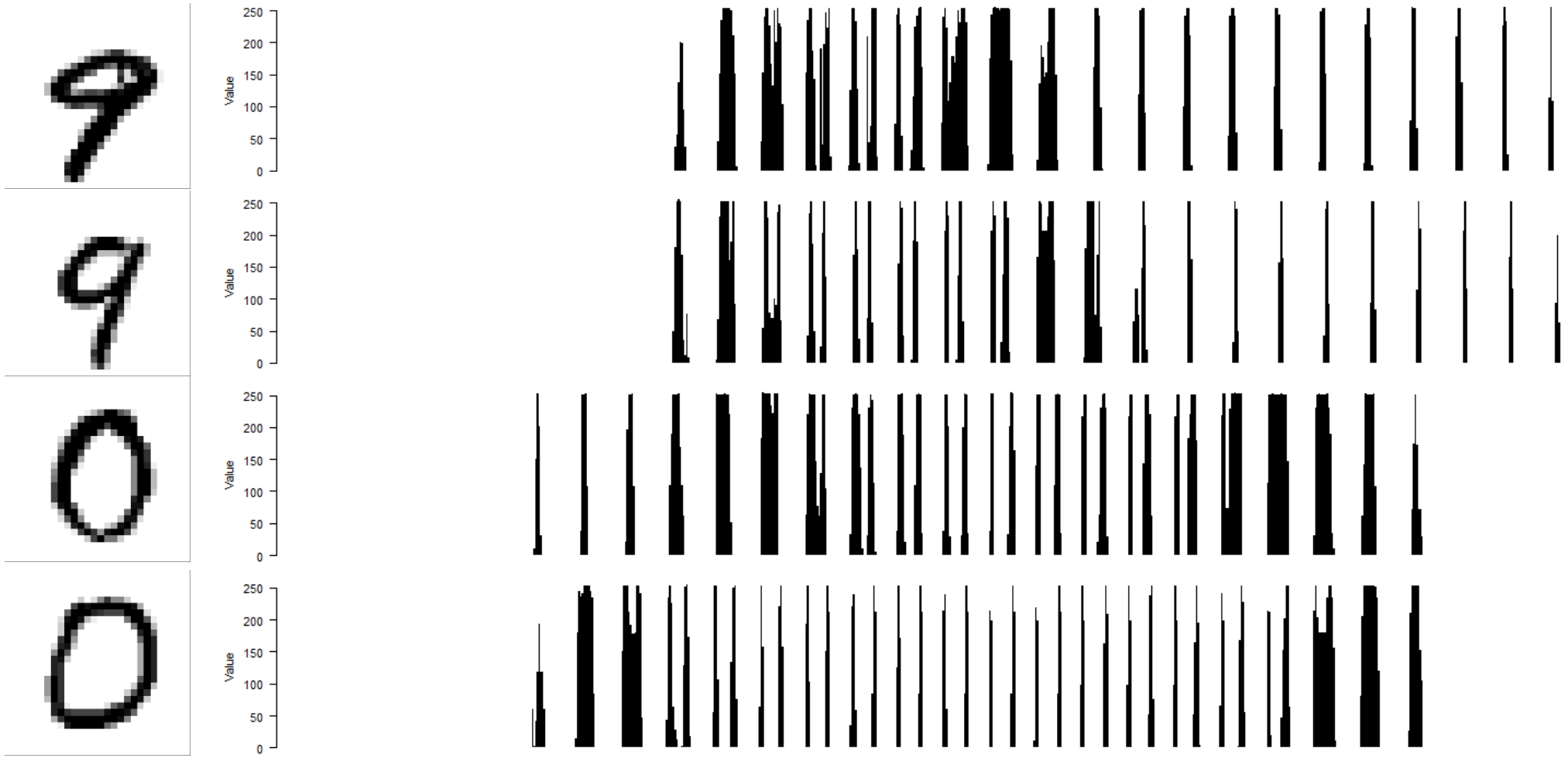
Neighbors

- t-SNE: stochastic neighbor embedding
  - Define neighbors in high-D space
  - Minimize a loss function
  - Many-body simulation
  - High initial learning rate (should scale with input)
- UMAP: uniform manifold approximation
  - Also minimizes neighbor distance, but by “folding space”

Neighbors in 784 dimensions

MNIST (70,000 hand-drawn digits, 28x28 pixels)

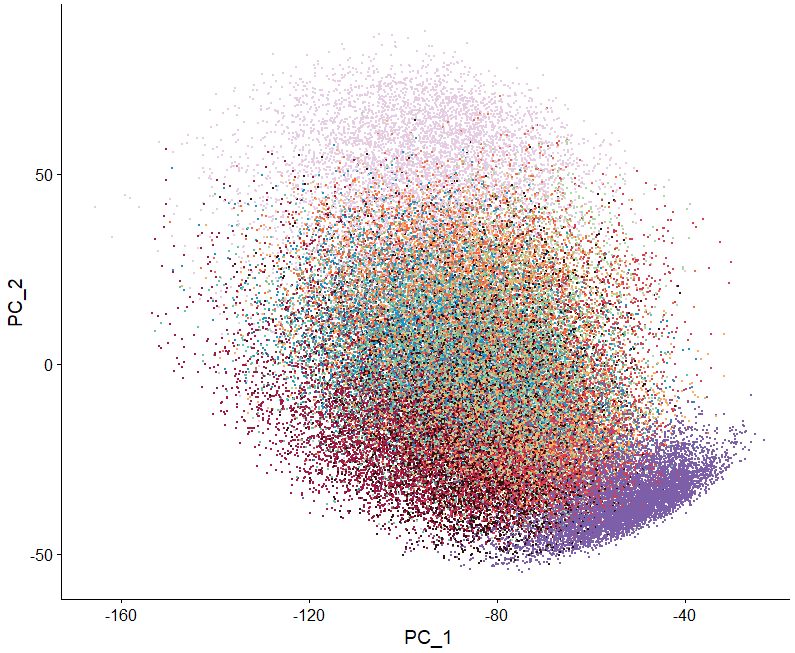
What dimensions are we reducing?

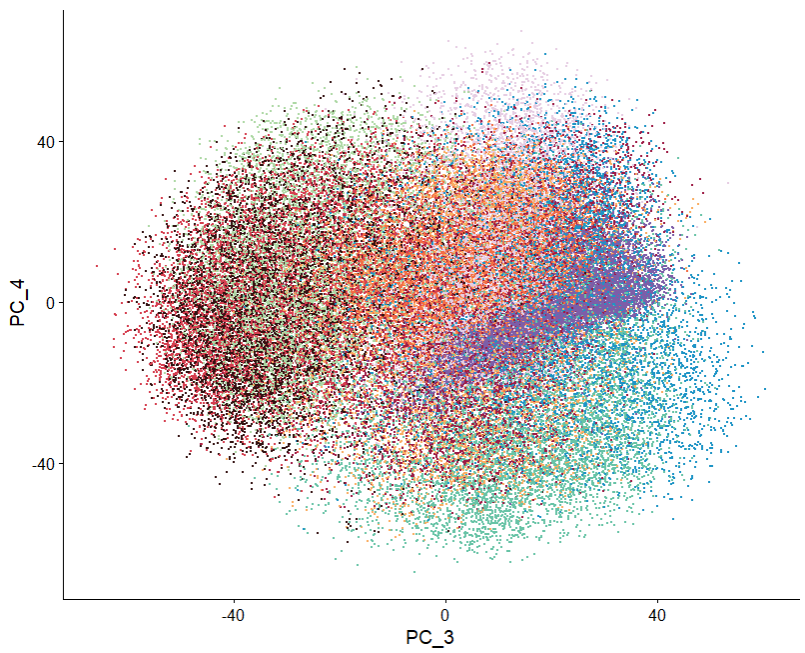


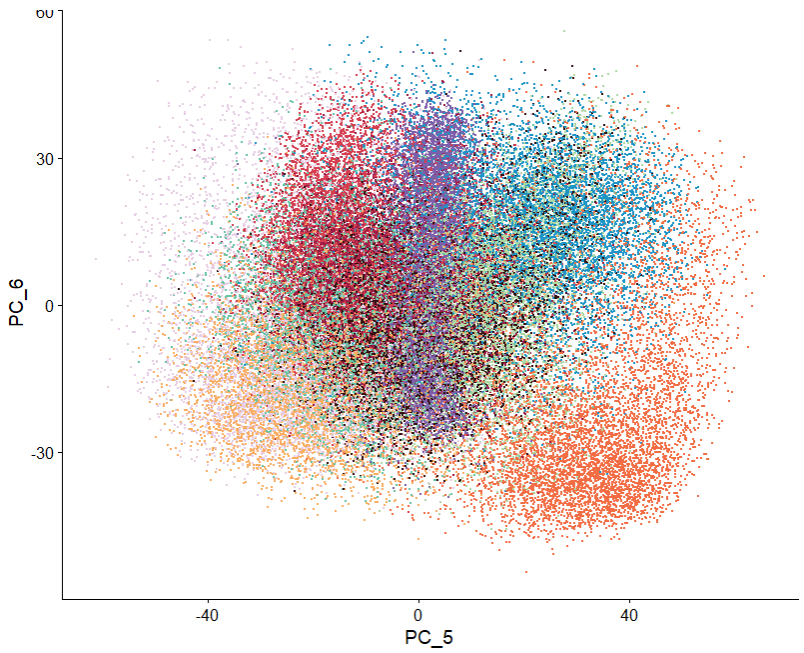
Pixel values

What dimensions are we reducing?

- "Traditional" DR technique: Principal component analysis (PCA)







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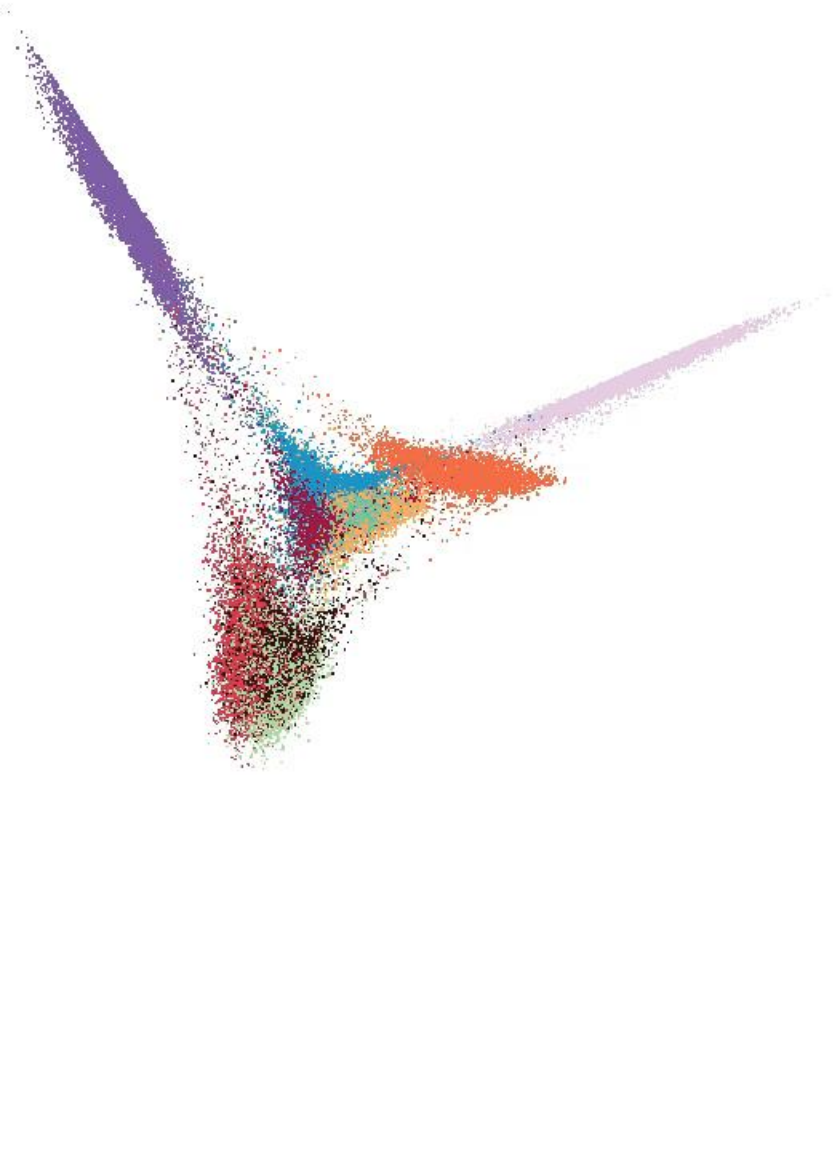
Dimensional reduction (UMAP)

- Begin with an embedding
  - PCA
  - "Spectral" embedding (Laplacian eigenvectors)
- Minimize a loss function that penalizes high distances between neighbors
- "High-energy" early, followed by annealing



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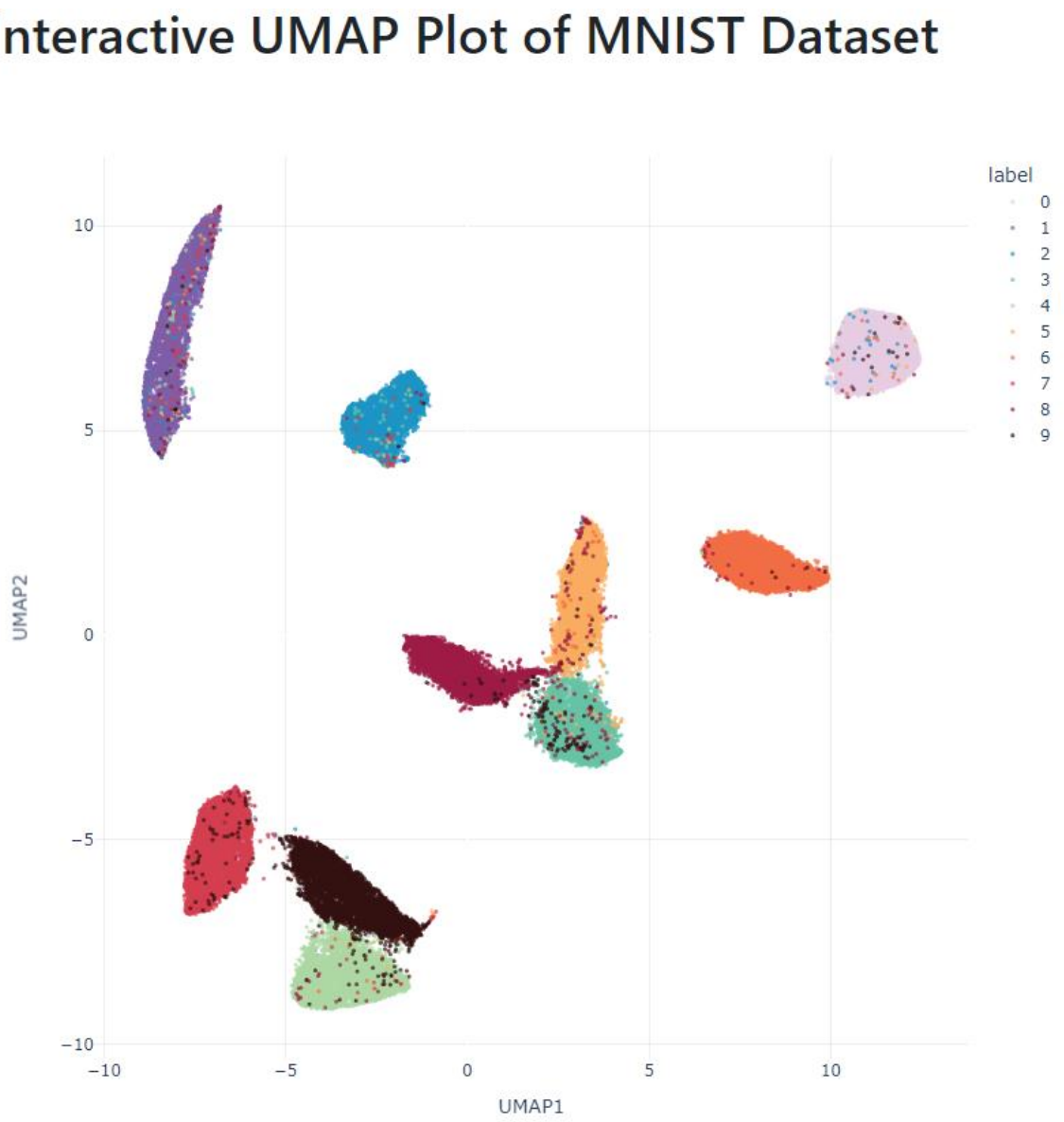

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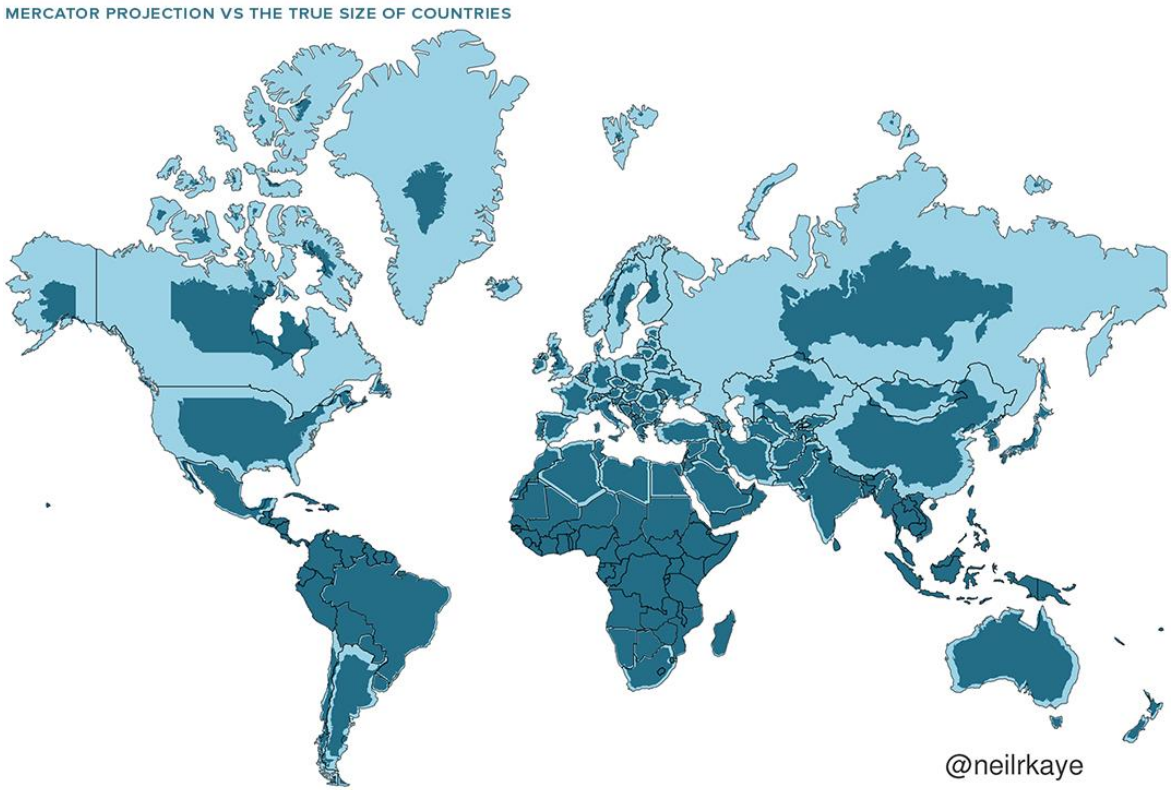
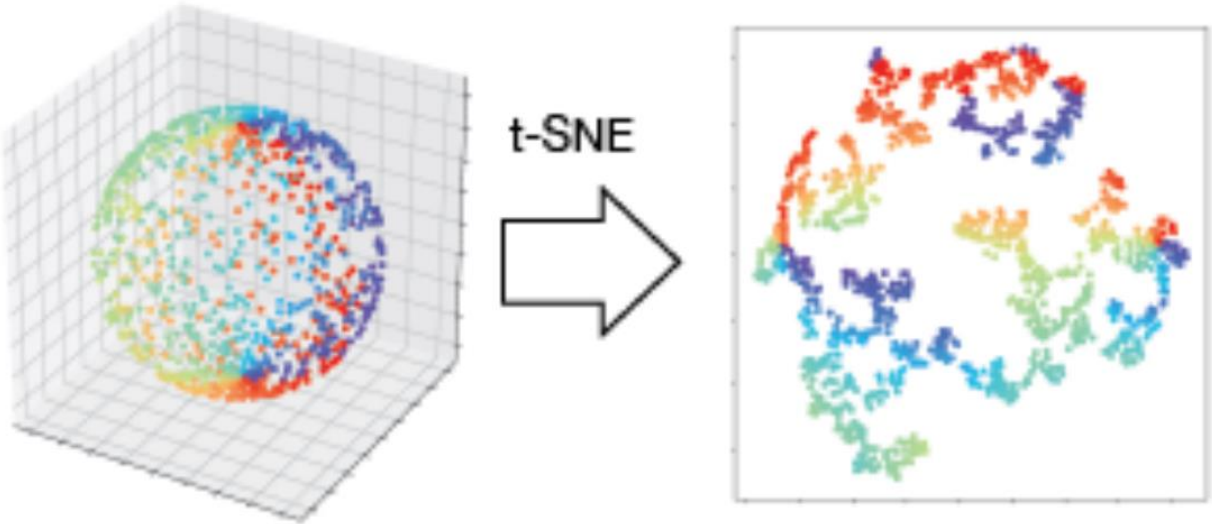
⚠ Reducing dimensions distorts distance





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Cooley et al. 2021 *bioRxiv*

Initialization matters!

