Gene count normalization in single-cell imaging-based spatially resolved transcriptomics

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Johns Hopkins University

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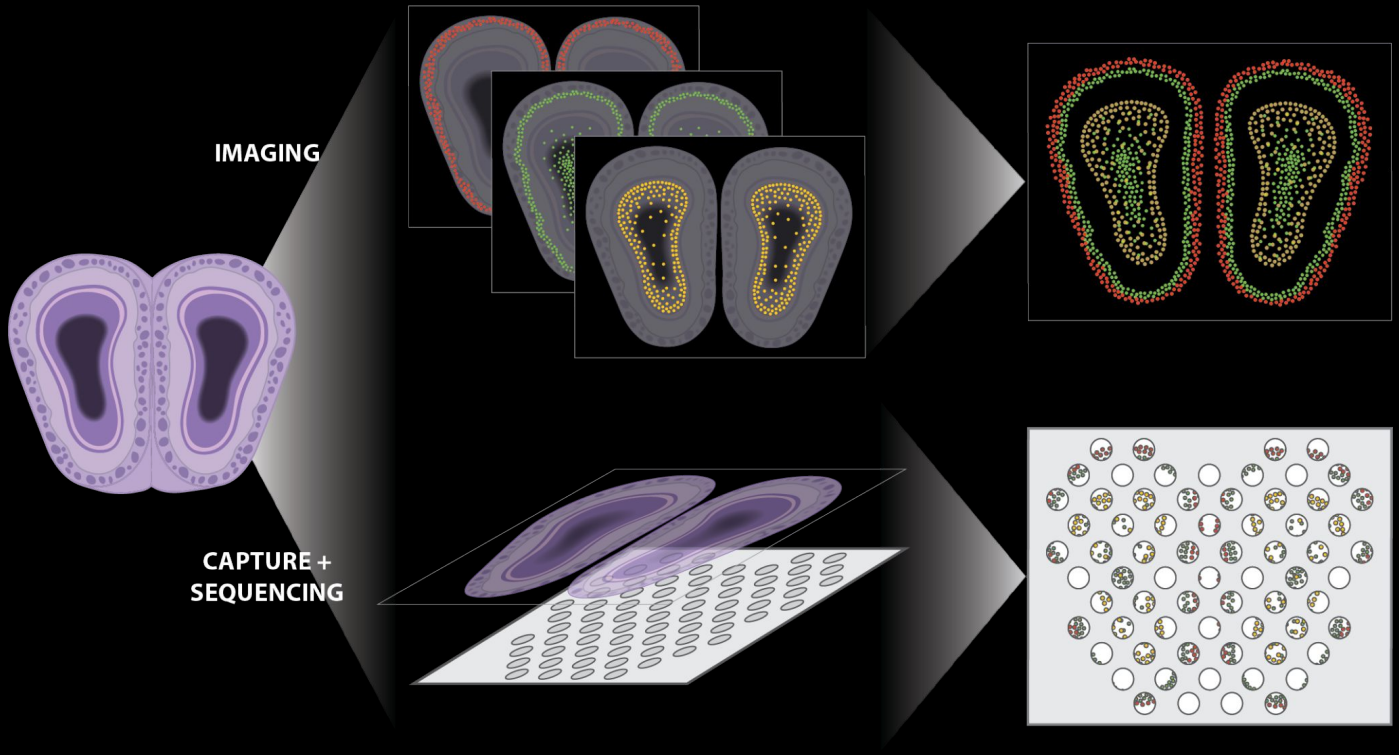
Spatially resolved transcriptomics (SRT)



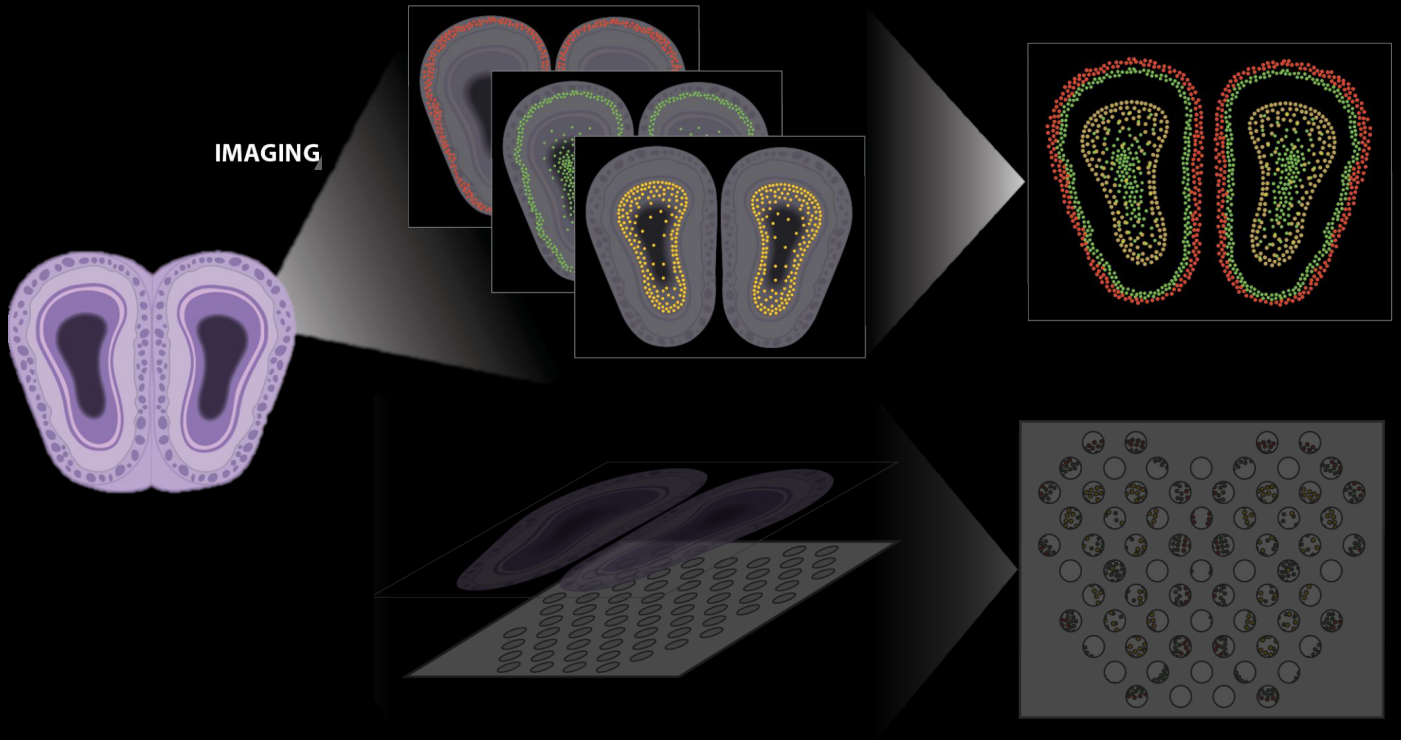
Imaging-based SRT

- Single molecule
- Targeted
- ~100-500 genes

Sequencing-based SRT

- Spot resolution
- Untargeted
- Whole transcriptome

Spatially resolved transcriptomics (SRT)