Kobak & Linderman 2021 *Nat Biotech*

Finding causes of variation

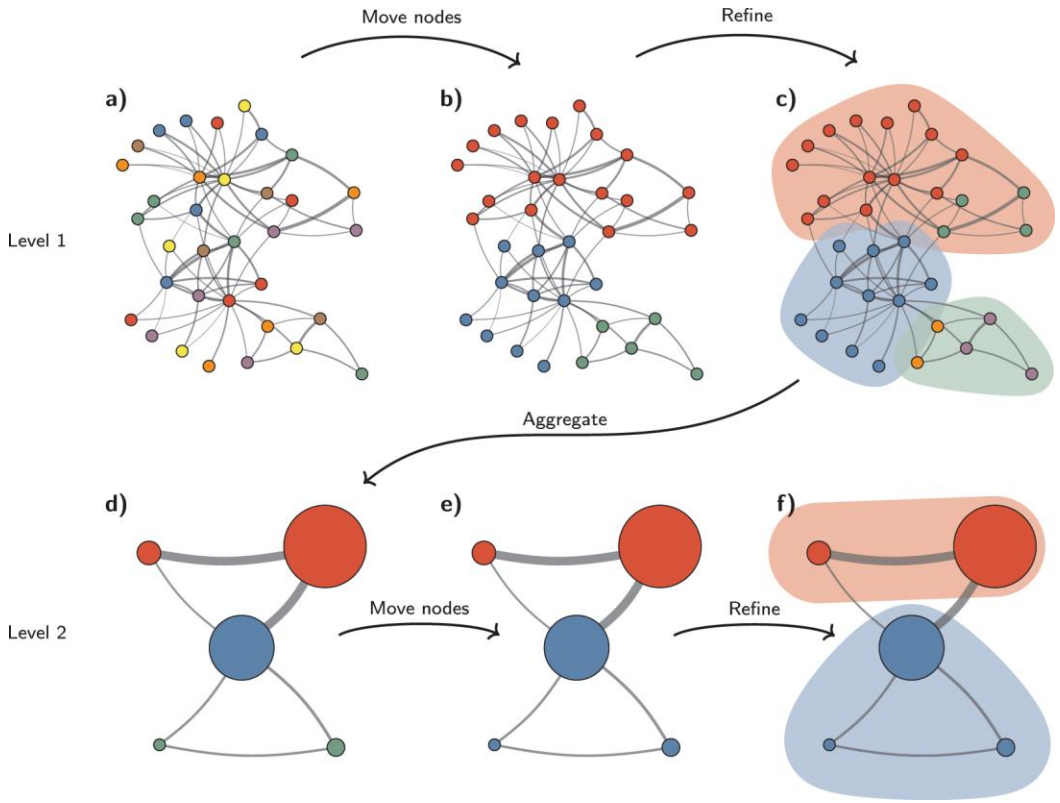
- Differences will either be **discrete** or **continuous**
  - Two unrelated cell types = **discrete**
    - Clustering → marker genes
  - Cellular differentiation = **continuous**
    - Pseudotime trajectories

⚠️ Not all variation is good variation!

- Mitochondrial / chloroplast RNA
- Doublets
- Dropouts
- Ambient RNA
- Cell cycle

Clustering

- Leiden algorithm
  - Graph traversal of k-nearest neighbors
  - Identifies well-connected communities
  - Higher resolution → more clusters



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Traag et al. 2019 *Sci Rep*

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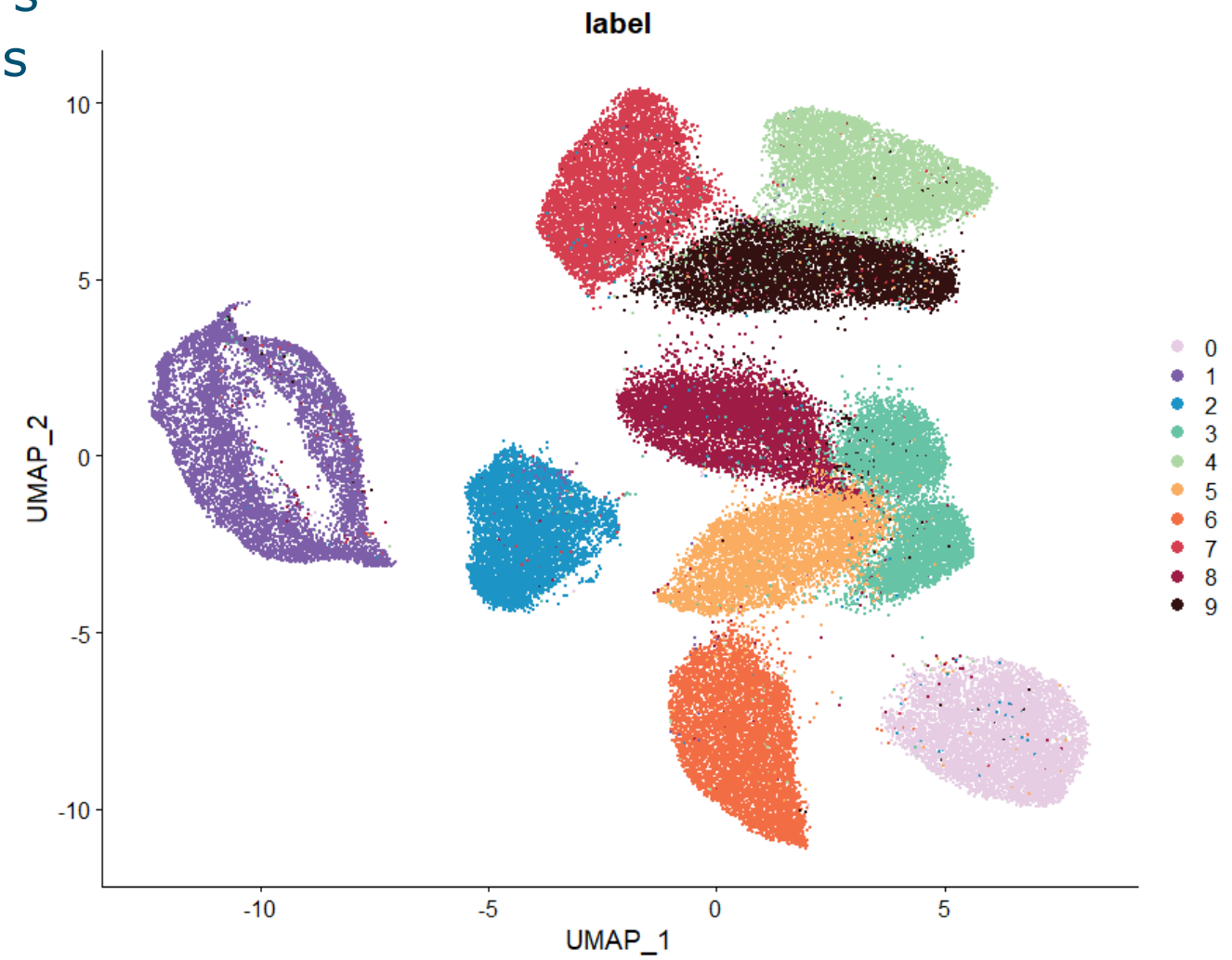

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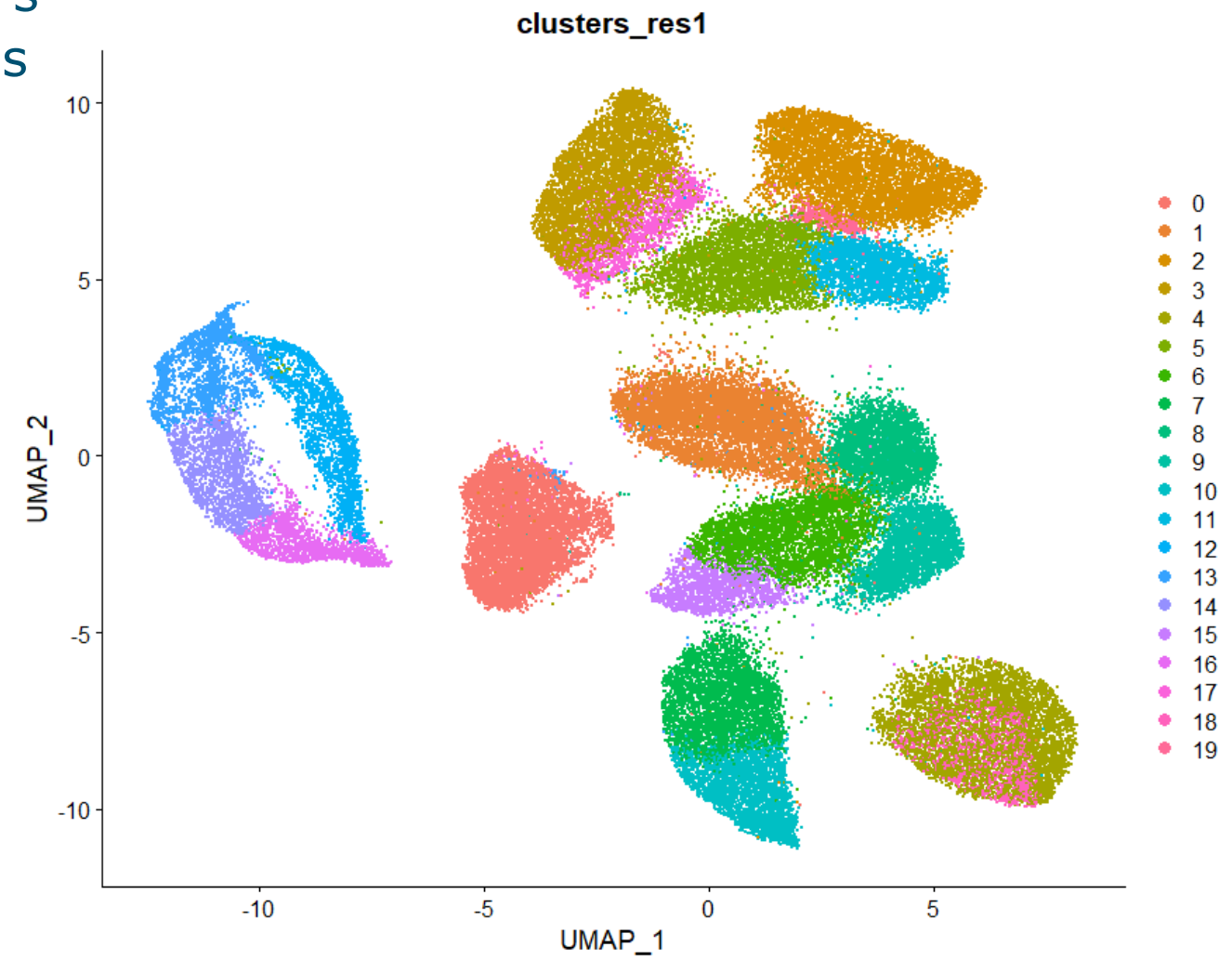

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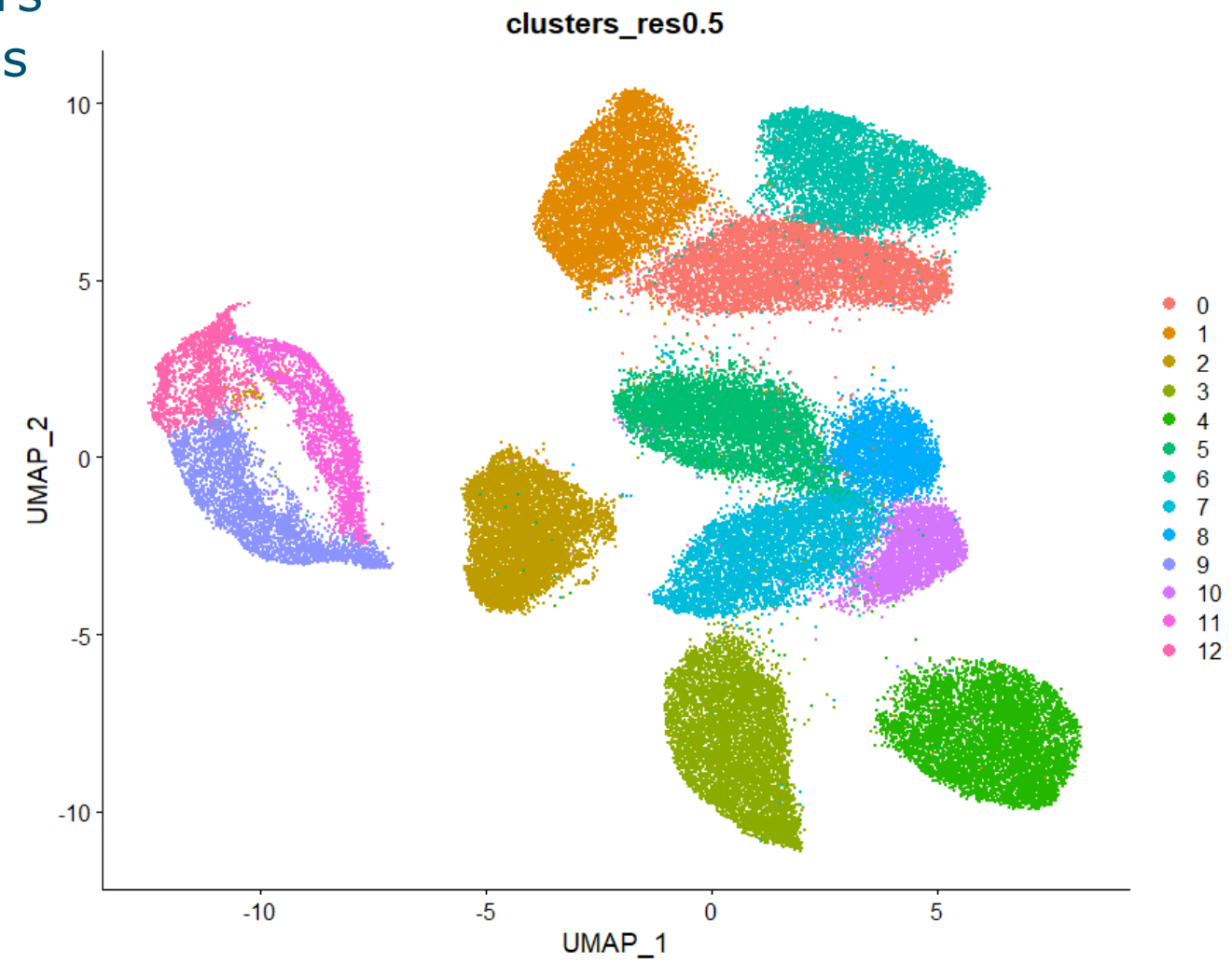

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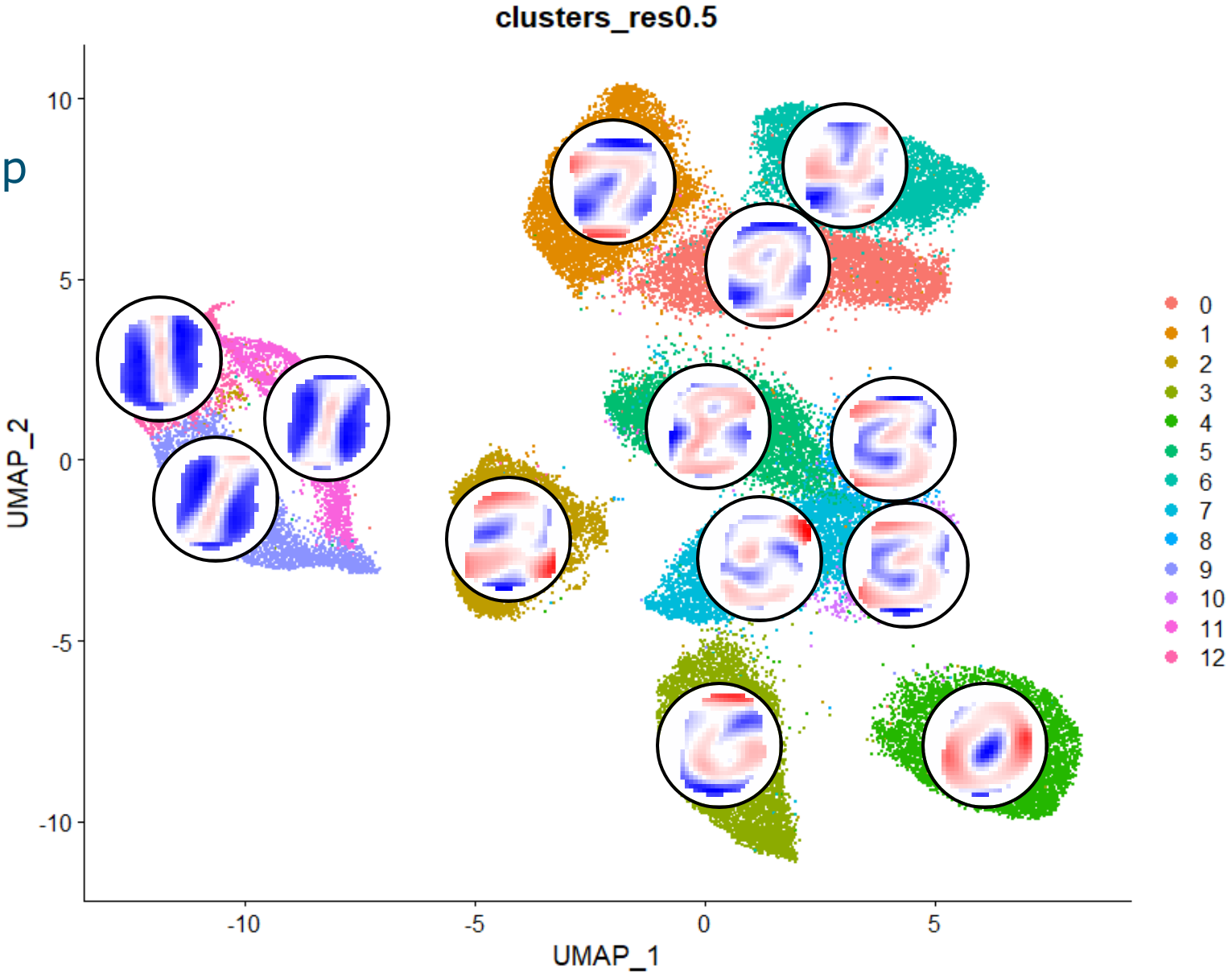
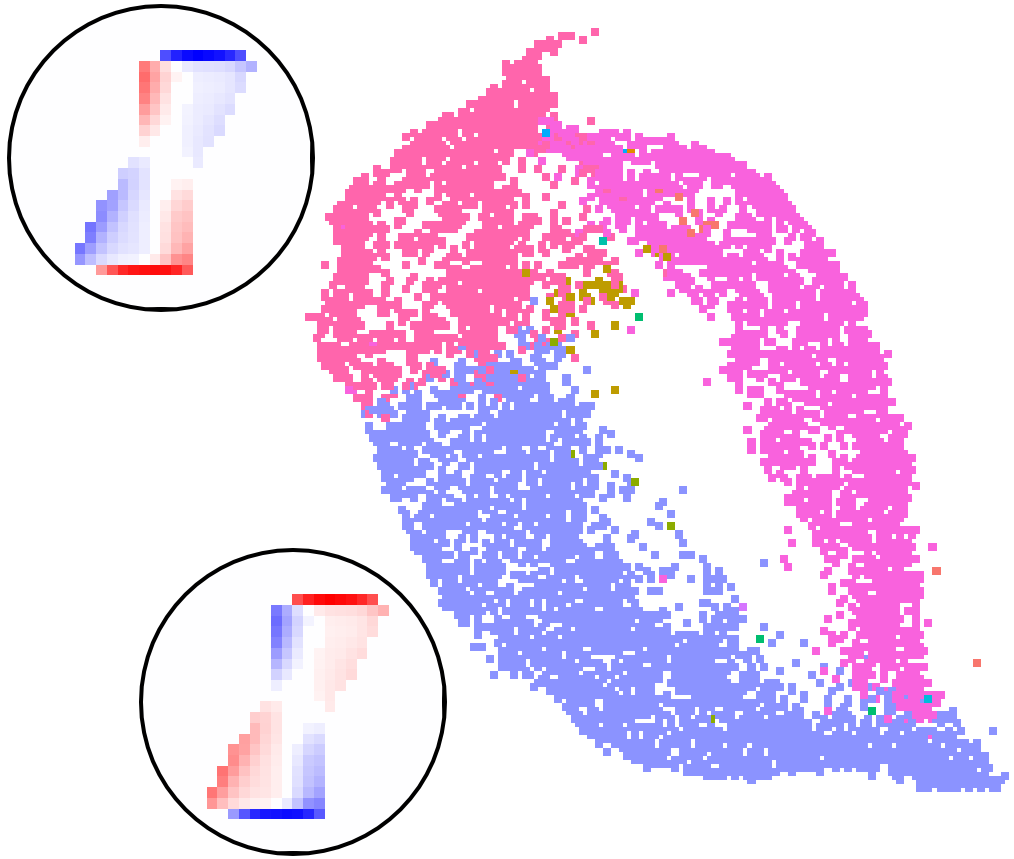
Marker genes

- One vs. all
  - Red – higher in cluster
  - Blue – higher in outgroup



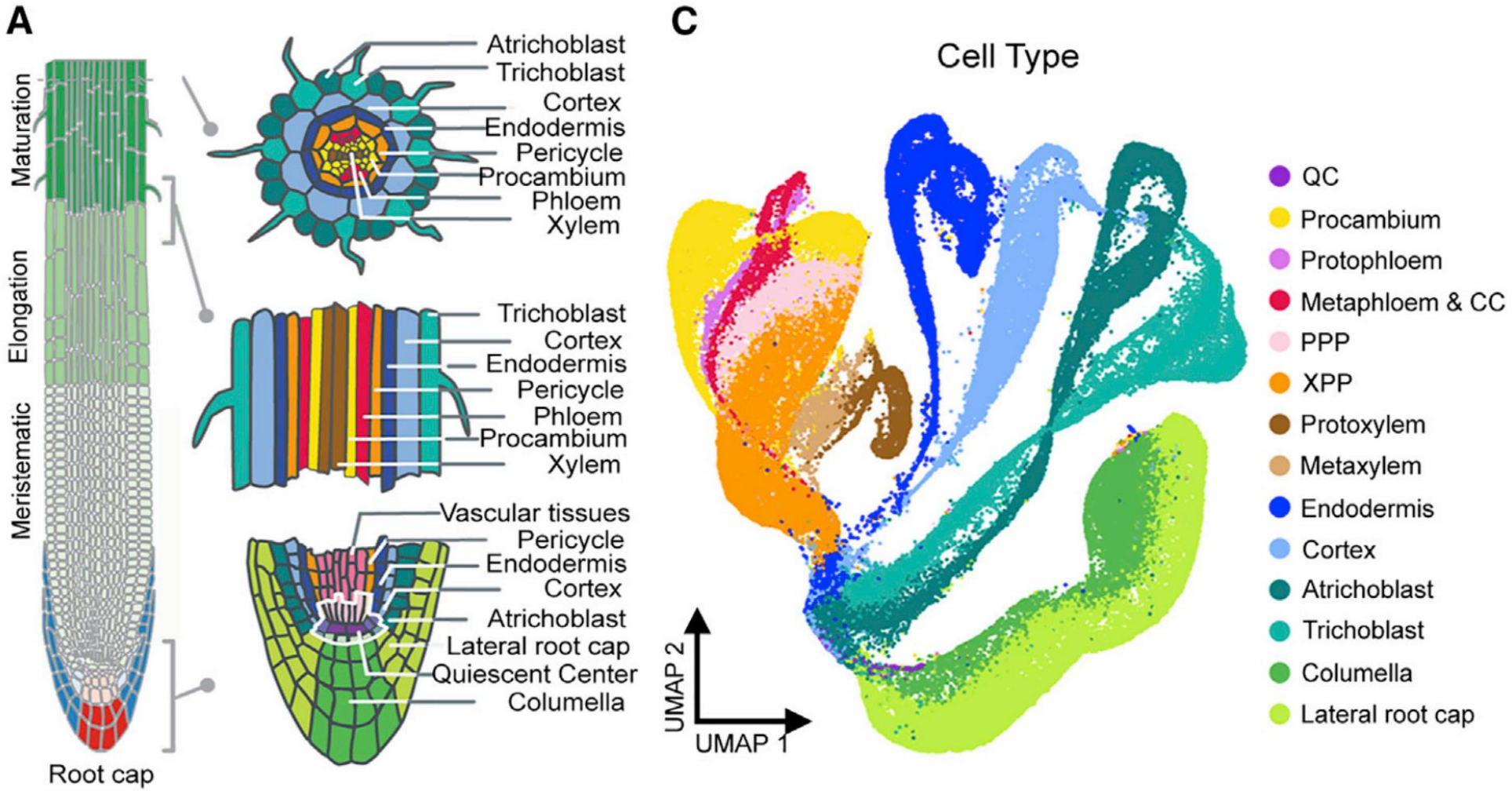
Marker genes

- One cluster vs. another
  - All other clusters are irrelevant
  - A vs. B = -(B vs. A)



Pseudotime trajectories

- Developing tissues show more **continuous** variation



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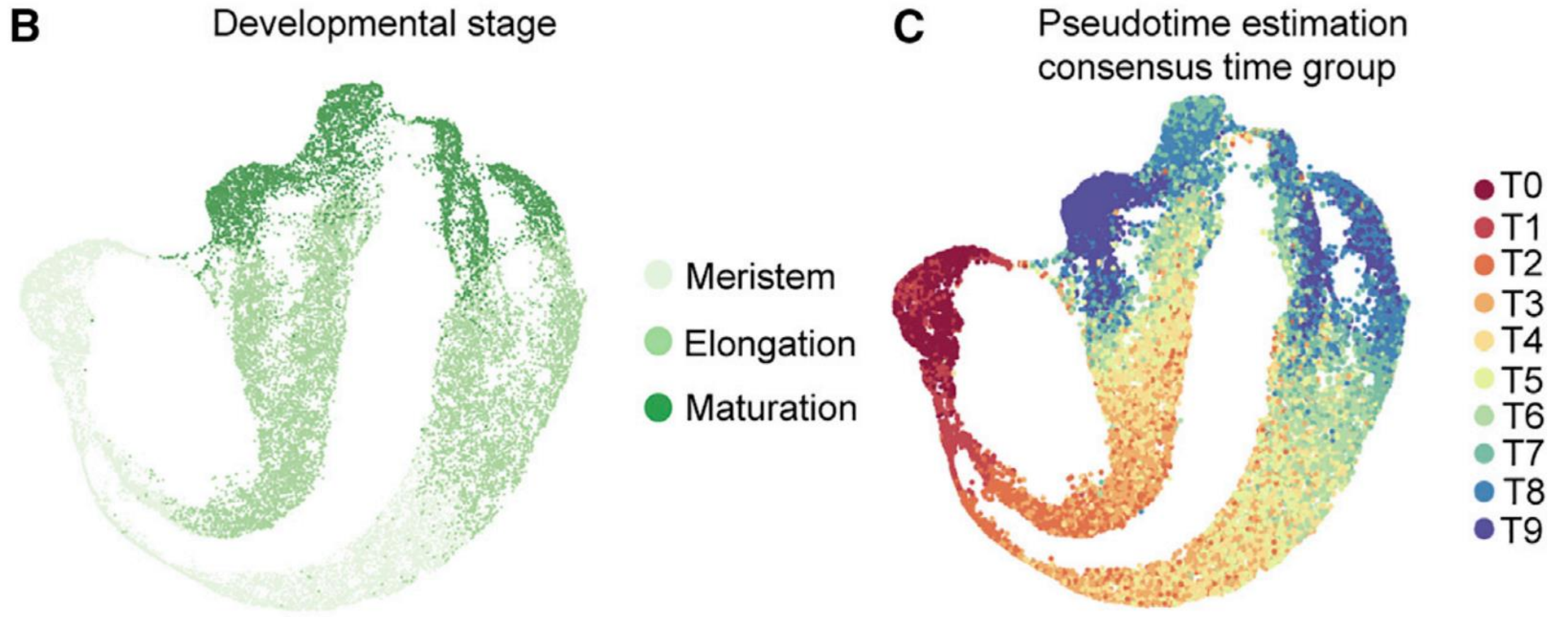
Shahan et al. 2022 *Dev Cell*

Pseudotime trajectories



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Shahan et al. 2022 *Dev Cell*

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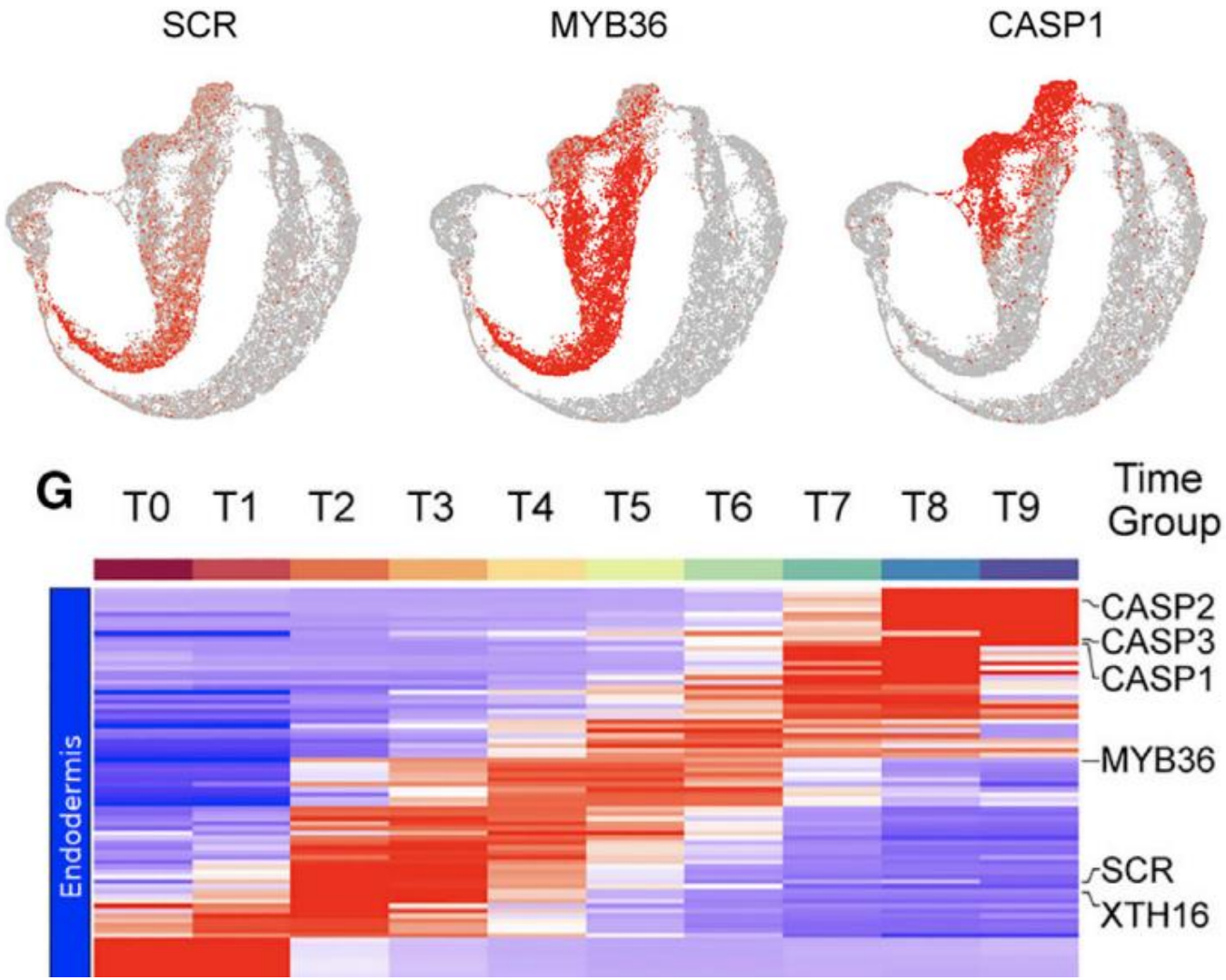

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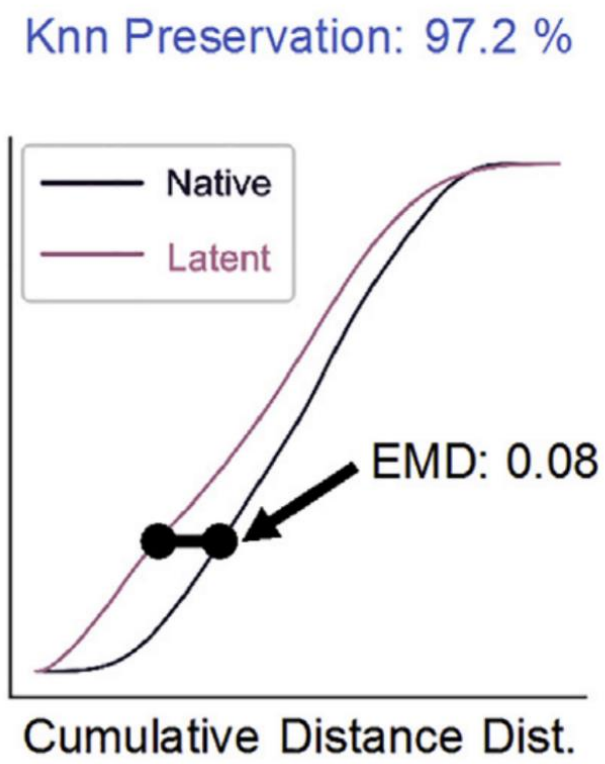
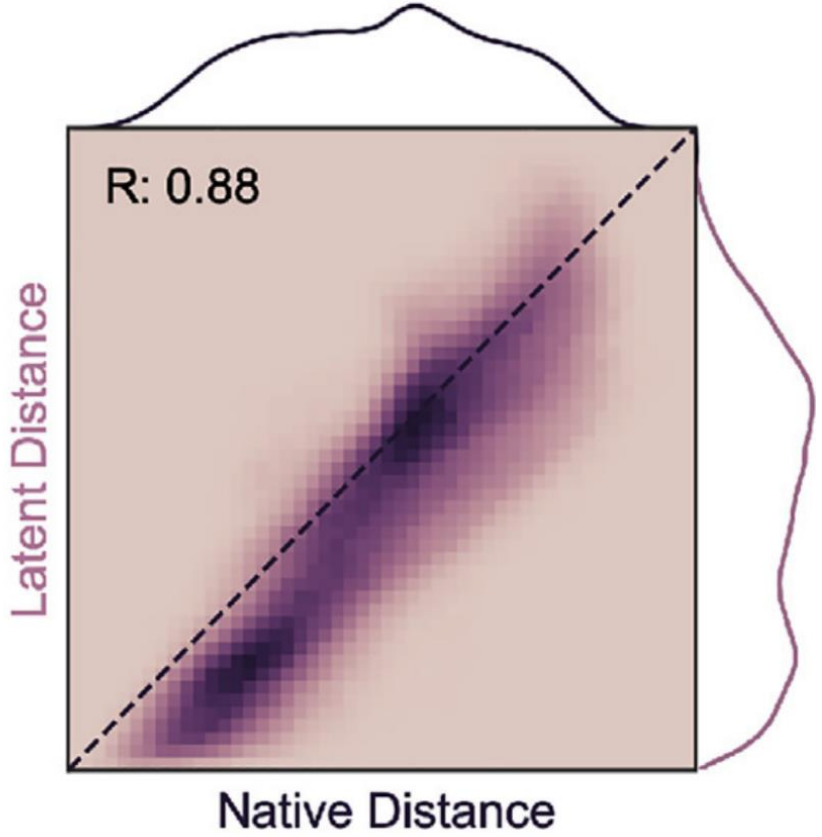
Shahan et al. 2022 *Dev Cell*