Imaging-based SRT

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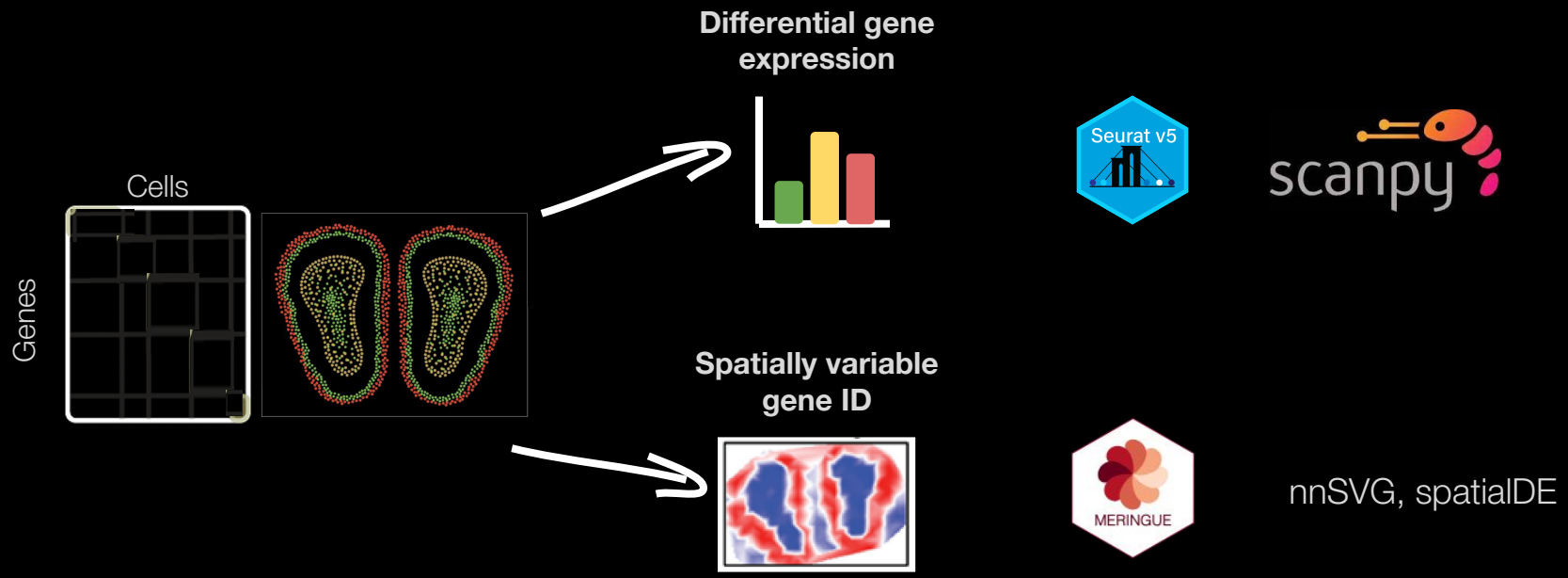
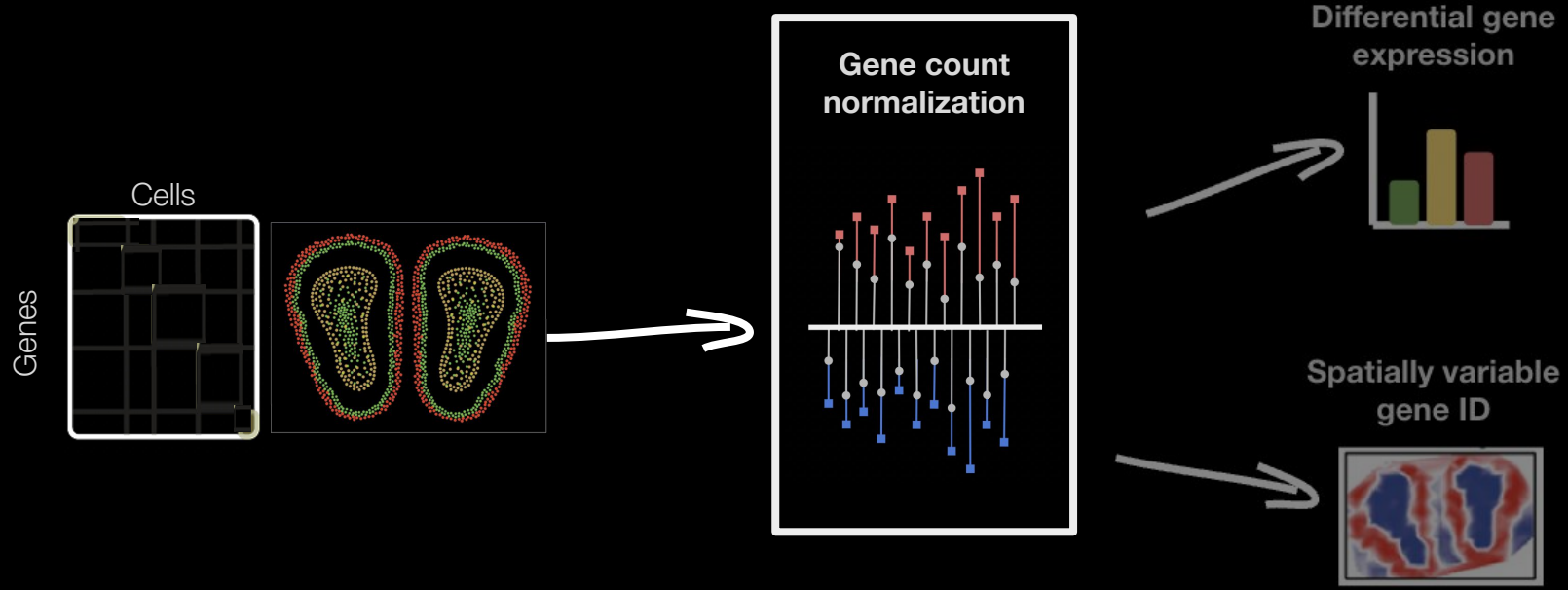
imSRT: computational methods to generate biological hypotheses



imSRT: gene count normalization is the first step to many downstream analyses



Count normalization in imaging-based SRT

- Goals of count normalization, current normalization approaches, evaluation criteria
- Case study: ventricle gene panel in mouse brain MERFISH imSRT
- Evaluating normalization across gene panels, technologies and tissues
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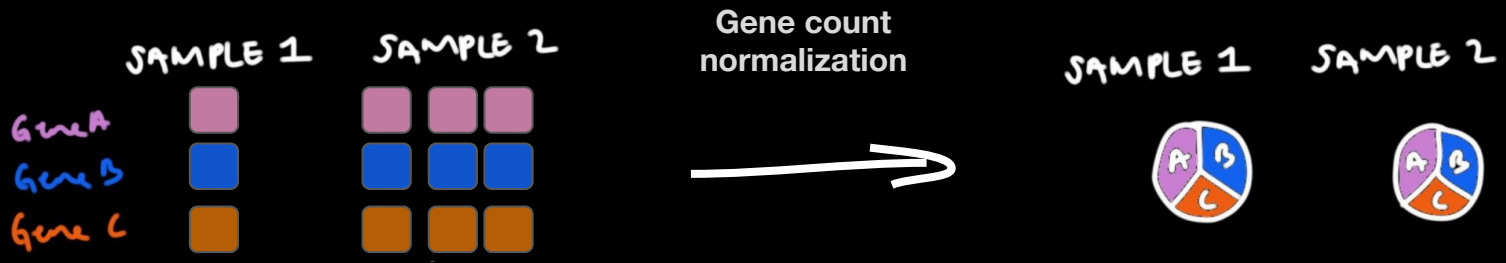
Gene count normalization: accounting for technical variation



- **Bulk RNA-seq:** PCR bias, reagent variation
- **scRNA-seq:** stochastic RNA capture
- **imSRT:** partial cell volume imaging

Gene count normalization: current approaches

Library size	DESeq2	TMM (edgeR)	scTransform	Cell volume/ cell area
Bulk RNA-seq	Bulk RNA-seq	Bulk RNA-seq		
scRNA-seq			scRNA-seq	
imSRT				imSRT

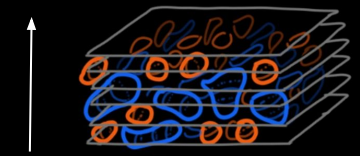
Gene count normalization: current approaches

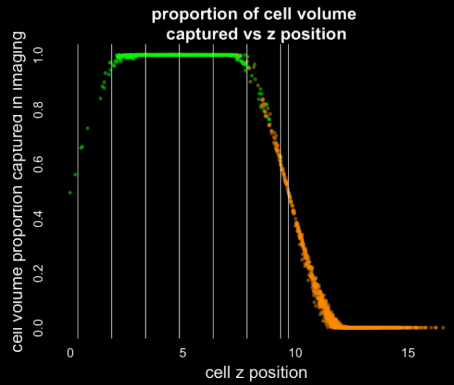
Library size	DESeq2	TMM (edgeR)	scTransform	Cell volume/ cell area
Bulk RNA-seq	Bulk RNA-seq	Bulk RNA-seq		
scRNA-seq			scRNA-seq	
imSRT				imSRT

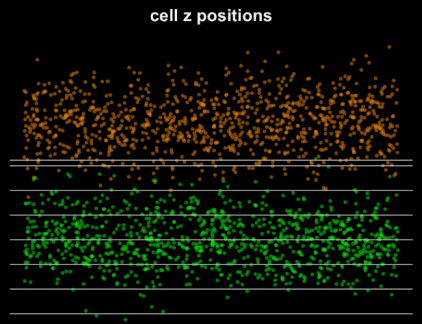
COUNT BASED NORMALIZATION

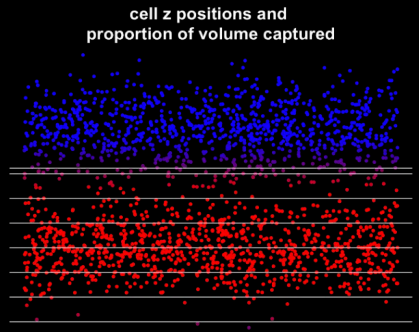
Gene count normalization: is it necessary?
Simulation to evaluate systematic RNA capture biases