Pseudotime trajectories

- But how can we know if a trajectory is accurate?
  - Calibrate against known marker genes
  - Measure distortions of "native" distances in the reduced space





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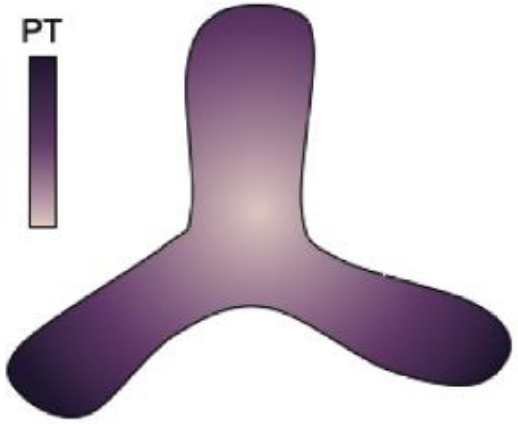
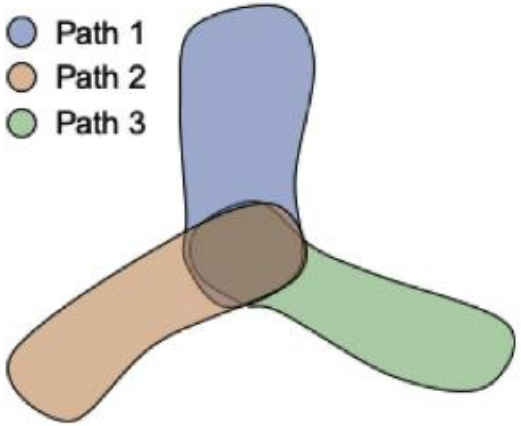
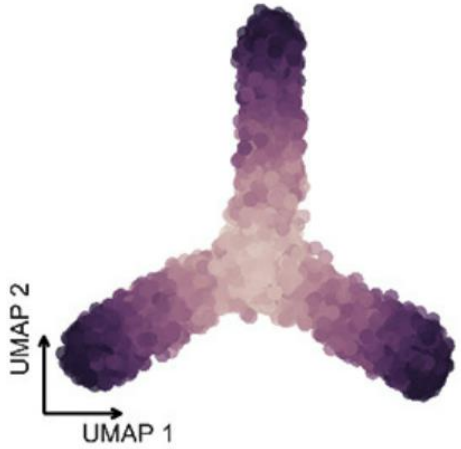
Heiser & Lau 2020 *Cell Reports*

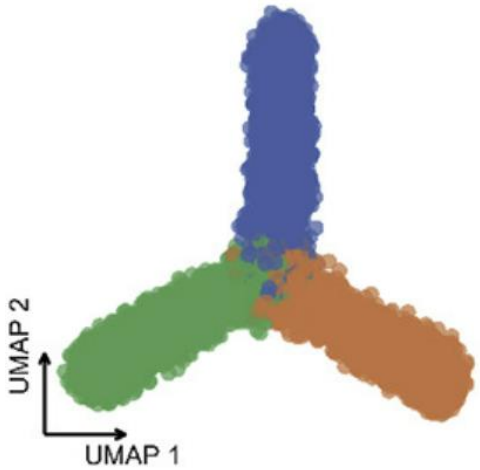
Pseudotime trajectories

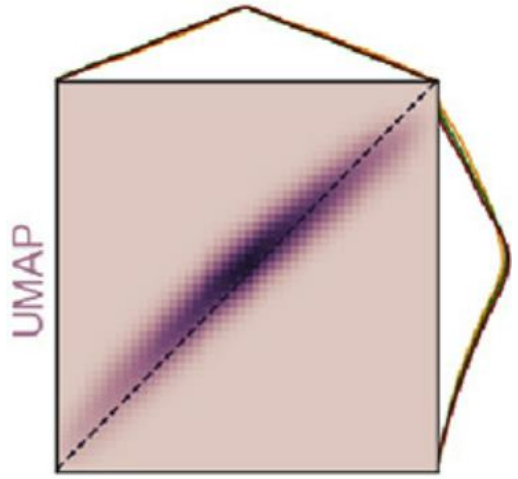
- UMAP excels when data are continuous and uniform











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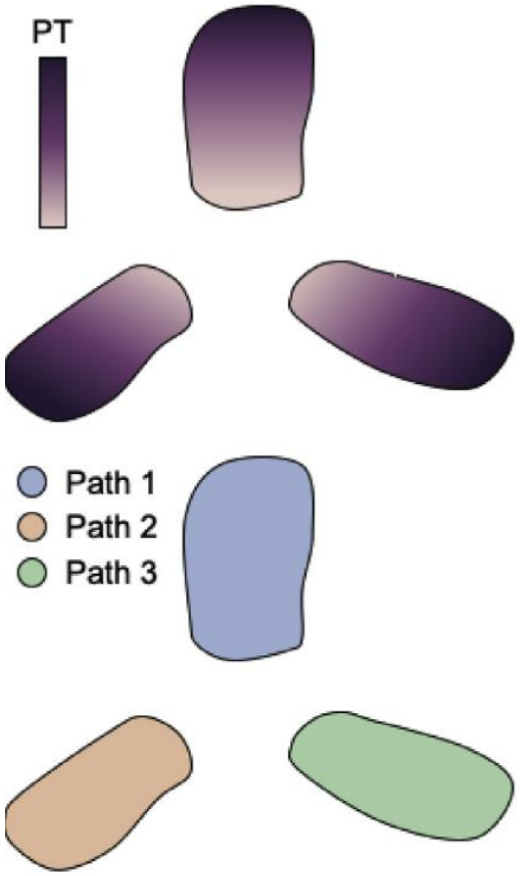
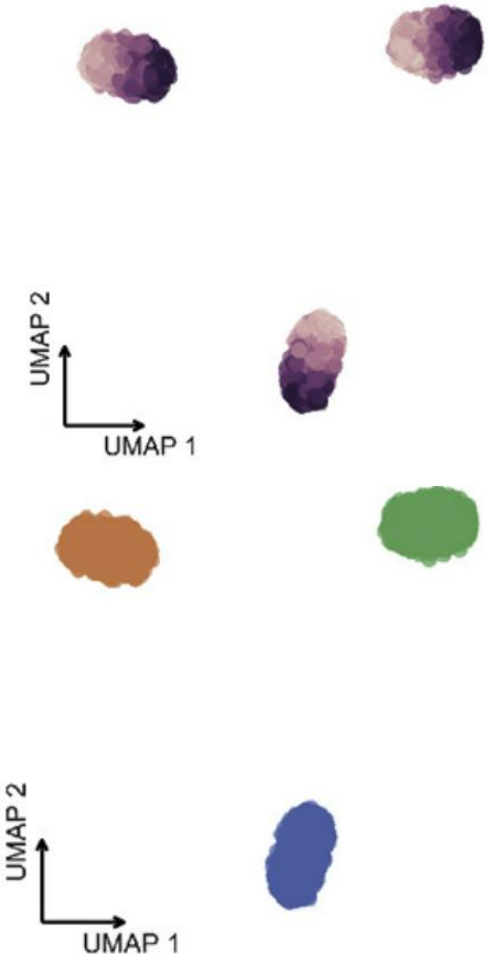
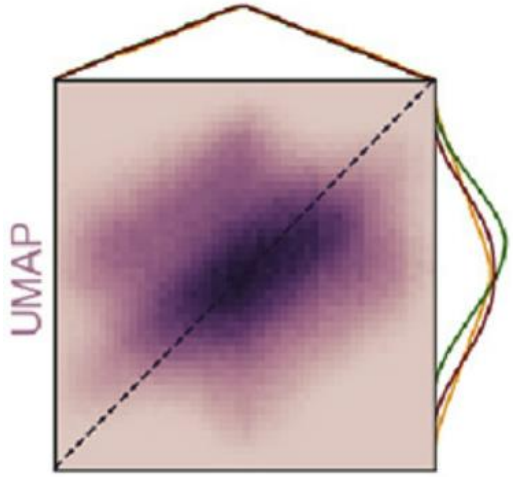
Heiser & Lau 2020 *Cell Reports*

Pseudotime trajectories

⚠ UMAP fails to globally orient discontinuous data







Heiser & Lau 2020 *Cell Reports*

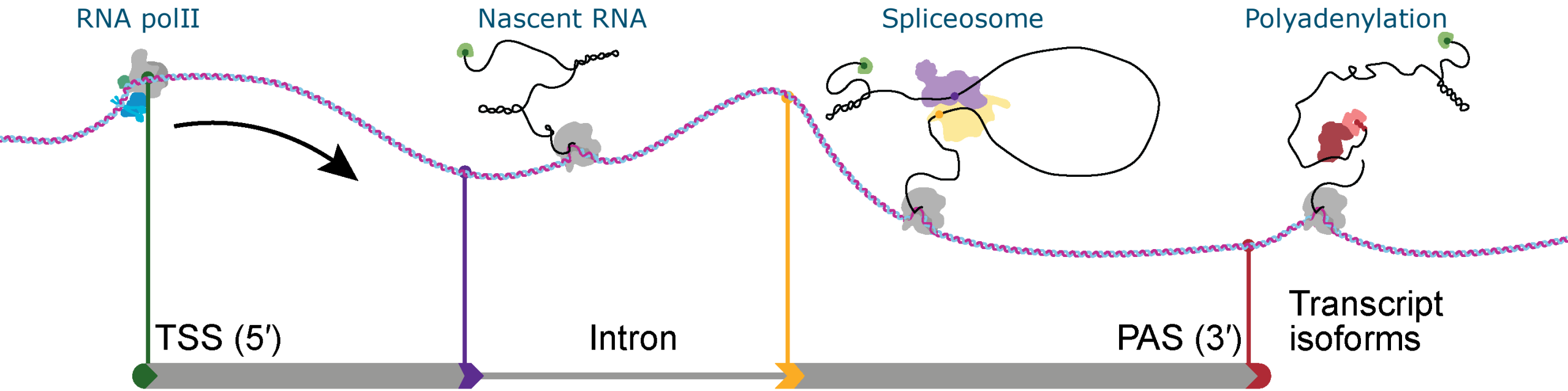
Pseudotime trajectories

⚠ Assumes a continuous path between cell types
- Incomplete data creates bad embeddings
- Not all heterogeneity is developmental!

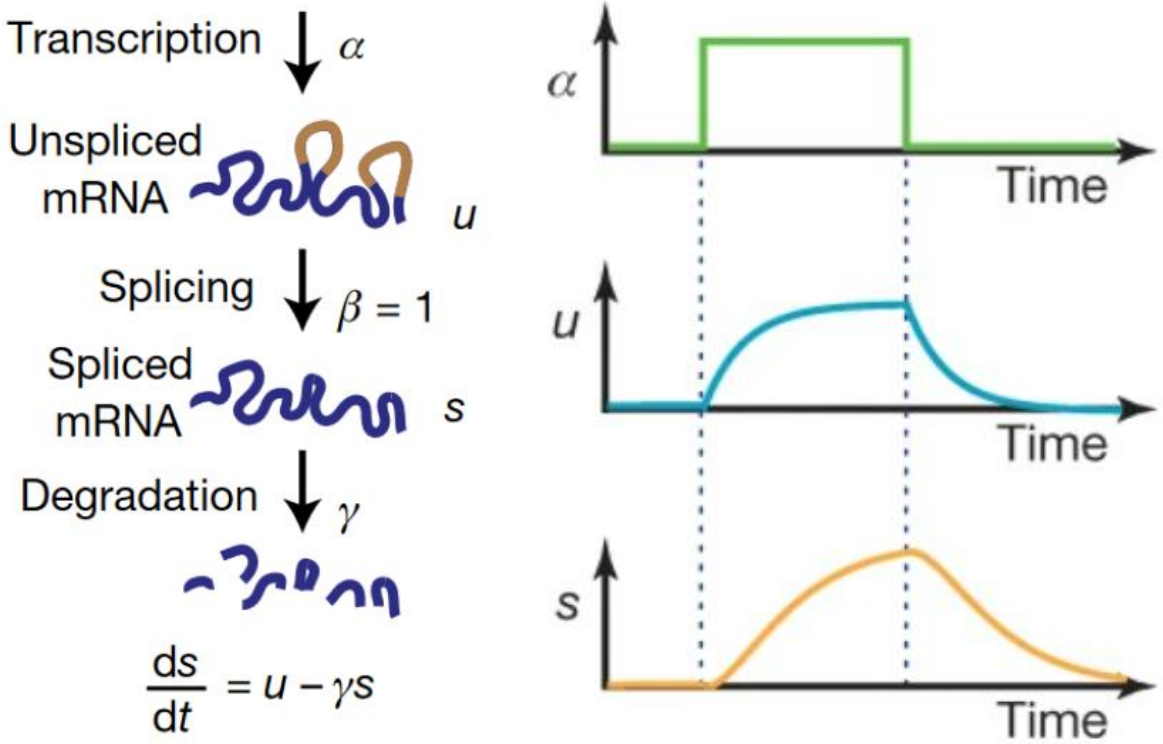
⚠ Operates in reduced dimensions
- 2D projections can have massive distortion!
- Need to be “anchored” in reality
- DR methods (UMAP, t-SNE) are not globally coherent

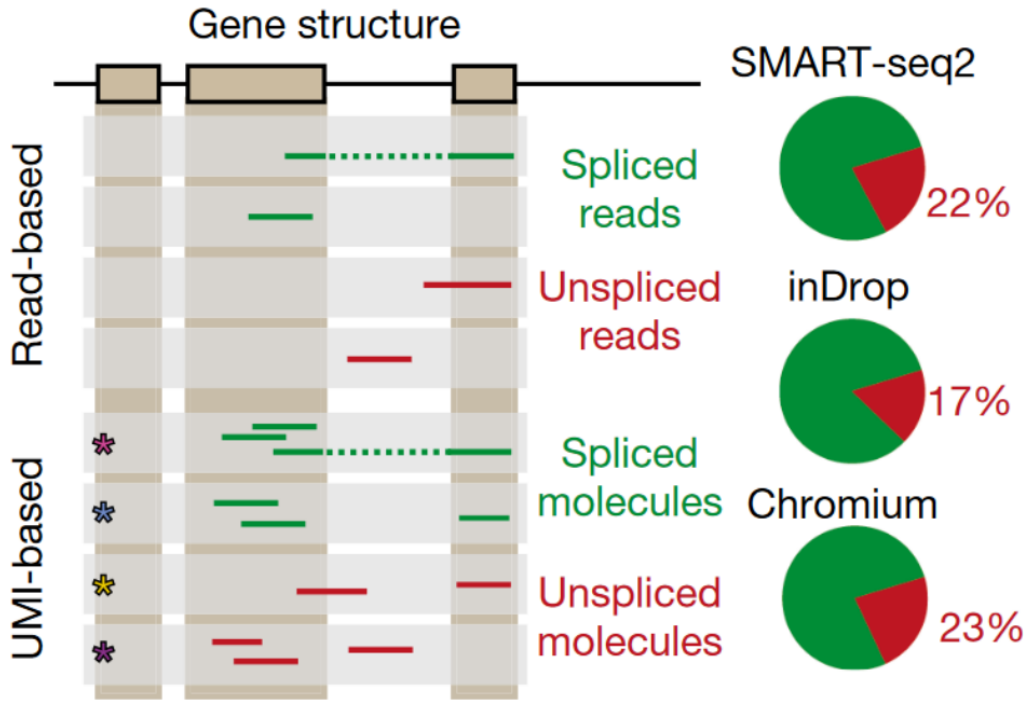
What else can we do with single-cell RNA?

Measuring RNA birth and death



RNA “velocity”: predicting the future?

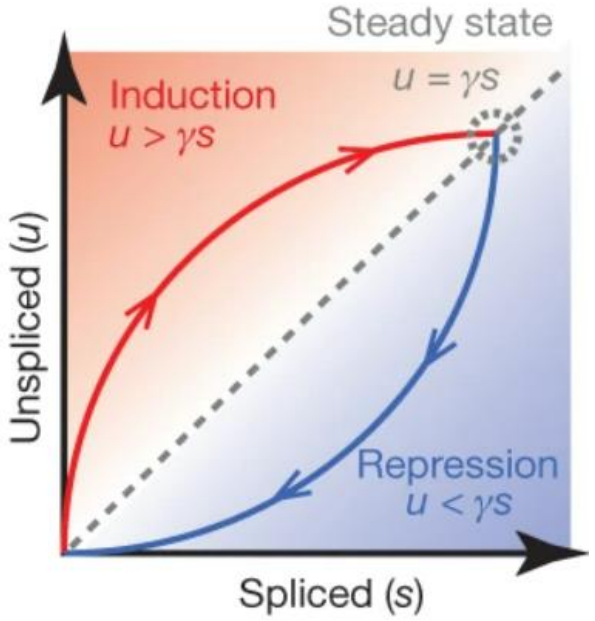




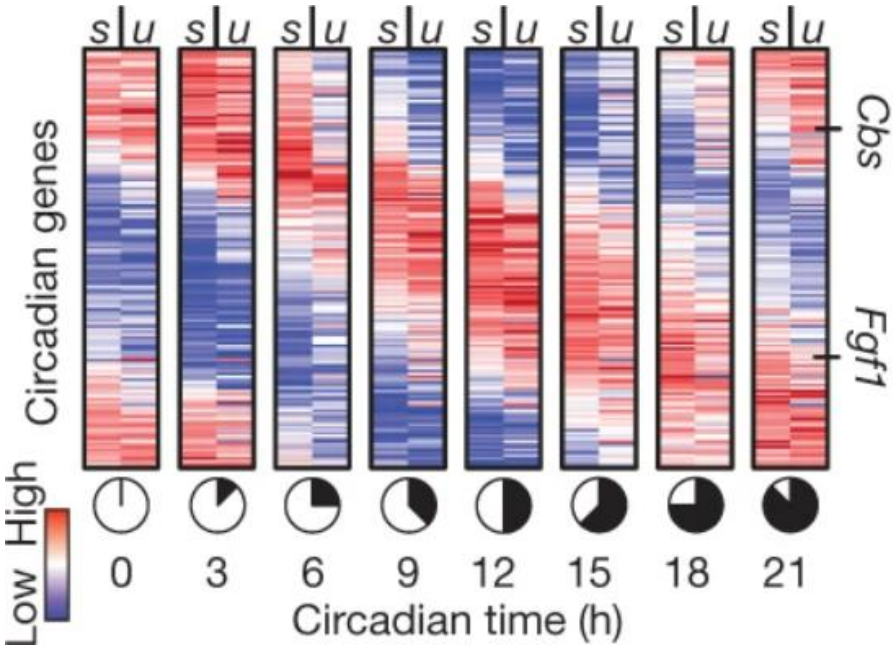
La Manno et al. 2018 *Nature*

RNA “velocity”: predicting the future?