One cell type, two z locations





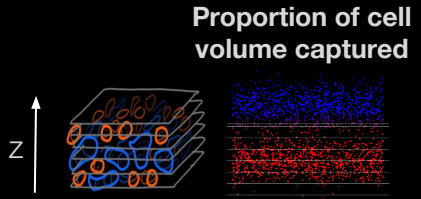


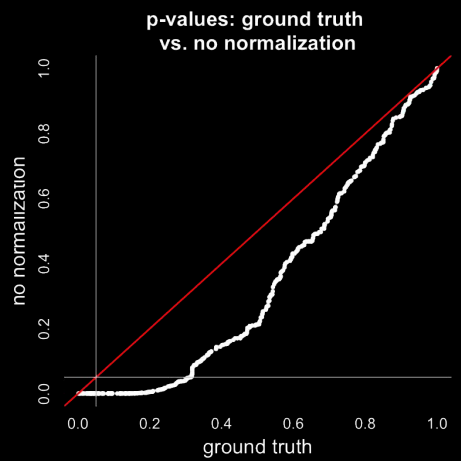


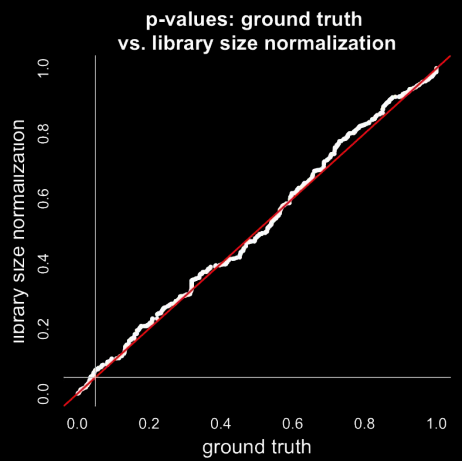
Gene count normalization: is it necessary?

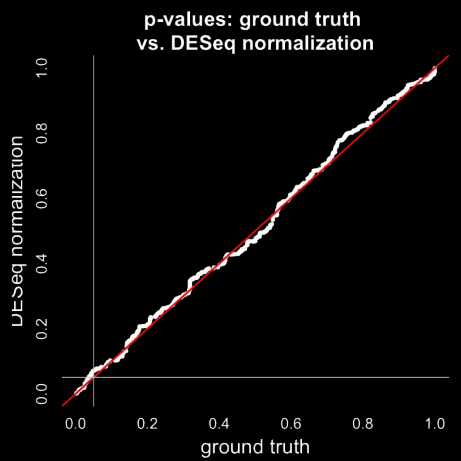
Simulation to evaluate systematic RNA capture biases

Partial cell capture in imaged volume: inflated Type I error rate without normalization



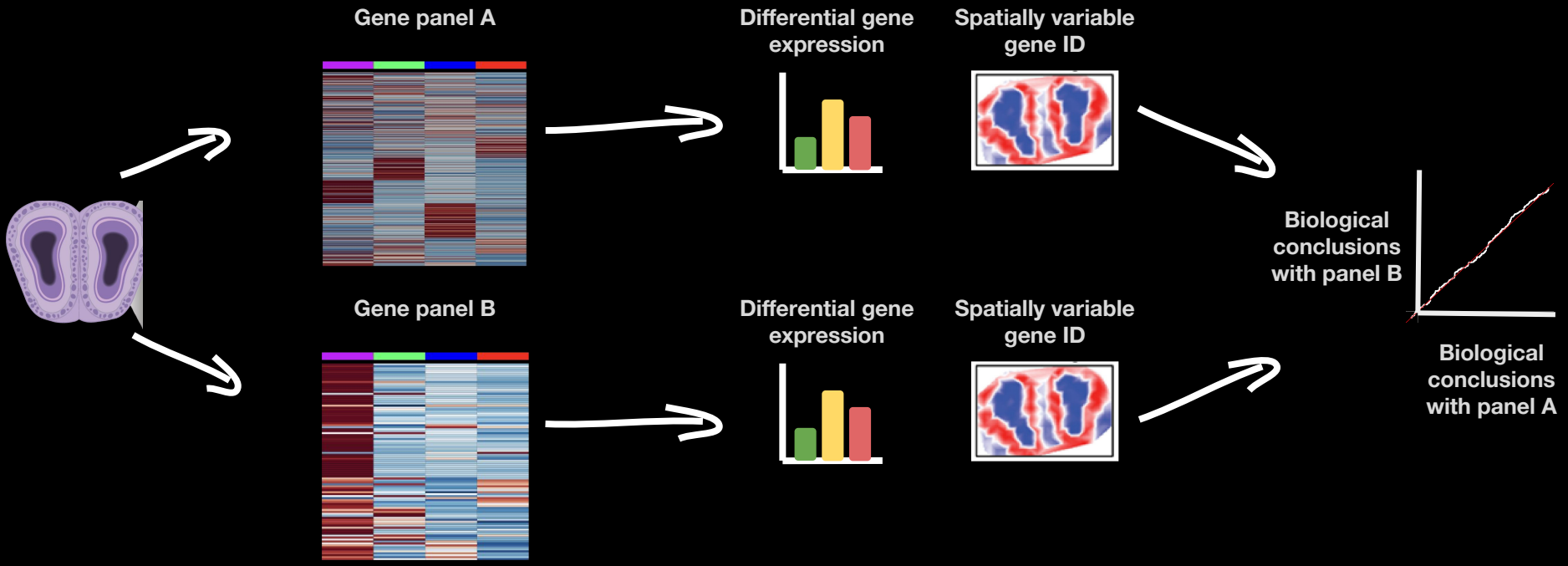






Gene count normalization: evaluation criteria

Robustness of downstream analyses with different gene panels



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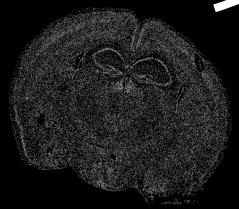
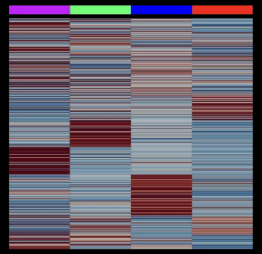
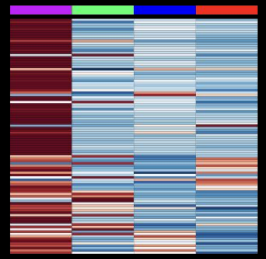
Comparing normalization methods in mouse brain MERFISH with different gene panels