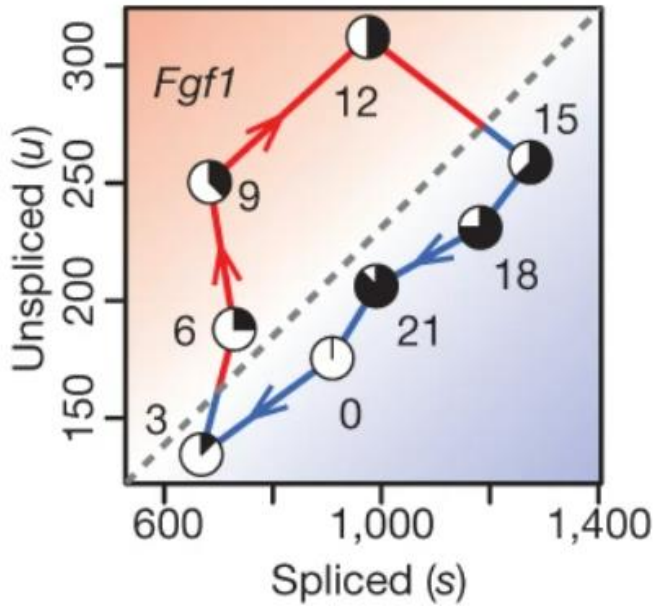
Model RNA kinetics



Unspliced RNA precedes spliced RNA



Kinetics of a single circadian gene



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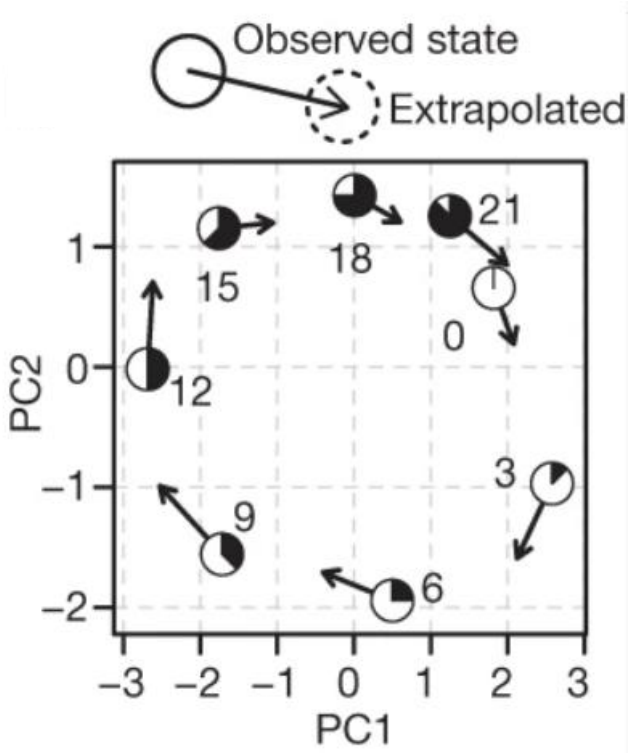
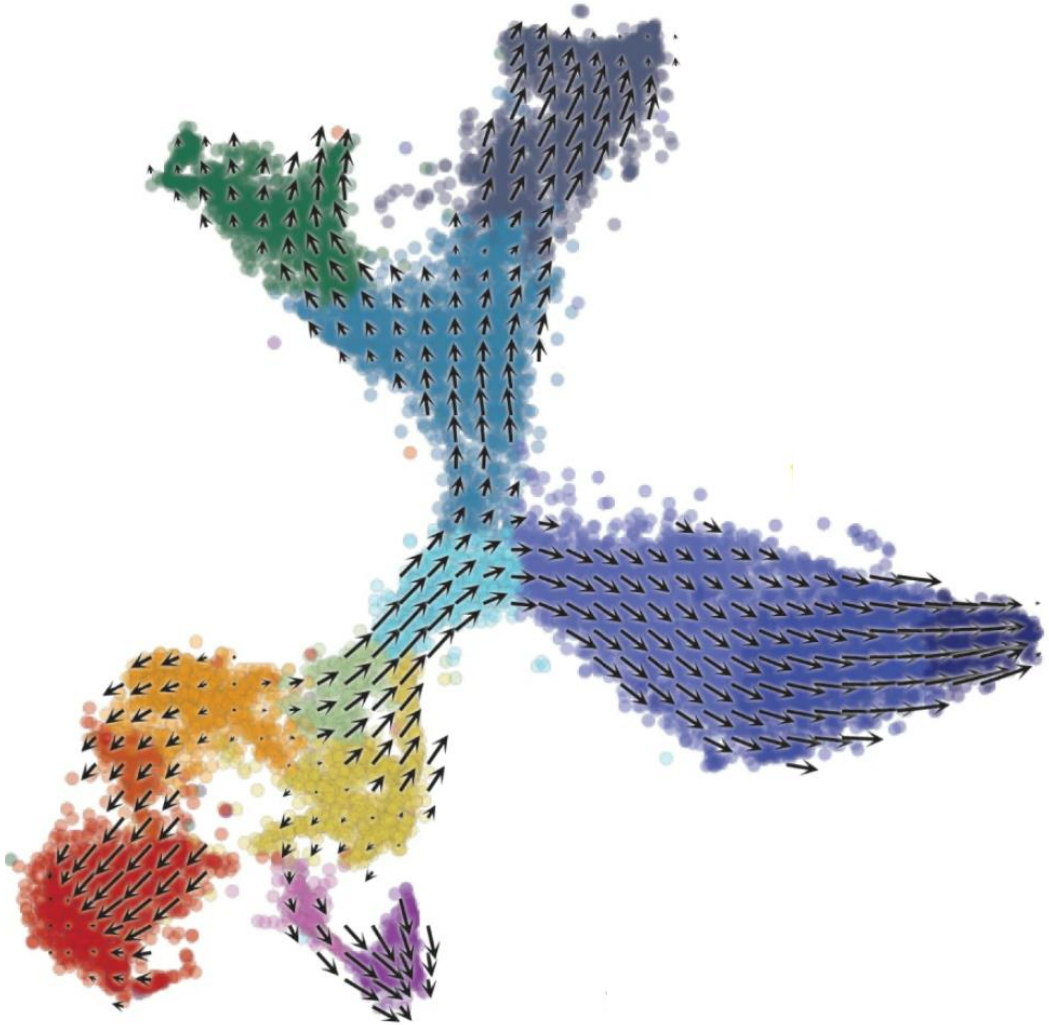
La Manno et al. 2018 *Nature*

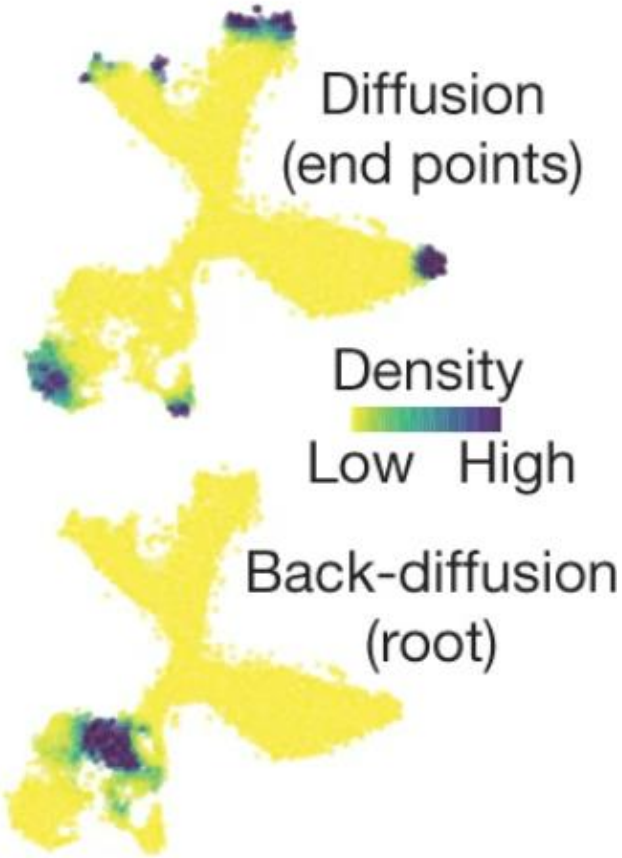
RNA “velocity”: predicting the future?

Circadian cycle



Developing hippocampus cells



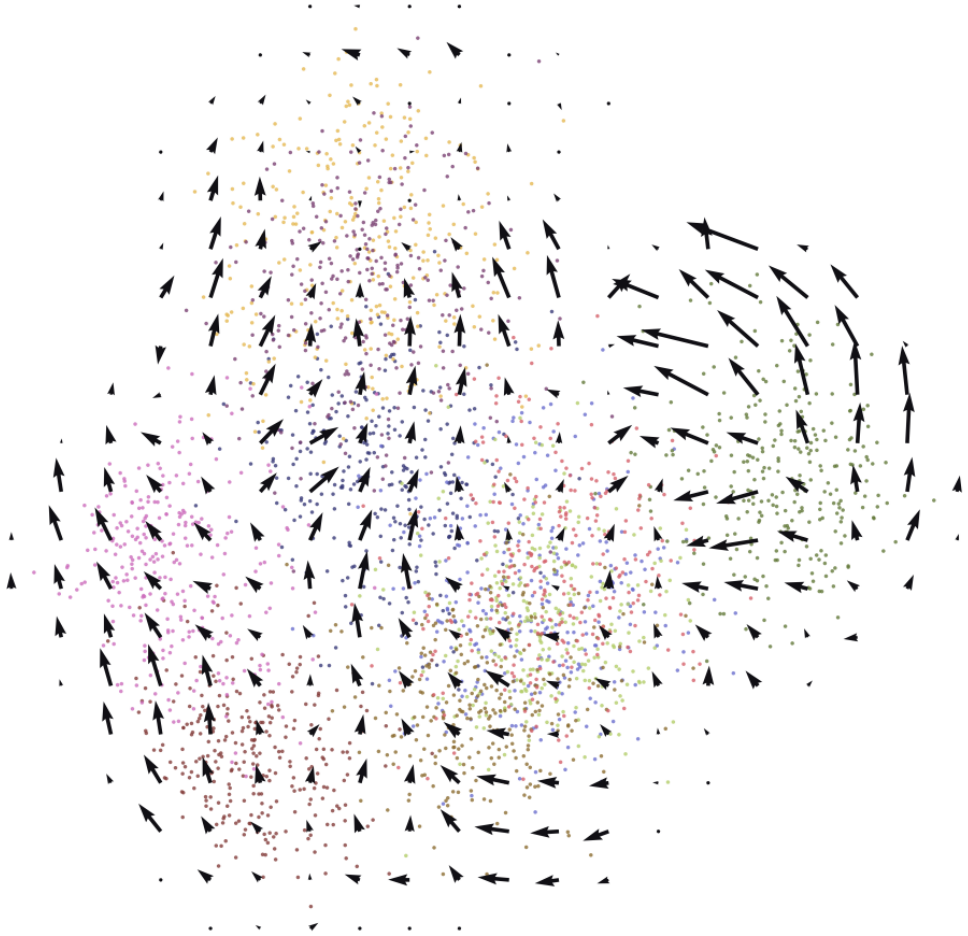


La Manno et al. 2018 *Nature*

RNA “velocity”: predicting the future?

⚠ Vectors will always be found, even when none exist

Noisy simulation of 10 discrete cell types



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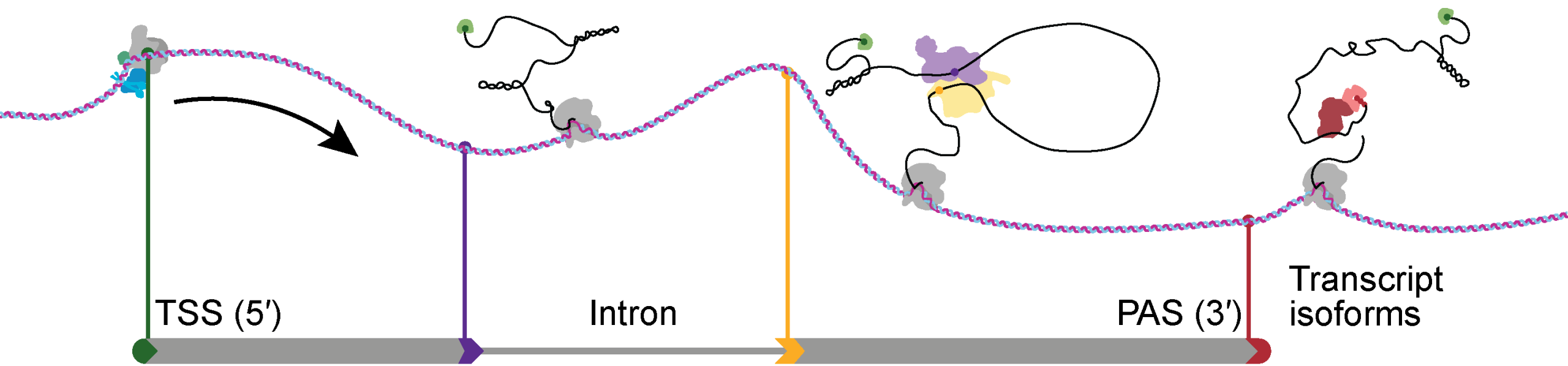
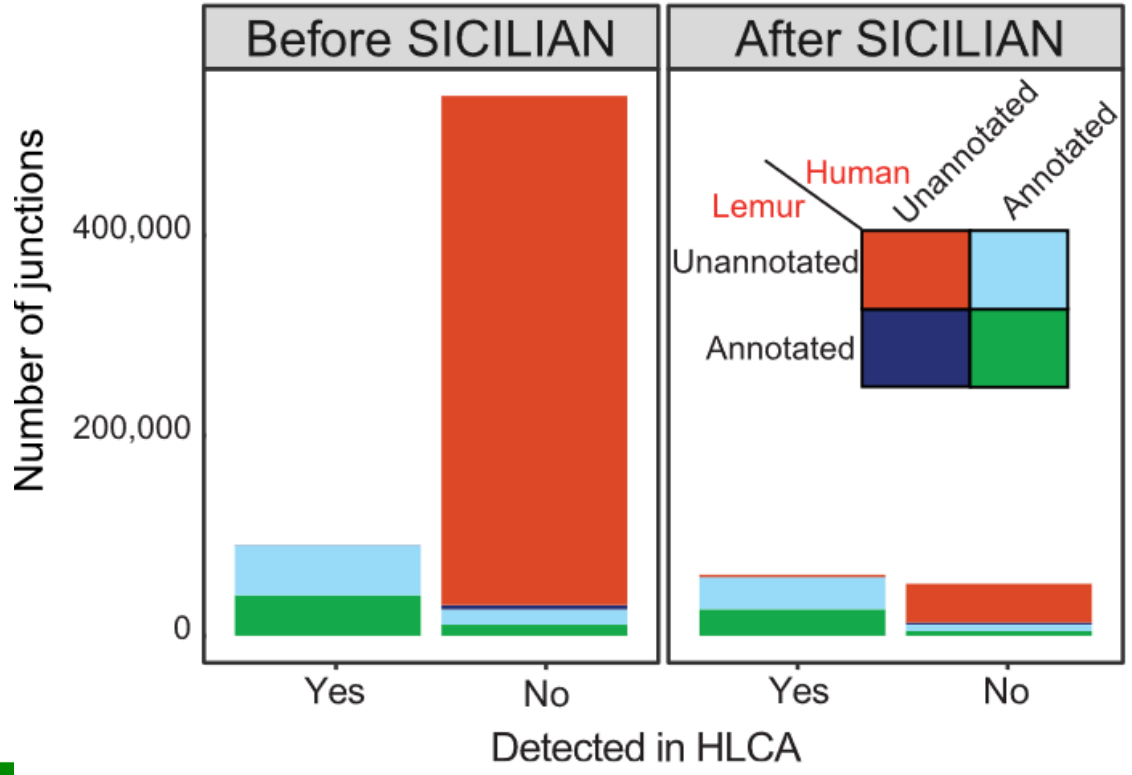
Gorin et al. 2022 *PLoS Comp Biol*

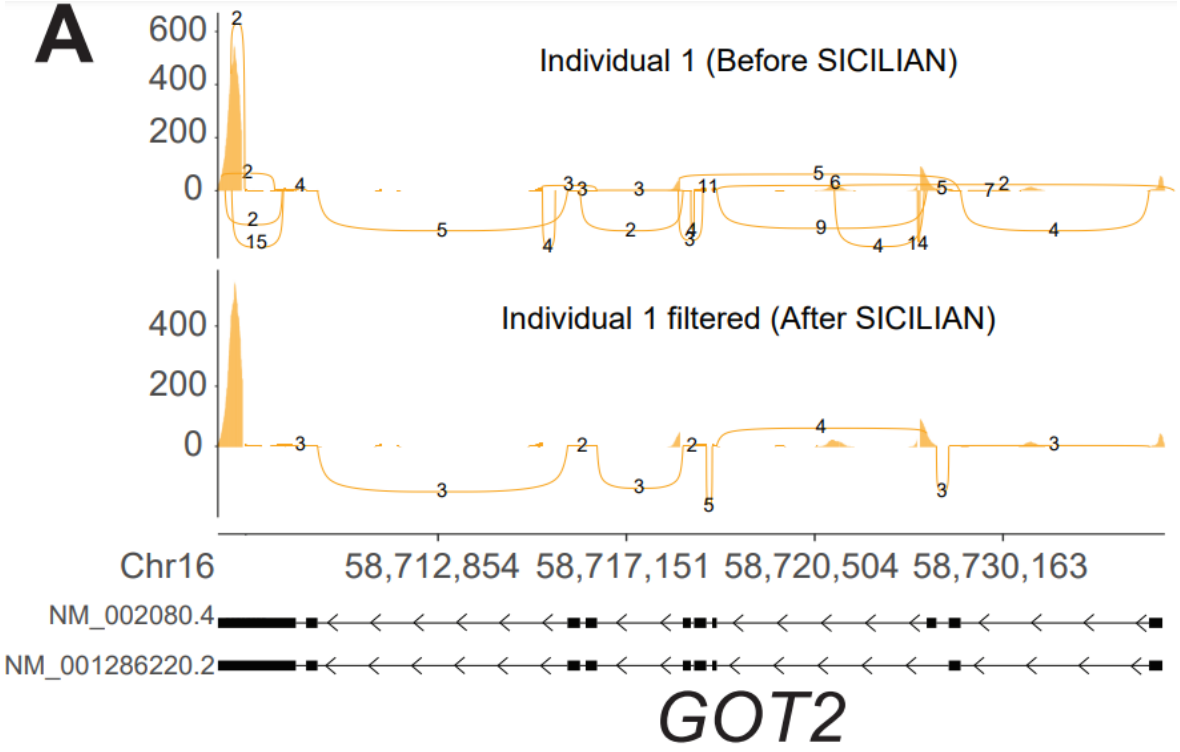
Genes → isoforms?



Limits to isoform identification

- ⚠ Single-cell sequencing is noisy
  - Gapped alignments yield more false splice junctions than true ones
  - SICILIAN filters splice junctions by sequence entropy
- ⚠ Most scRNA-seq data is heavily biased to 3’ ends (10X Genomics)





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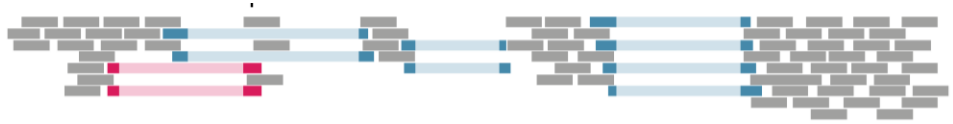
Dehghannasiri et al. 2021 *Genome Biology*

Single-cell isoform assembly?

- Full-length single cell sequencing? (Smart-seq)
  - Transcript assemblers have abysmal precision (4 false transcripts per true transcript)



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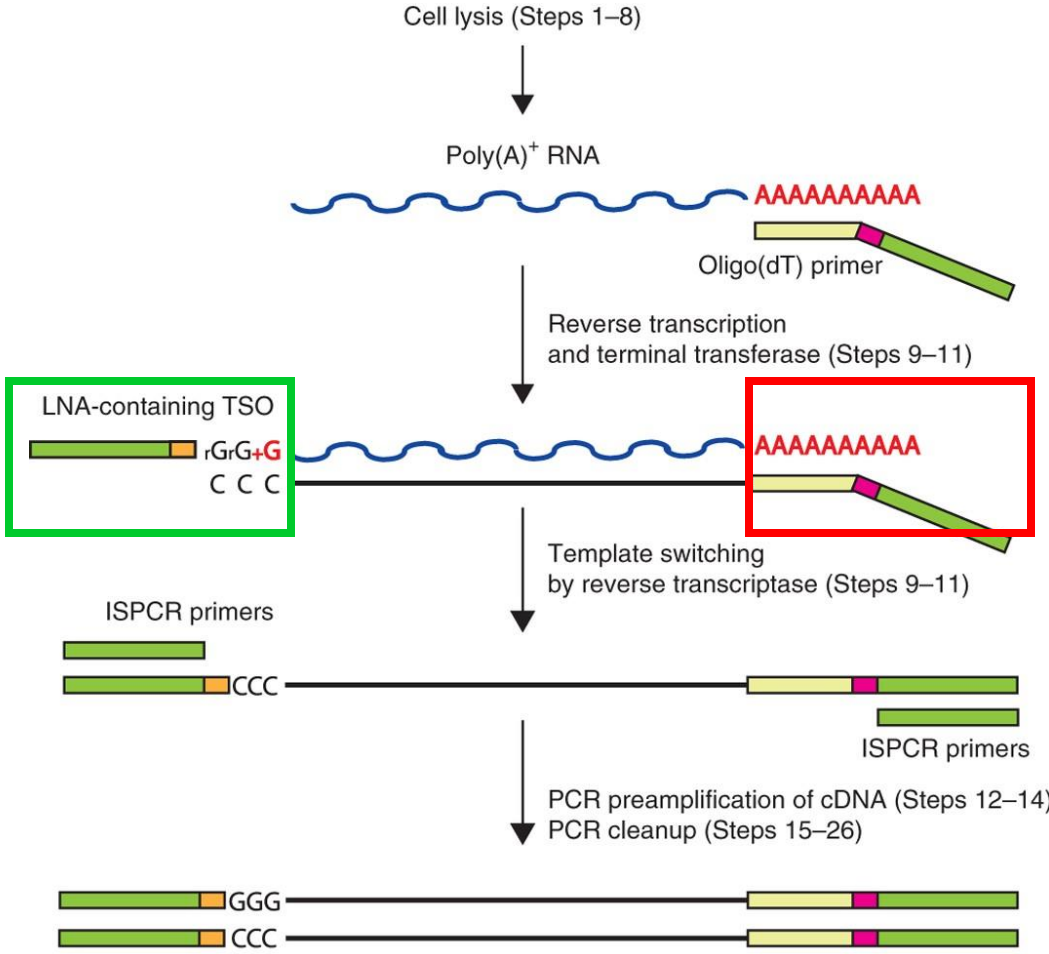
Schon et al. 2022 *Genome Biology*

Something is missing...

Smart-seq2 protocol

Template-switching oligo (TSO)



Poly(A) tail

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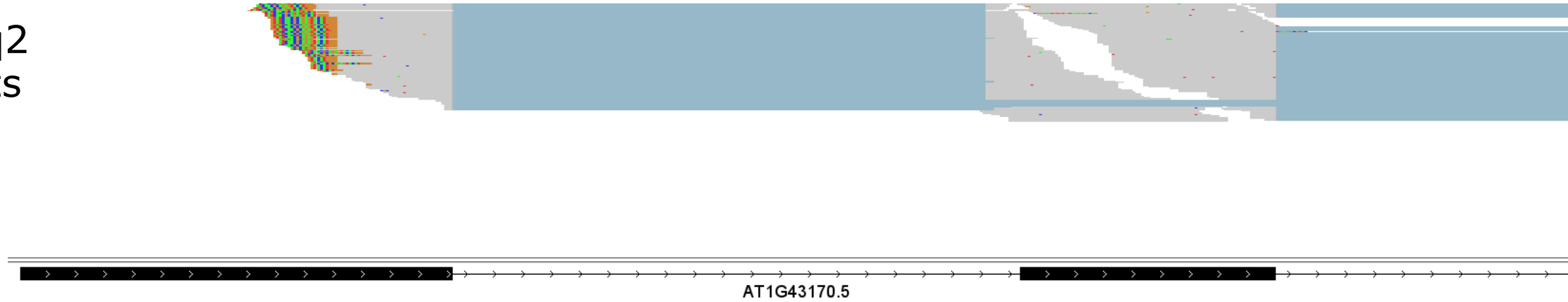
Picelli et al. 2014 *Nature Protocols*

Something is missing...

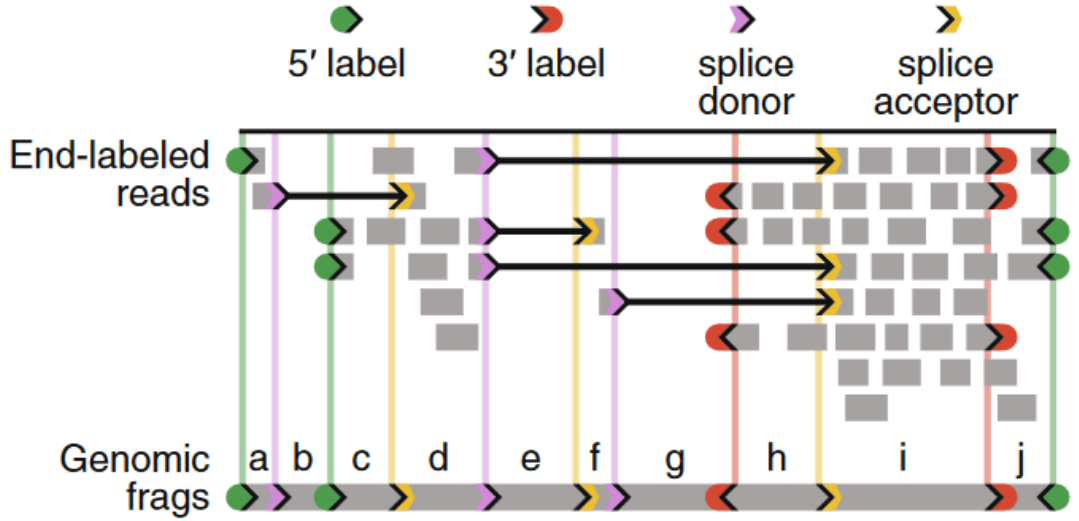
TSO positions

Smart-seq2 alignments



- Could RNA end labels improve transcript assembly?

End-guided assembly



bookend

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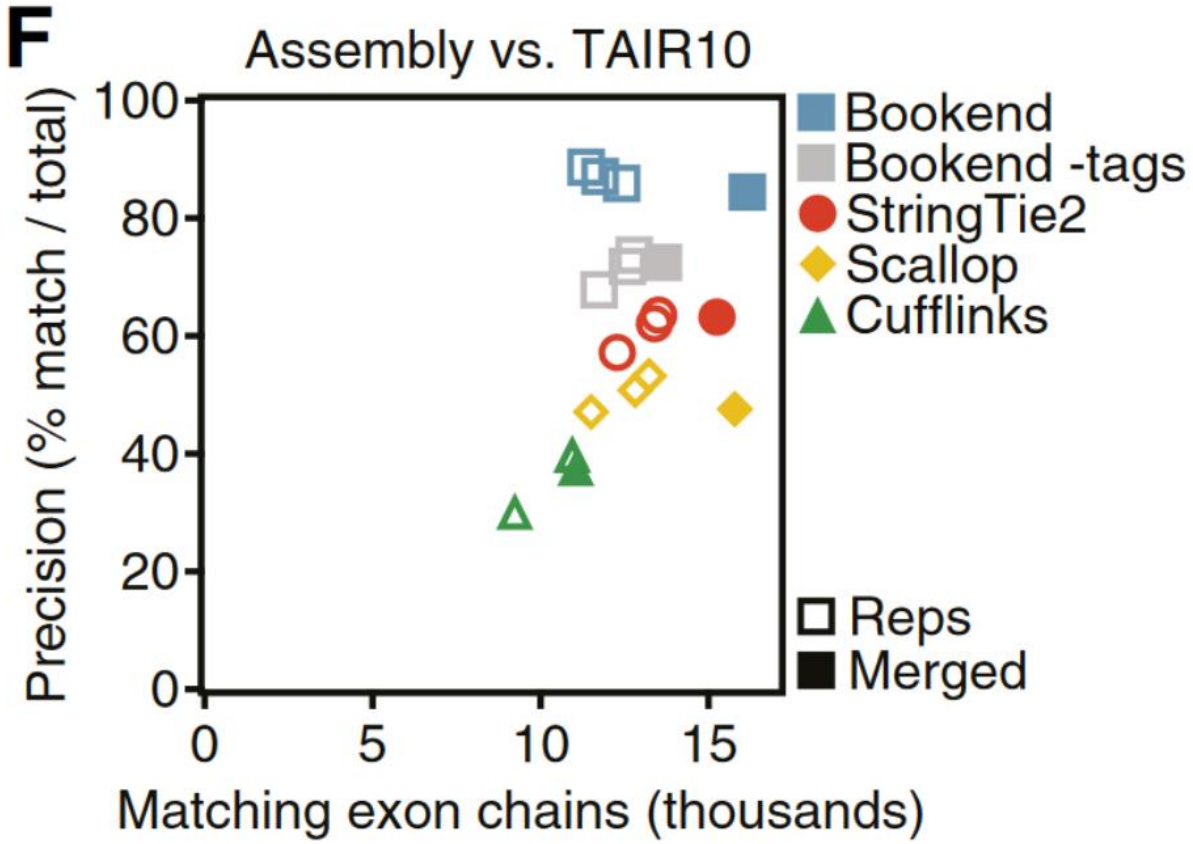
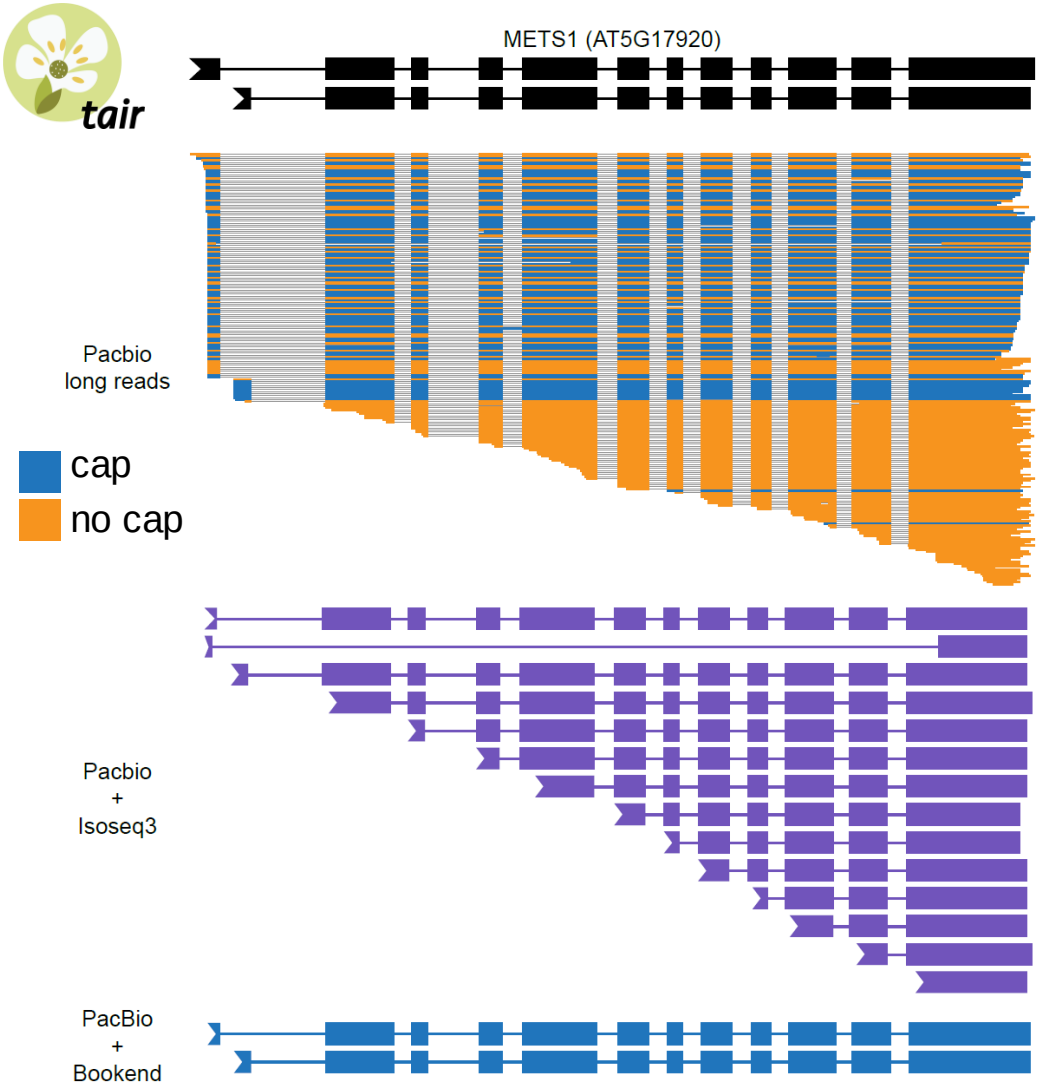
Schon et al. 2022 *Genome Biology*

End-guided assembly





Schon et al. 2022 *Genome Biology*

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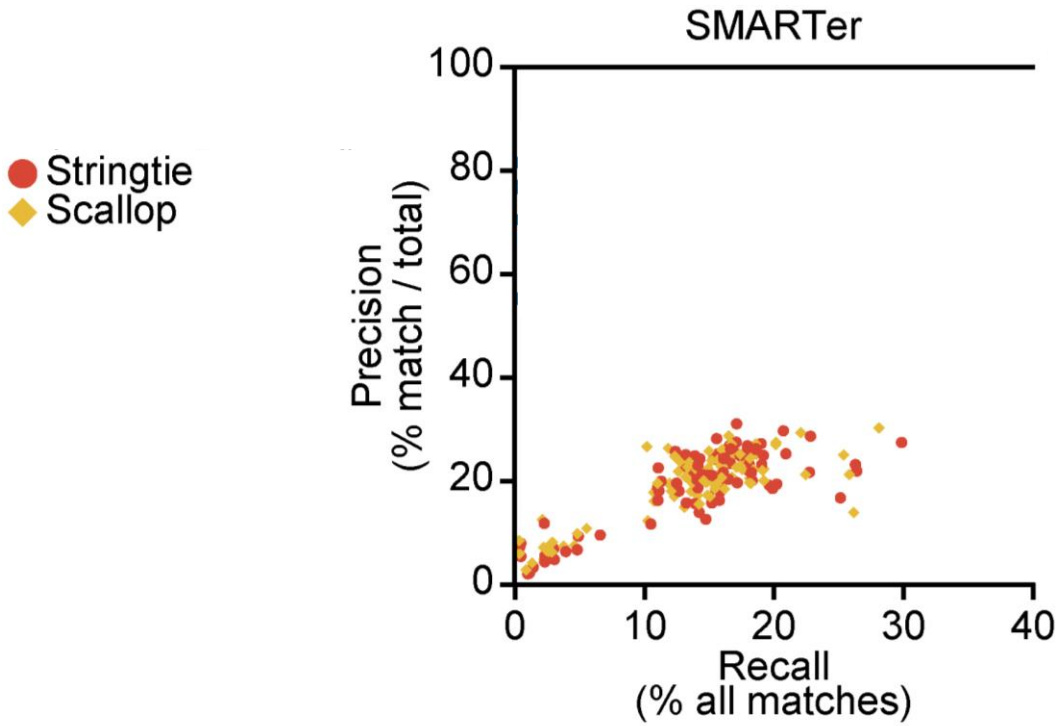
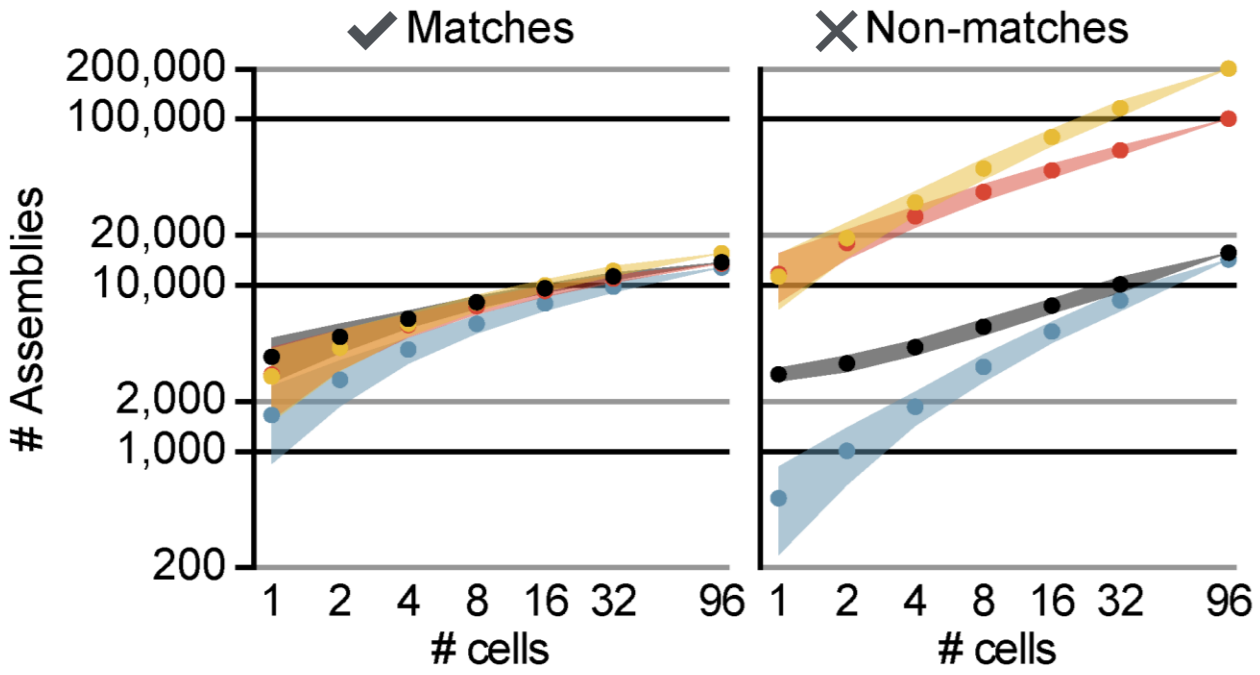
End-guided assembly