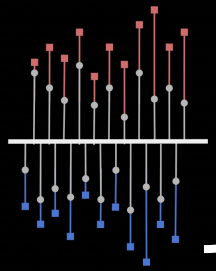
Full gene panel
481 genes



Ventricle gene panel
100 ventricle genes



Gene count normalization



Library size
scTransform
DESeq2
TMM
Cell volume

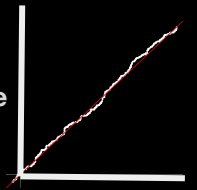
Downstream analyses

Differential gene expression + fold change

Spatially variable gene ID

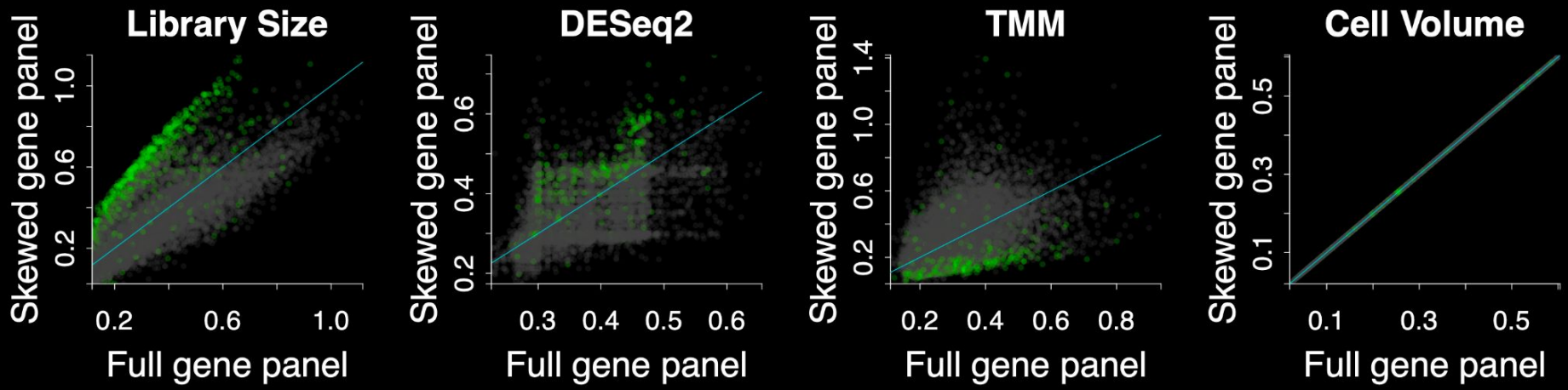
Compare results

Ventricle skewed gene panel



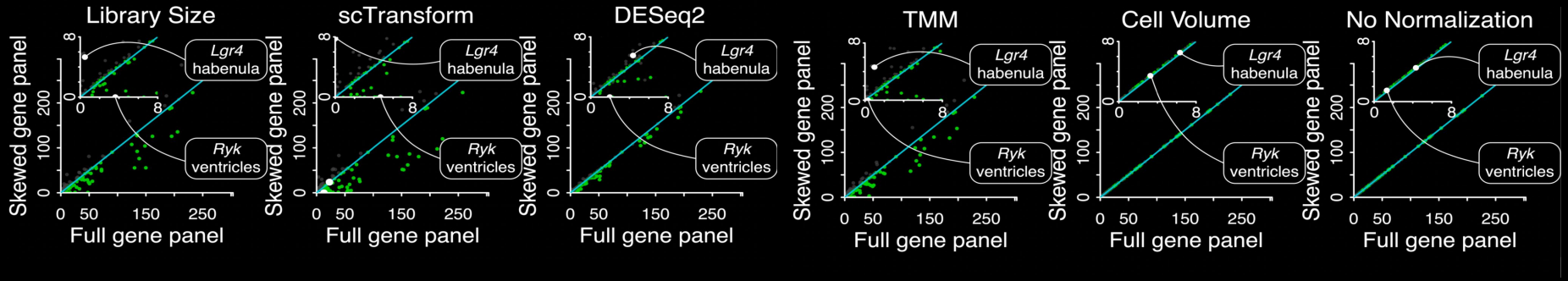
Full gene panel

Normalizing scaling factors: tissue region specific bias with ventricle gene panel



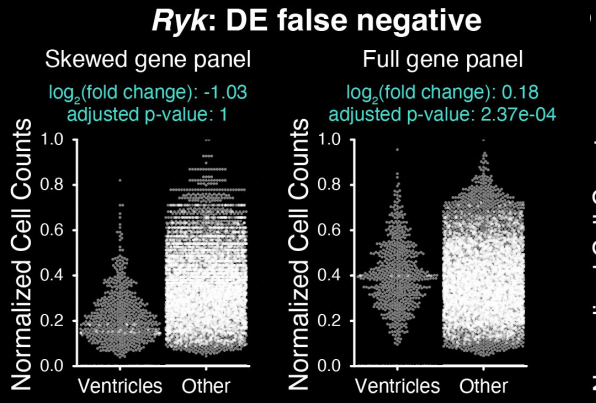
COUNT BASED NORMALIZATION

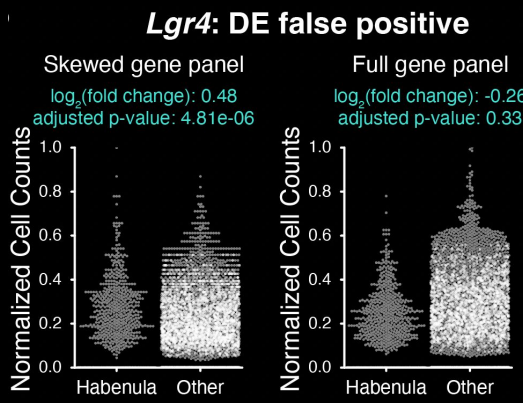
Differential gene expression: tissue region specific bias with ventricle gene panel

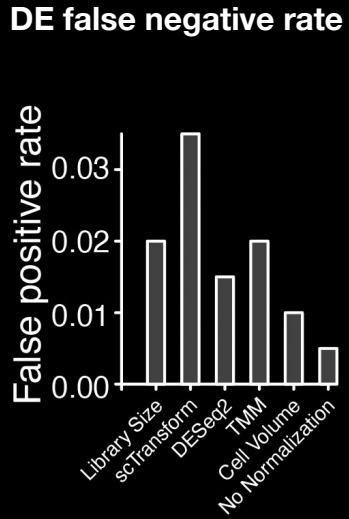


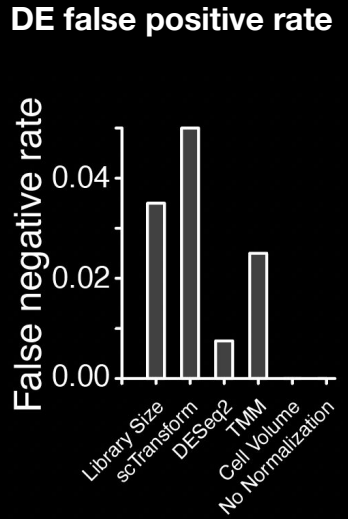
COUNT BASED NORMALIZATION

Differential gene expression: tissue region specific bias with ventricle gene panel results in false positives and negatives

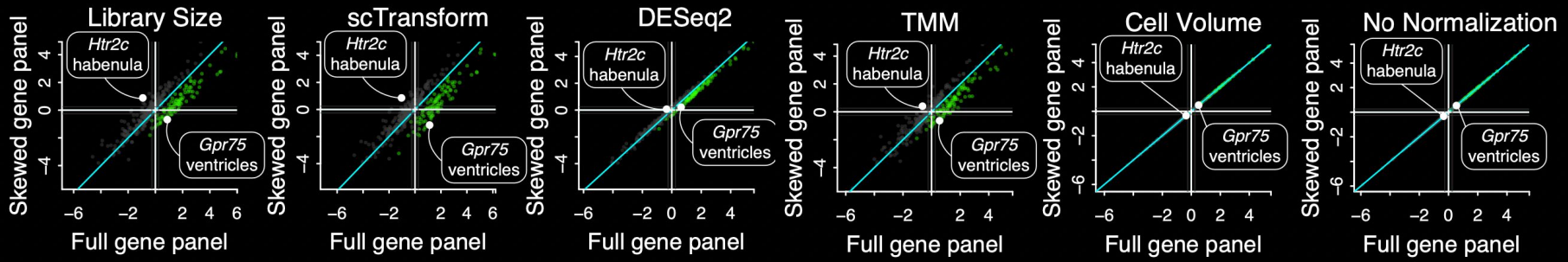






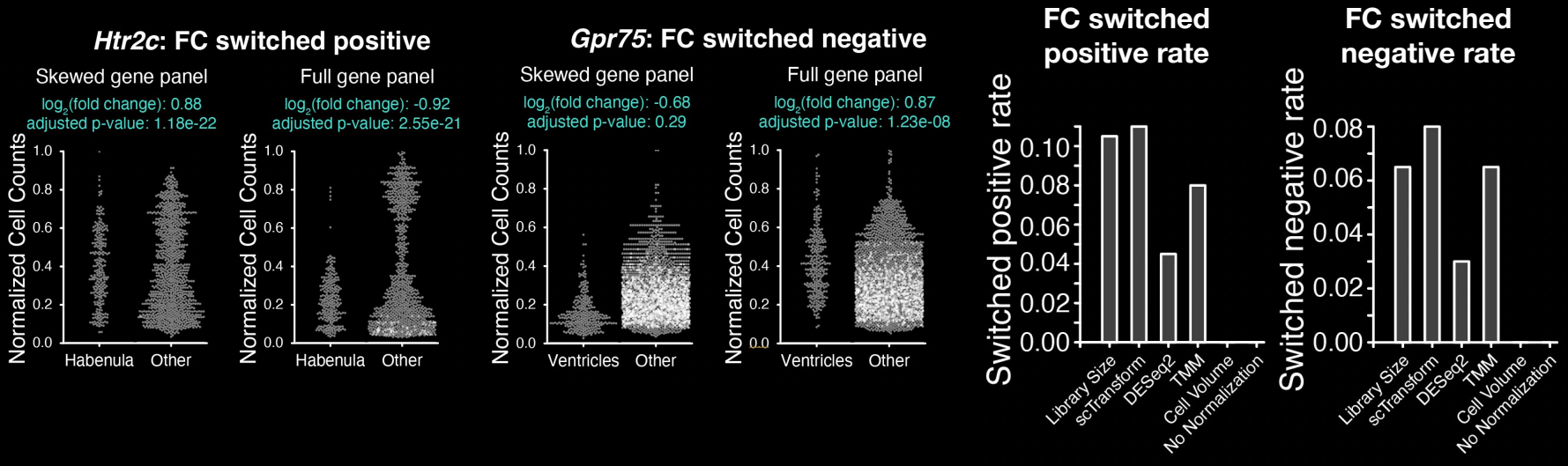


Differential gene expression: tissue region specific bias with ventricle gene panel