Mouse embryonic stem cell assemblies
(96 single cells)



Multi-cell assemblies
vs. reference (RefSeq)



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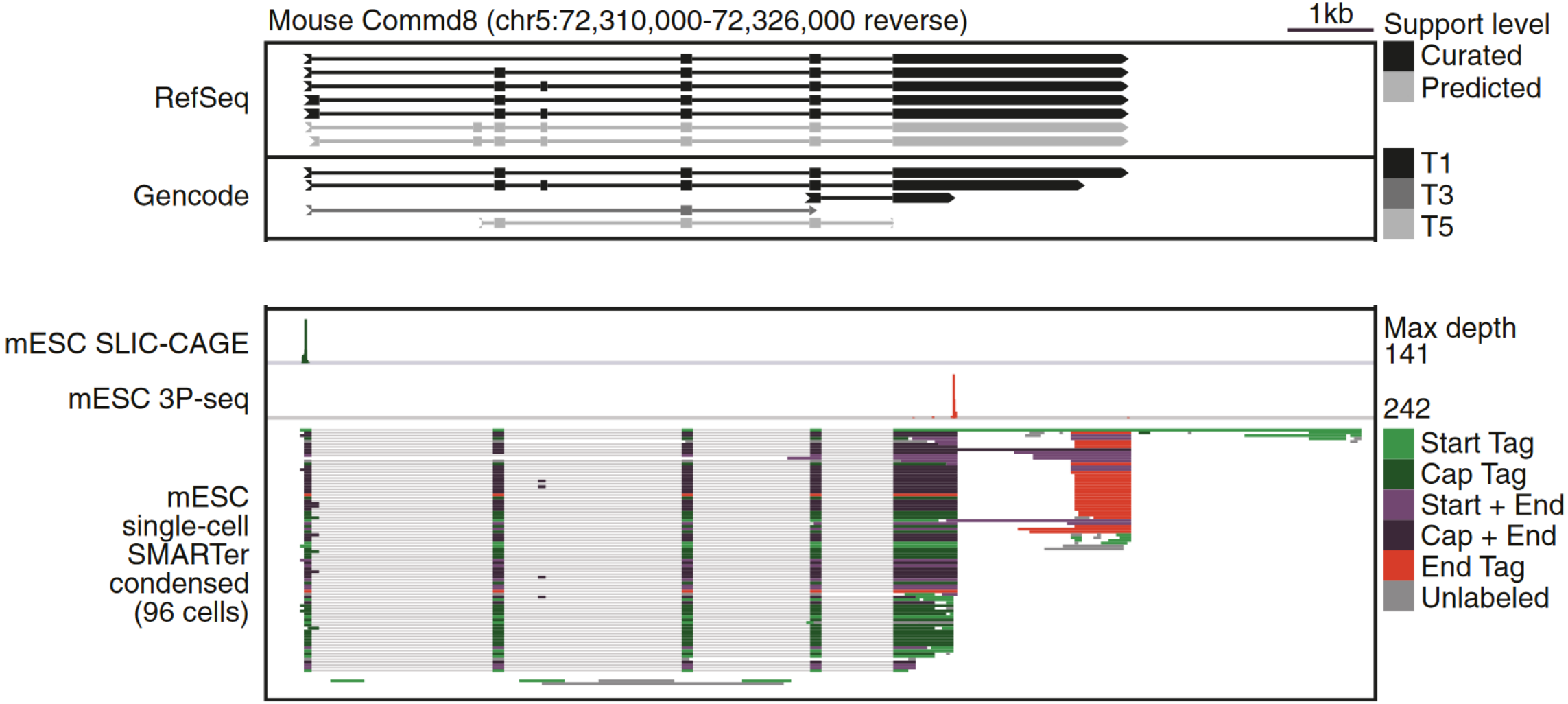
Schon et al. 2022 *Genome Biology*

Meta-assembly: each cell is unique



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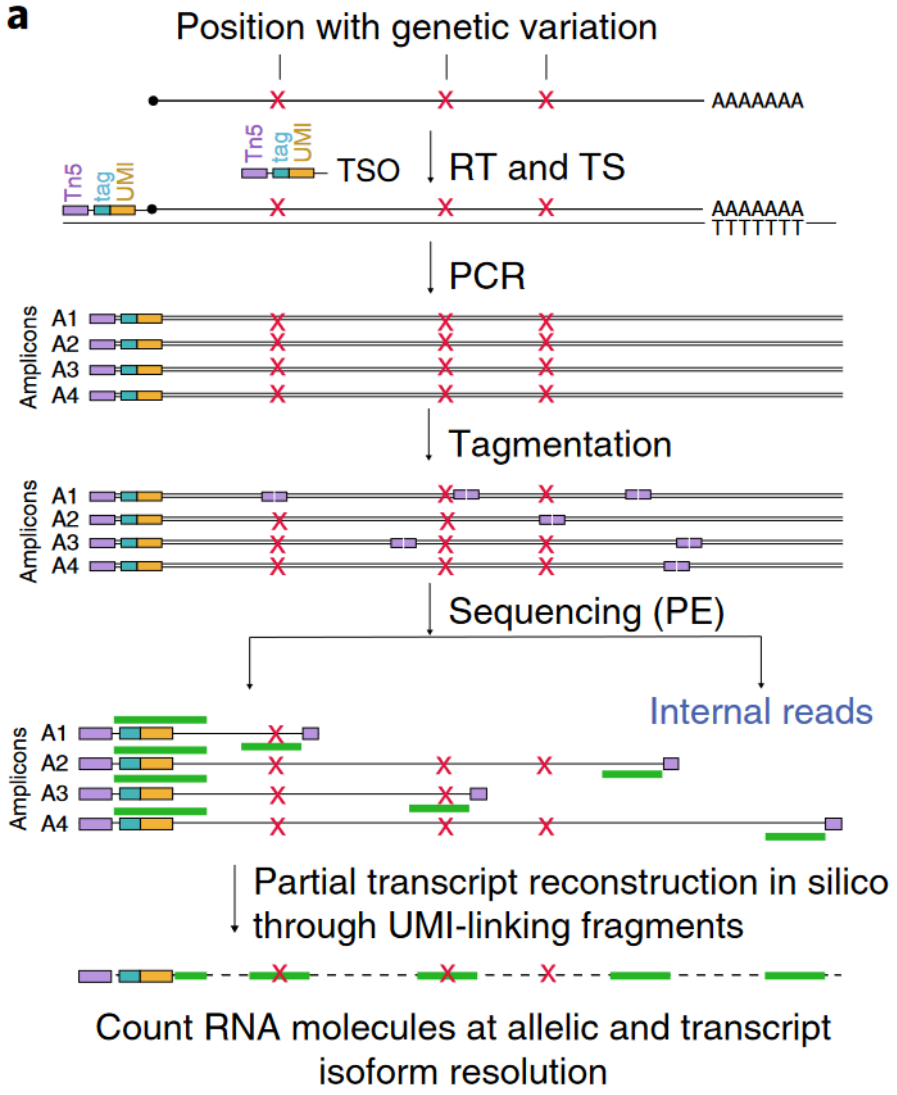
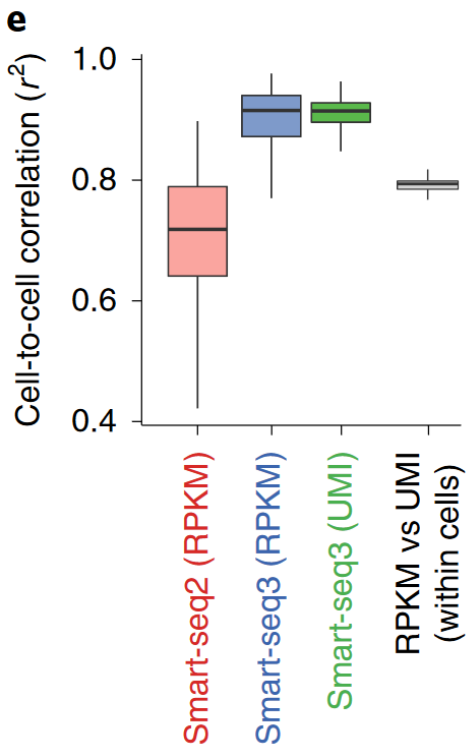
Schon et al. 2022 *Genome Biology*

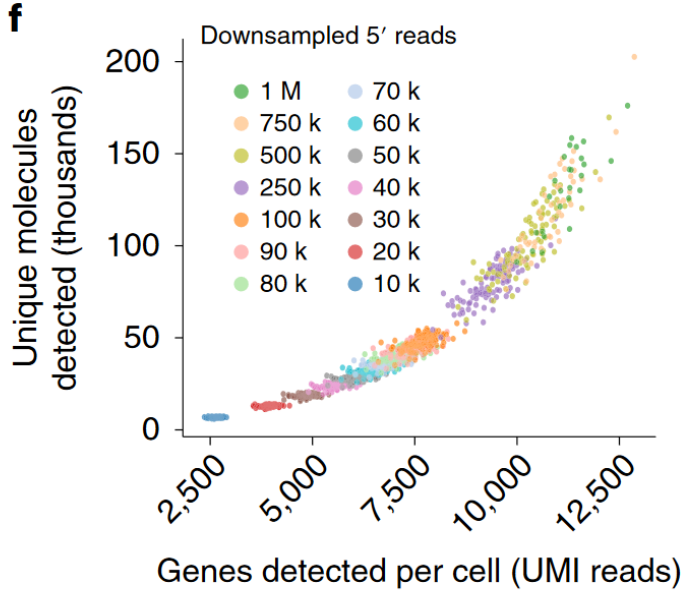
Partial molecule reconstruction



Smart-seq3

- Tn5-UMI-TSO → 20-30% 5’ PE reads
- Can capture >100,000 UMIs/cell
  ~25% of smFISH!





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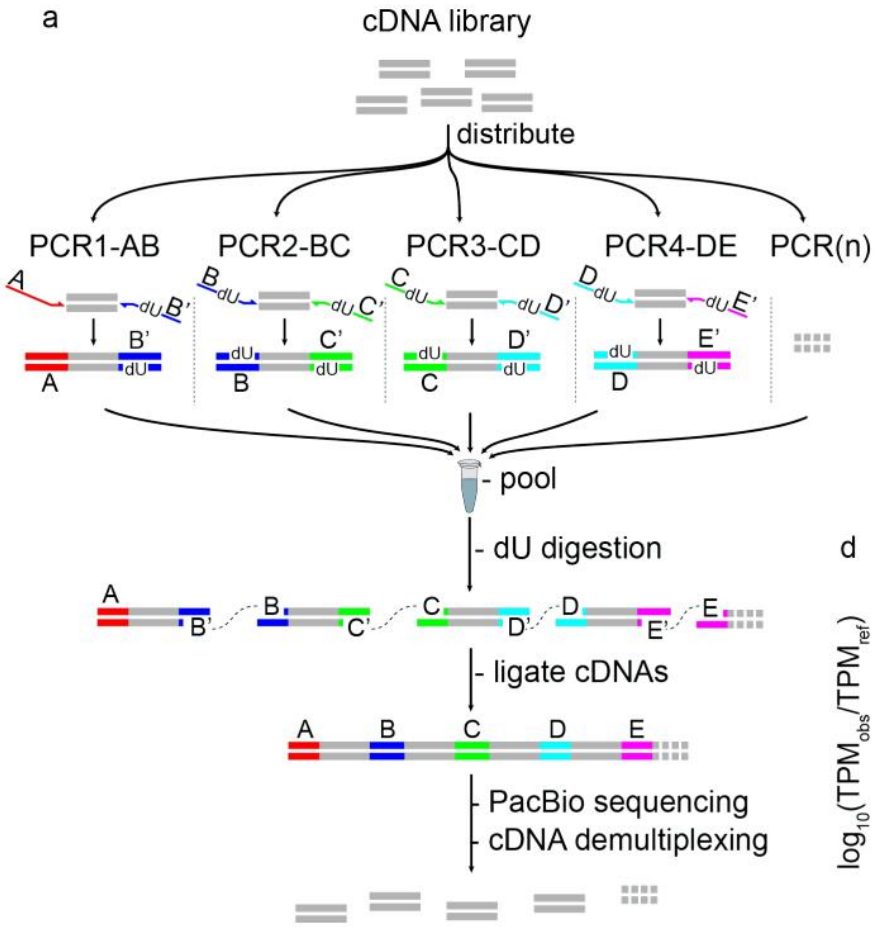
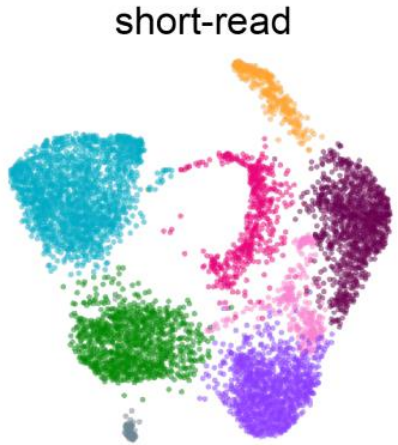
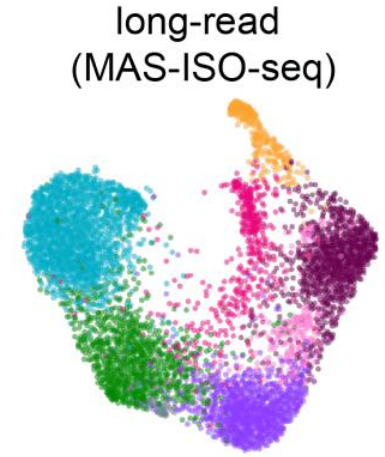
Hagemann-Jensen et al. 2020 *Nature Biotechnology*

Long reads in single cells







d

$\log_{10}(TPM_{obs}/TPM_{ref})$

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Al'Khafaji et al. 2021 *bioRxiv*

Conclusions

- Development is a cellular process
- We now have many methods for sequencing many RNAs x many cells
- Downstream analysis is both art and science
  - Well-grounded in reality:
    marker genes
    timepoints
    validation
  - "Number of clusters" is (partly) subjective
  - Continuous methods are possible, but prone to distortions
- Not all data is created equal!
  - Make sure the protocol is sufficient to answer your question
  - Isoform analysis requires full-length sequencing:
    Smart-seq (2,3,xpress)
    MAS-ISO-SEQ

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Thesis Reviewers
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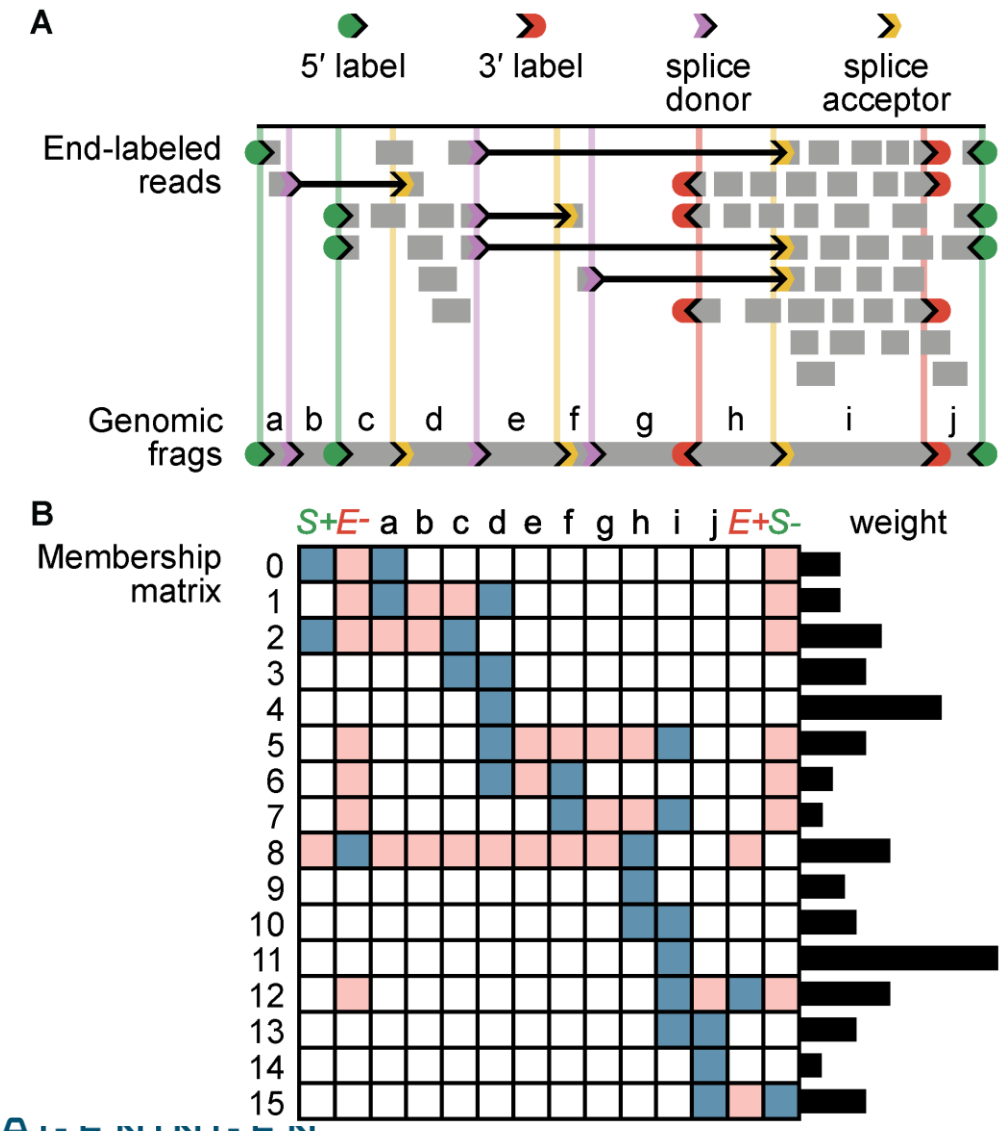
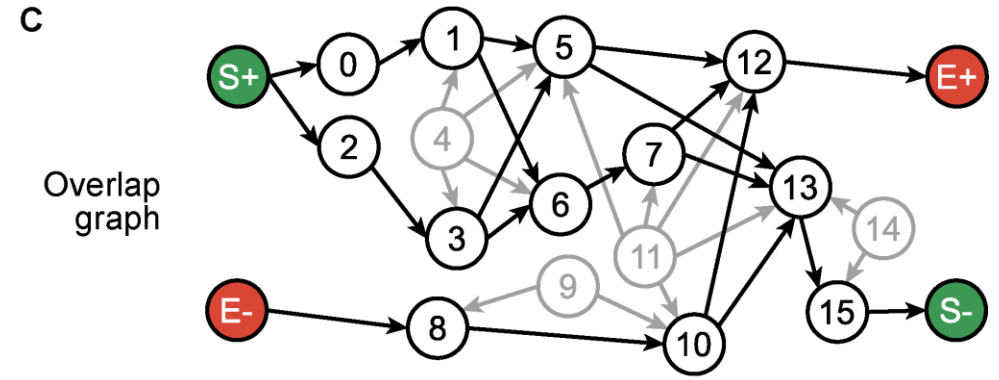
Dolf Weijers