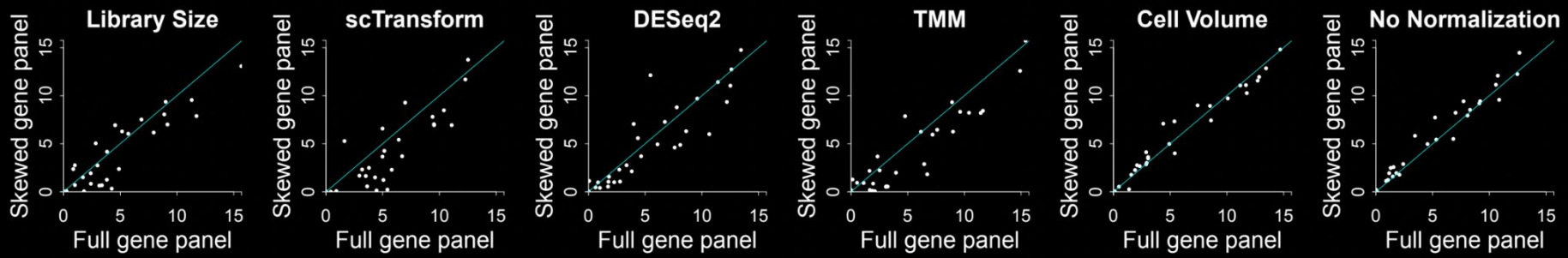


COUNT BASED NORMALIZATION

Differential gene expression: tissue region specific bias with ventricle gene panel results in switched gene fold changes



Spatially variable gene identification with ventricle gene panel results in false positives and negatives



COUNT BASED NORMALIZATION

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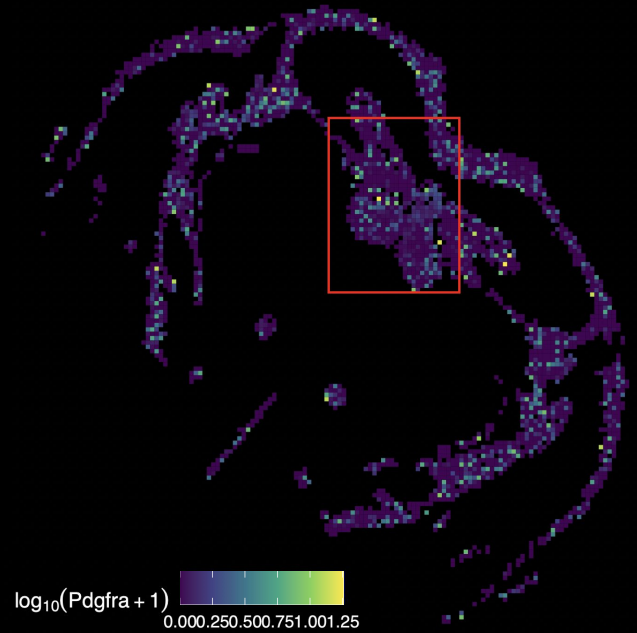
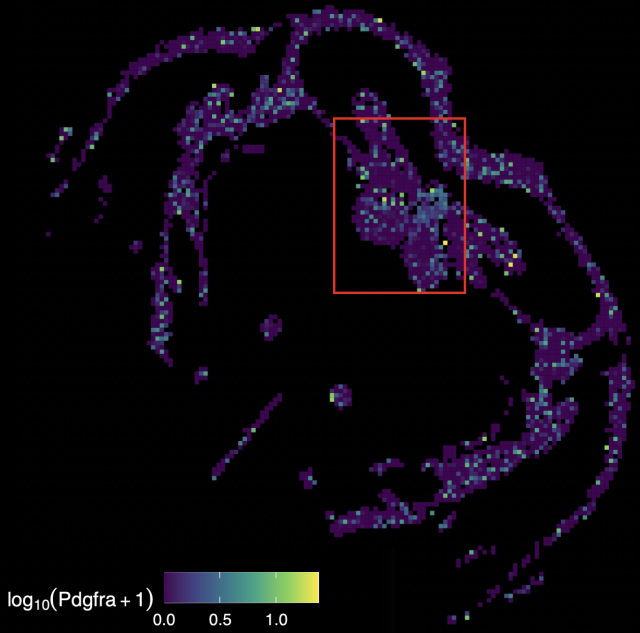
Pdgfra, library size normalization

Ventricle gene panel



Full gene panel



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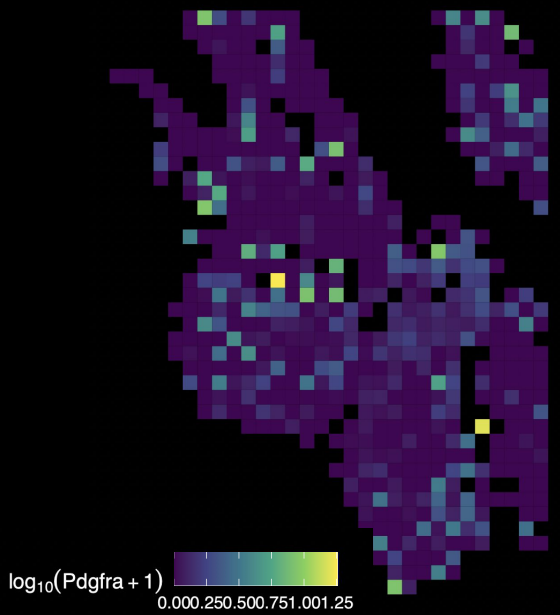

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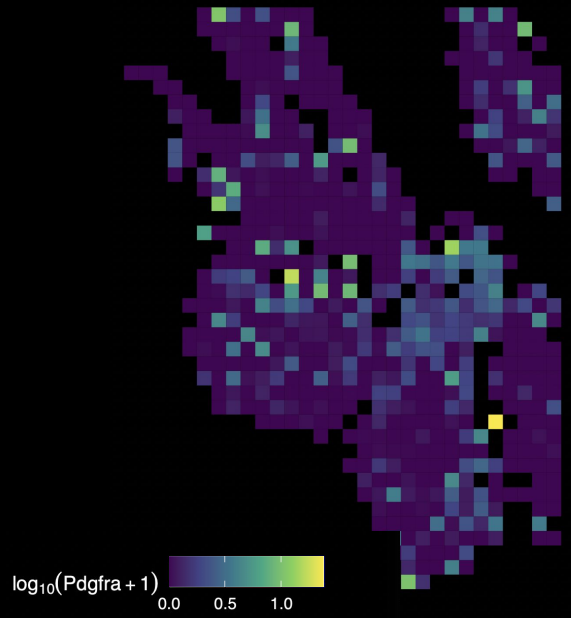

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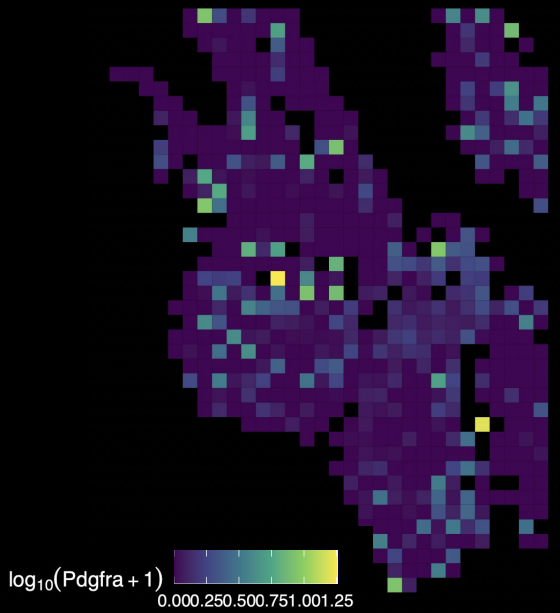

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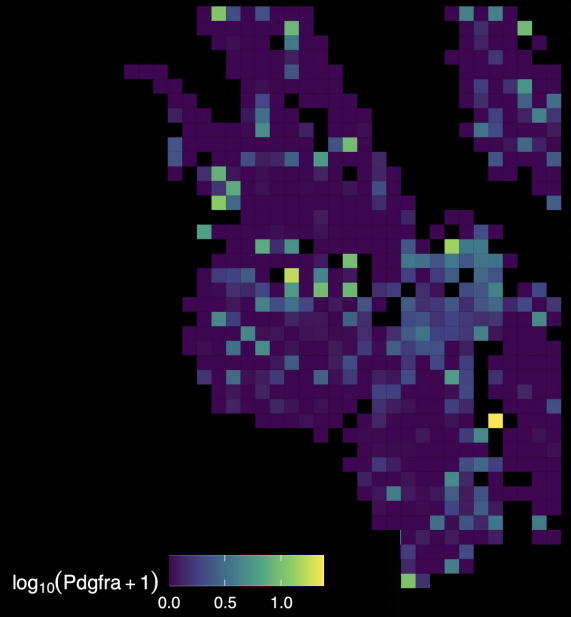

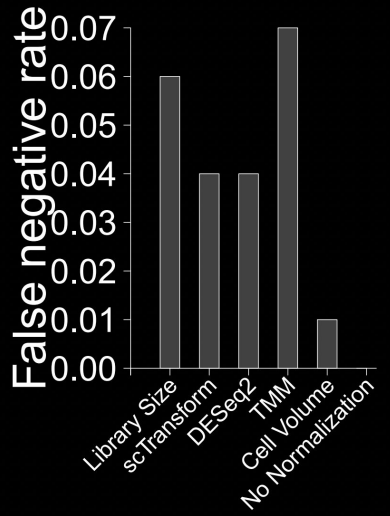

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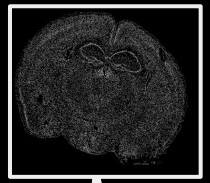
Region-specific biases in normalized gene expression generalize across gene panels

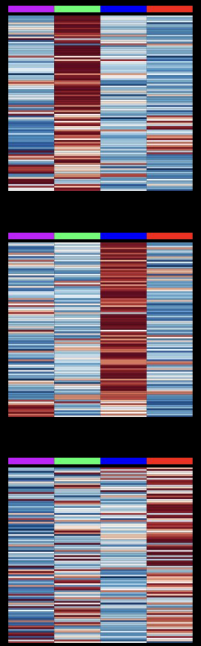


Habenula panel

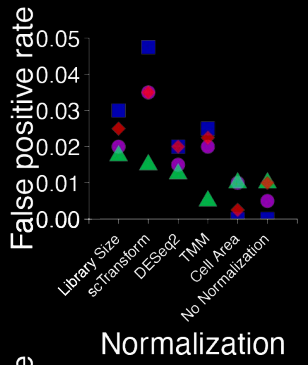
Fiber tracts panel

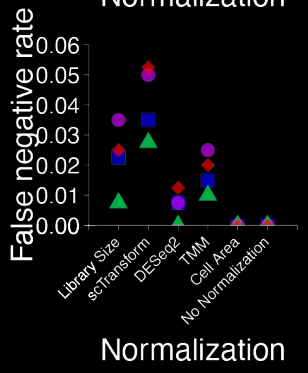
Dentate gyrus panel

Ventricle panel

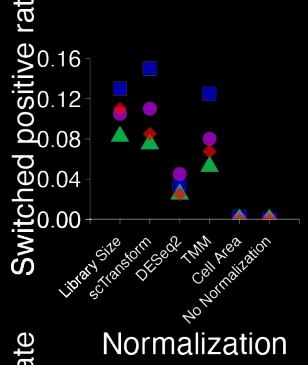


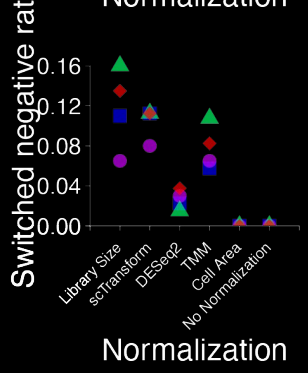
DE error rates



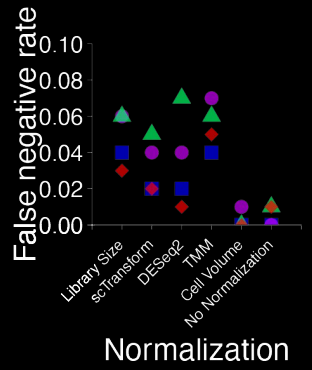


FC switch rates



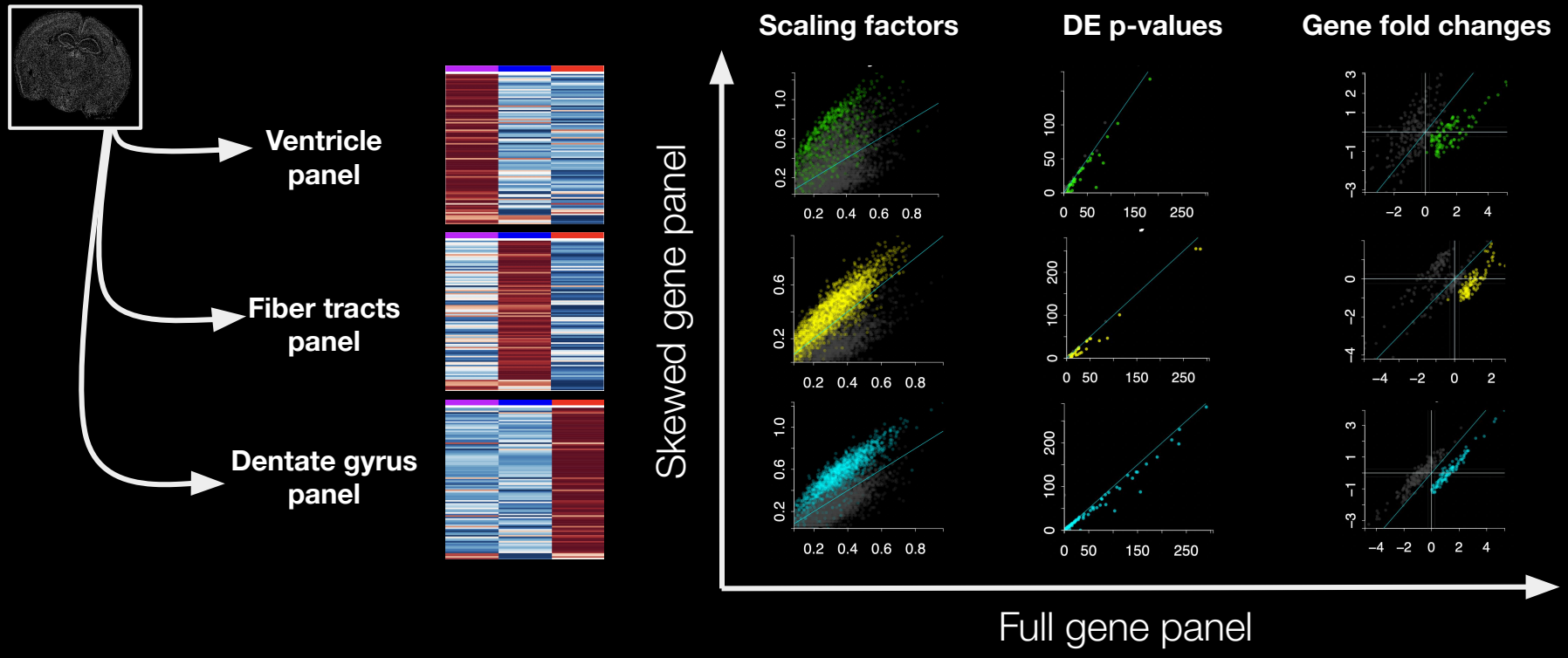


SVG error rates