VBCF
Next Generation Sequencing
Andreas Sommer
Ido Tamir

The Bookend framework

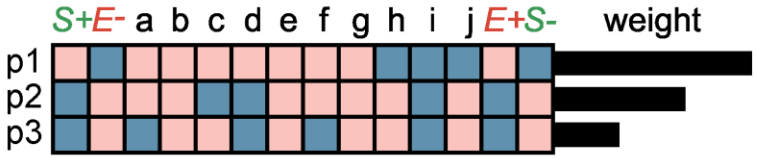




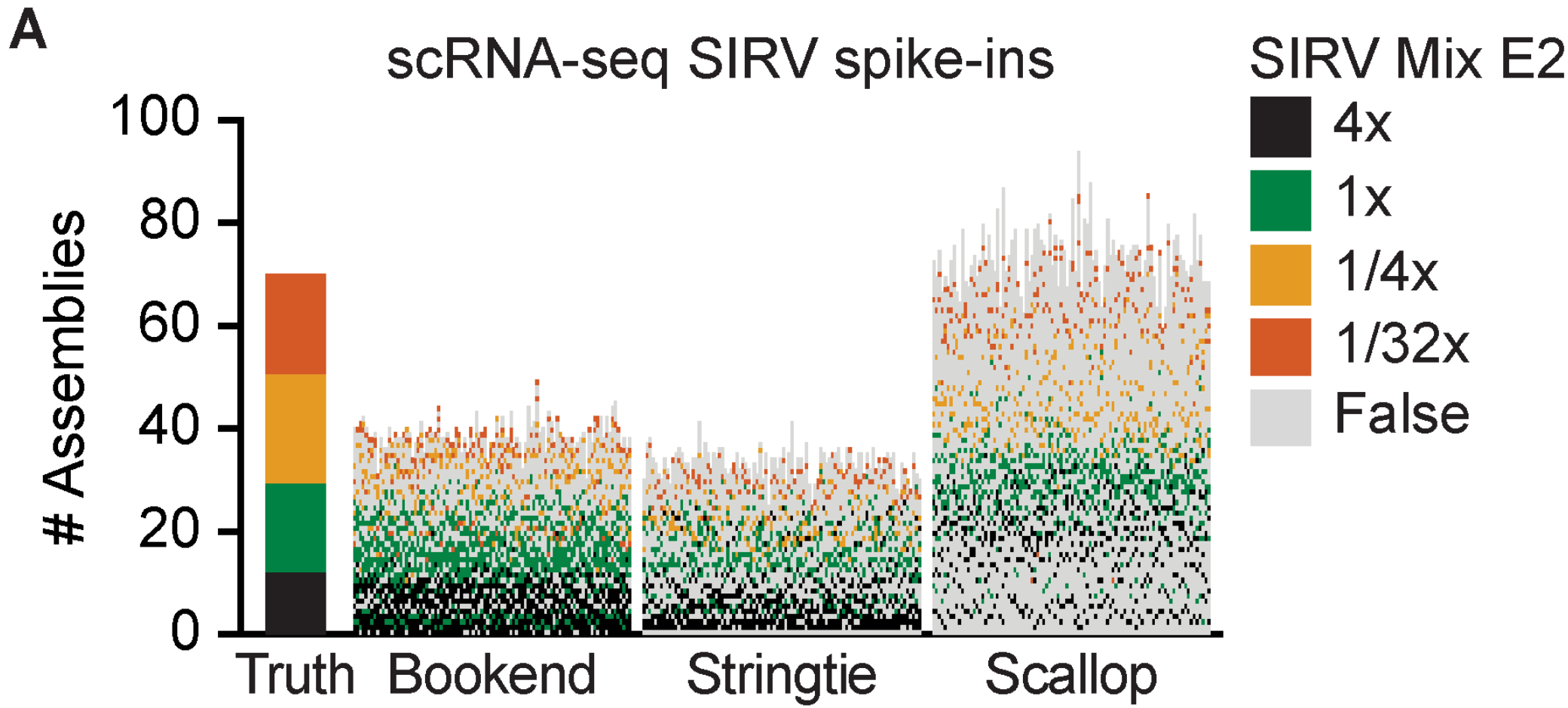
D

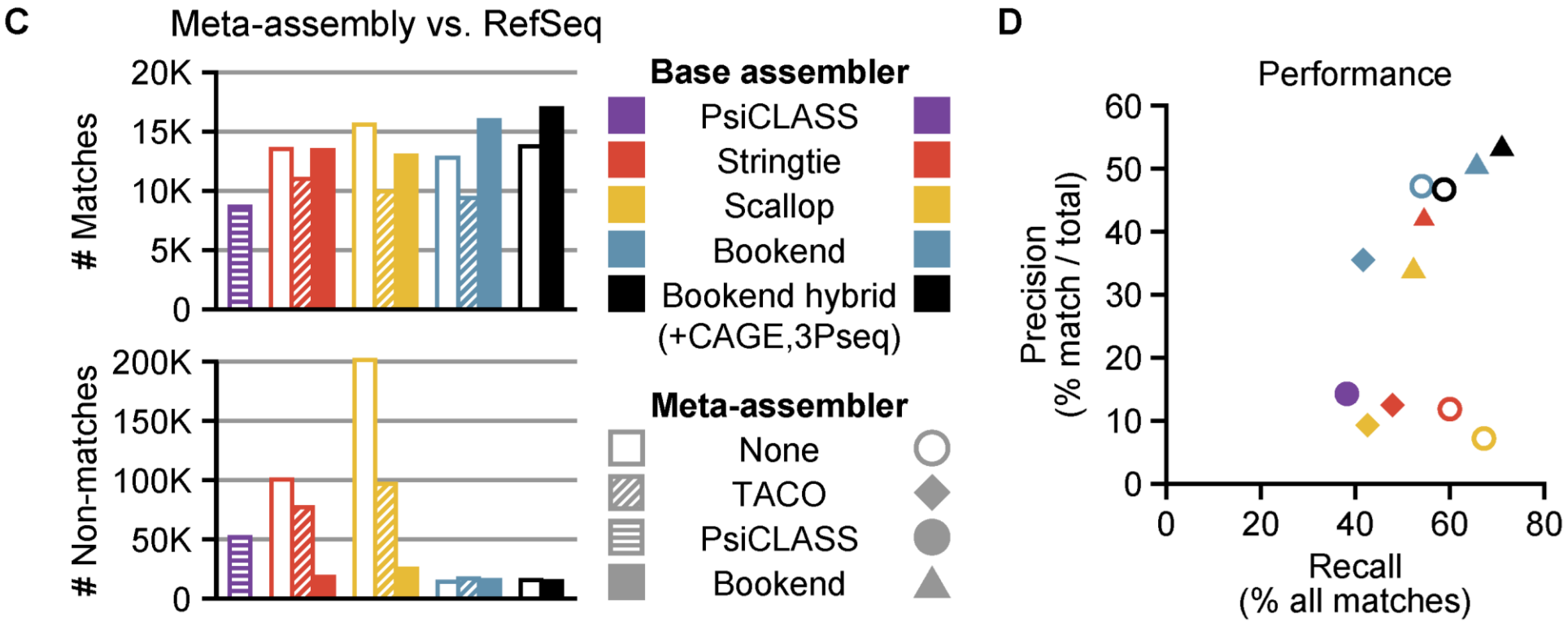
Greedy paths

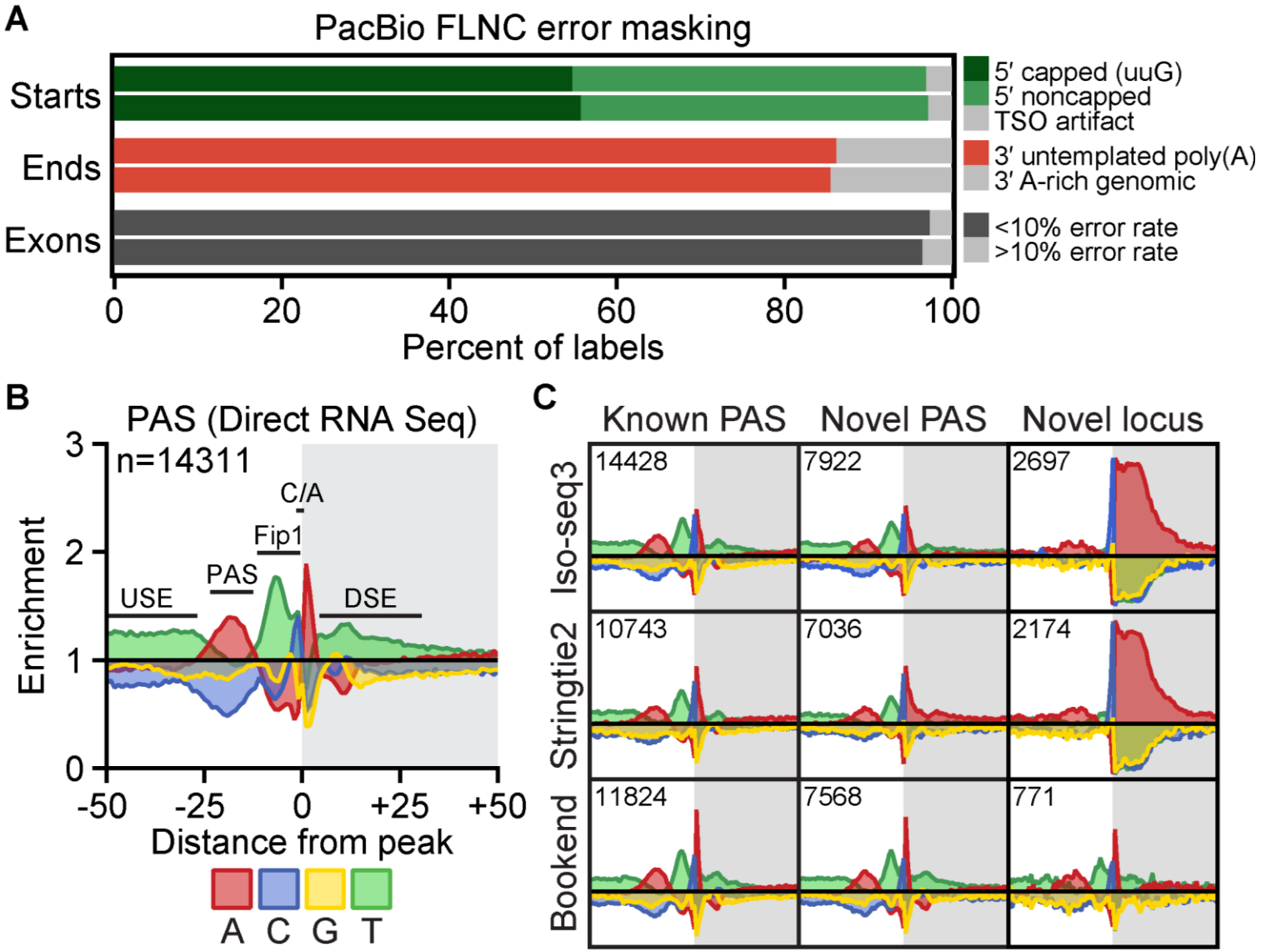
p1: *E*-→8/9→10/11→13/14→15→*S*-
p2: *S*+→2→3/4→5/11→12→*E*+
p3: *S*+→0→1/4→6→7/11→12→*E*+

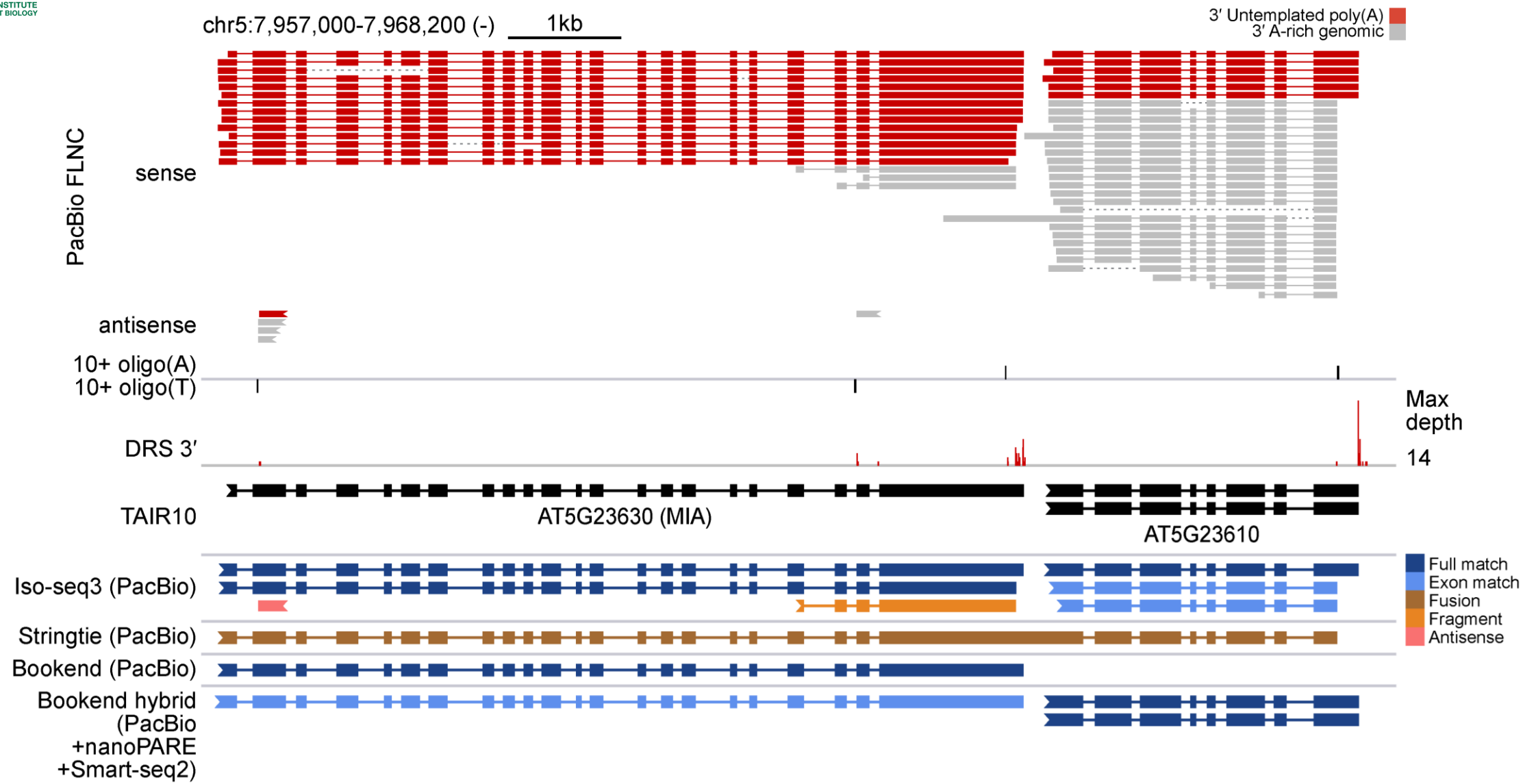


bookend


A
scRNA-seq SIRV spike-ins
Assemblies
100
80
60
40
20
0
Truth
Bookend
Stringtie
Scallop
SIRV Mix E2
4x
1x
1/4x
1/32x
False


C
Meta-assembly vs. RefSeq
Matches
20K
15K
10K
5K
0
Non-matches
200K
150K
100K
50K
0
Base assembler
PsiCLASS
Stringtie
Scallop
Bookend
Bookend hybrid
(+CAGE,3Pseq)
Meta-assembler
None
TACO
PsiCLASS
Bookend
D
Performance
Precision
(% match / total)
60
50
40
30
20
10
0
0
20
40
60
80
Recall
(% all matches)


A
PacBio FLNC error masking
Starts
Ends
Exons
0
20
40
60
80
100
Percent of labels
5′ capped (uuG)
5′ noncapped
TSO artifact
3′ untemplated poly(A)
3′ A-rich genomic
<10% error rate
>10% error rate
B
PAS (Direct RNA Seq)
n=14311
Enrichment
C/A
Fip1
PAS
USE
DSE
-50
-25
0
+25
+50
Distance from peak
A C G T
C
Known PAS
Novel PAS
Novel locus
Iso-seq3
Stringtie2
Bookend
14428
7922
2697
10743
7036
2174
11824
7568
771


chr5:7,957,000-7,968,200 (-)
1kb
3′ Untemplated poly(A)
3′ A-rich genomic
PacBio FLNC
sense
antisense
10+ oligo(A)
10+ oligo(T)
Max depth
14
DRS 3′
TAIR10
AT5G23630 (MIA)
AT5G23610
Iso-seq3 (PacBio)
Stringtie (PacBio)
Bookend (PacBio)
Bookend hybrid (PacBio +nanoPARE +Smart-seq2)
Full match
Exon match
Fusion
Fragment
Antisense