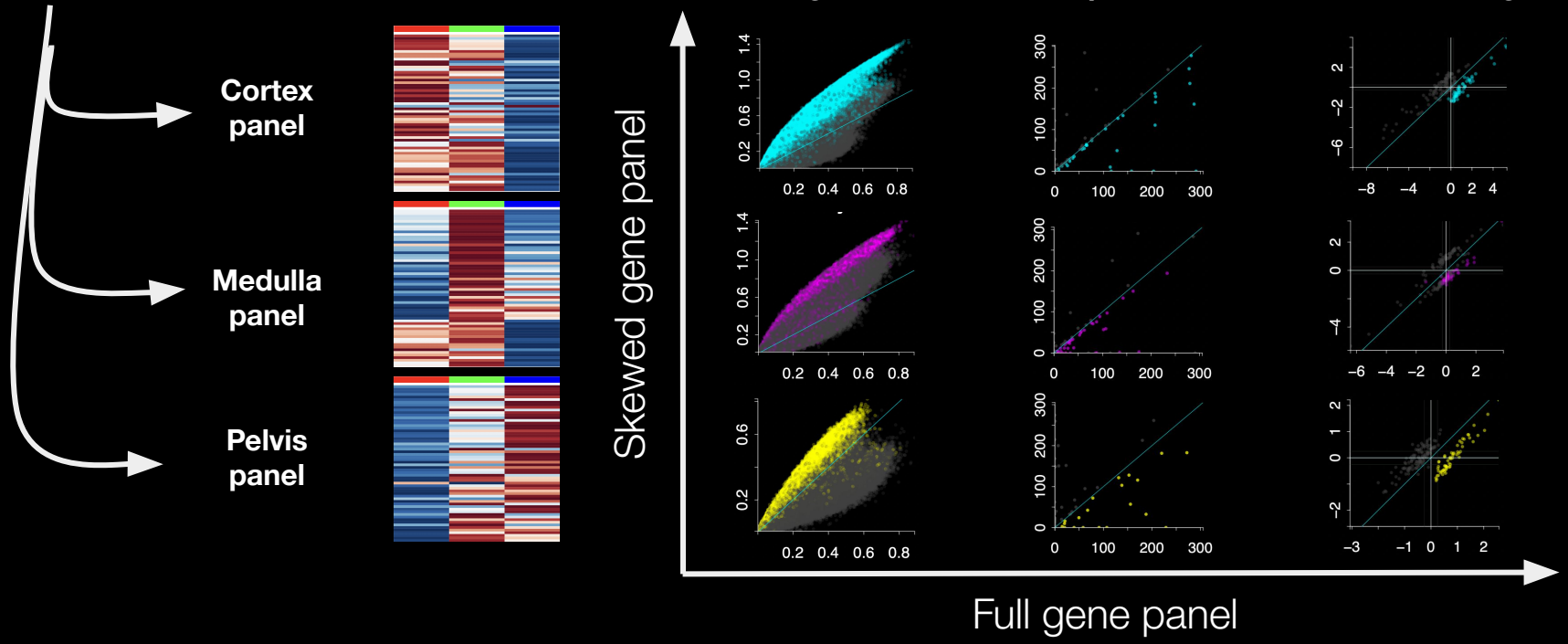
Region-specific biases in normalized gene expression generalize across imSRT technologies

Library size normalization with STARmapPLUS in mouse brain



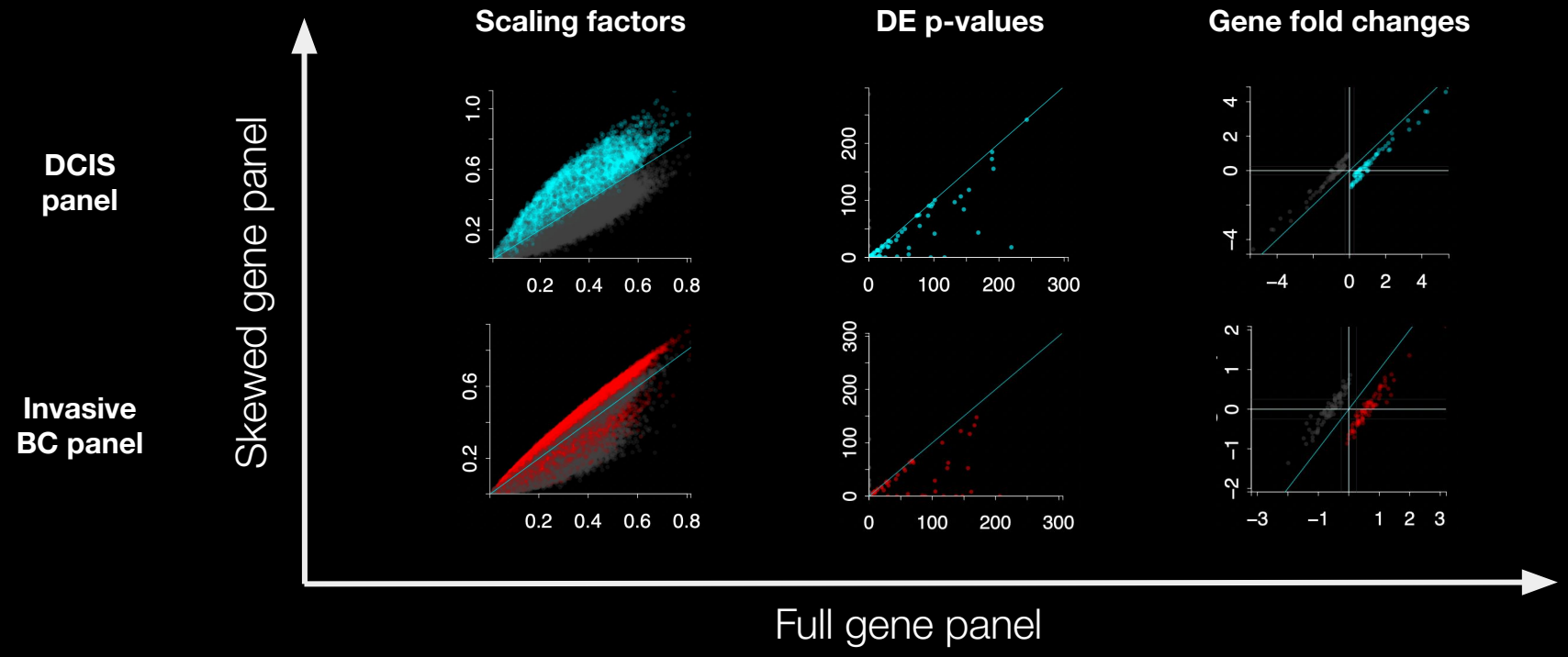
Region-specific biases in normalized gene expression generalize across tissue types

Library size normalization with seqFISH in mouse kidney



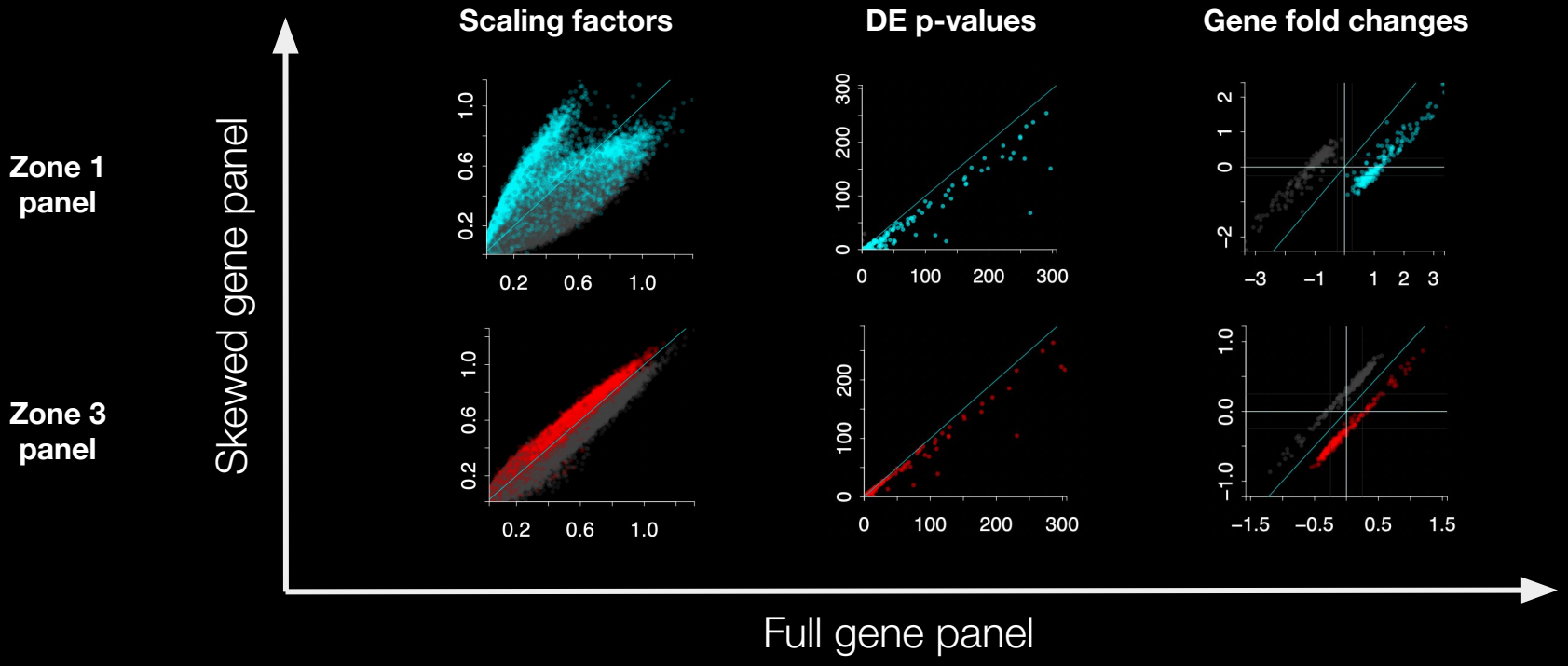
Region-specific biases in normalized gene expression generalize across tissue types

Library size normalization with 10X Xenium in human breast cancer



Region-specific biases in normalized gene expression generalize across tissue types

Library size normalization with CosMx in human liver



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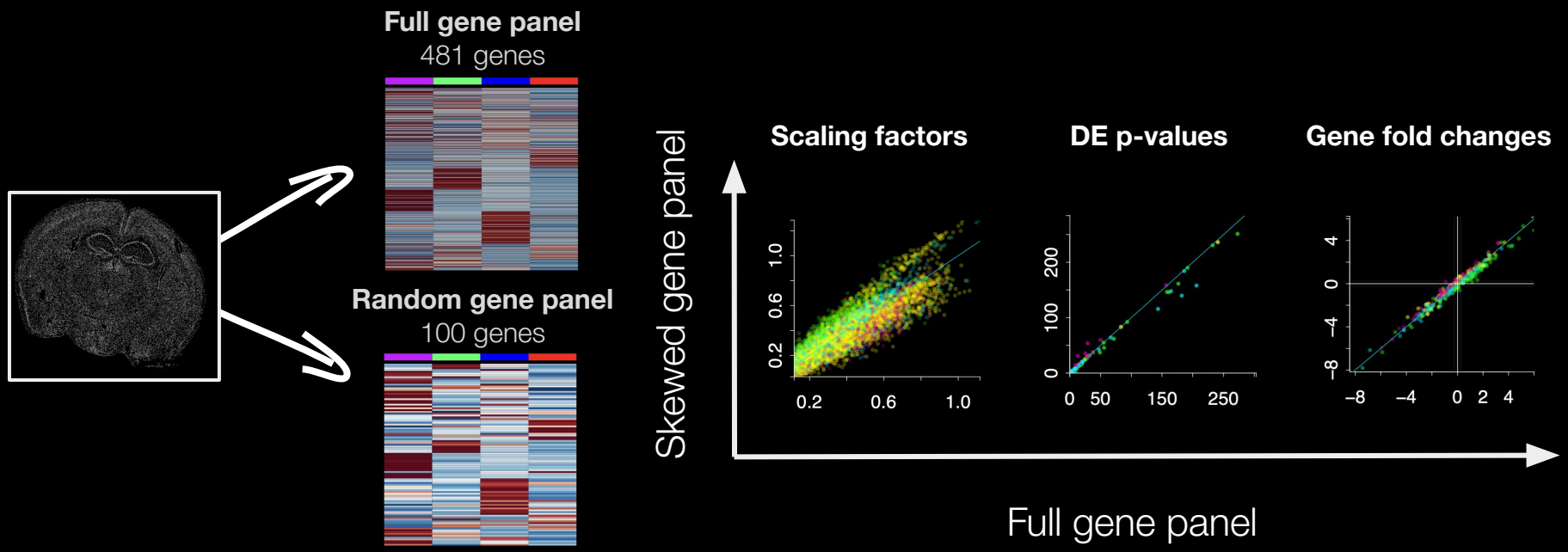
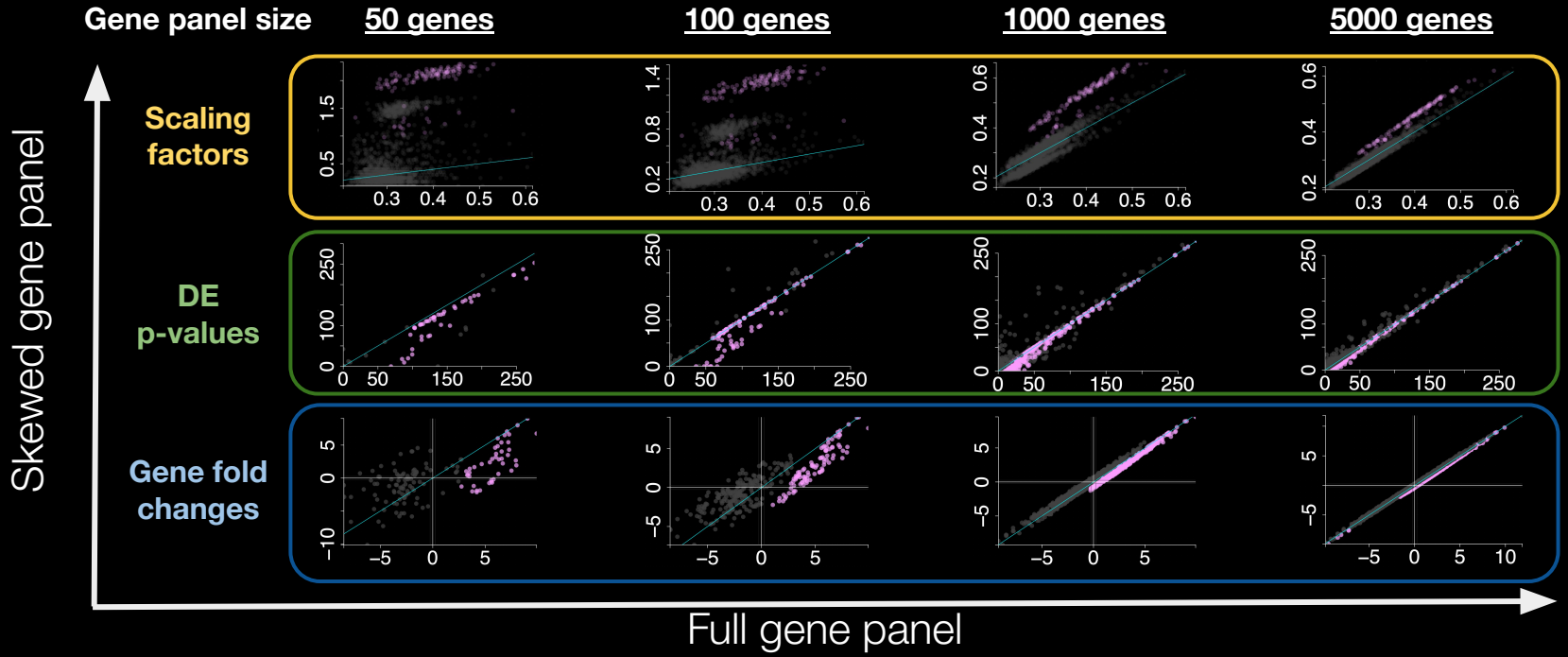
Region-specific biases can be mitigated with more representative gene panels

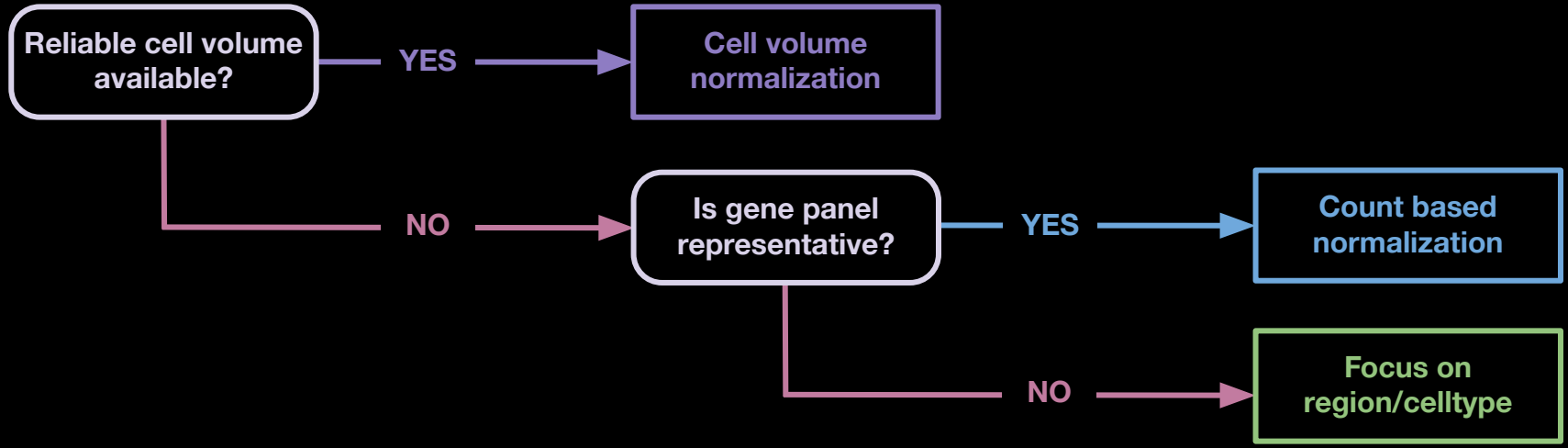


Region-specific biases can be mitigated with larger gene panels

Library size normalization with monocyte-skewed gene panels simulated from sorted PBMS scRNA-seq



Recommendations



Gene count normalization in single-cell imaging-based spatially resolved transcriptomics

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