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Supplementary Material

Extended Similarity Methods for Efficient Data Mining in Imaging Mass Spectrometry

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Contents

Extra IMS datasets and extended similarity results

All Python scripts used to calculate indices and generate plots can be found at: https://github.com/Prentice-lab-UF/Extended-Similarity-Indices-pyIMS.git.

For extra data not shown here, visit respective links for each data set:

https://github.com/Prentice-lab-UF/Extended-Similarity-Indices-mouse-brain-image-publication-supplemental-data

https://github.com/Prentice-lab-UF/Extended-Similarity-Indices-mouse-brain-image-A

https://github.com/Prentice-lab-UF/Extended-Similarity-Indices-mouse-brain-image-B

https://github.com/Prentice-lab-UF/Extended-Similarity-Indices-mouse-brain-image-C

Lipids for Principal Component Analysis

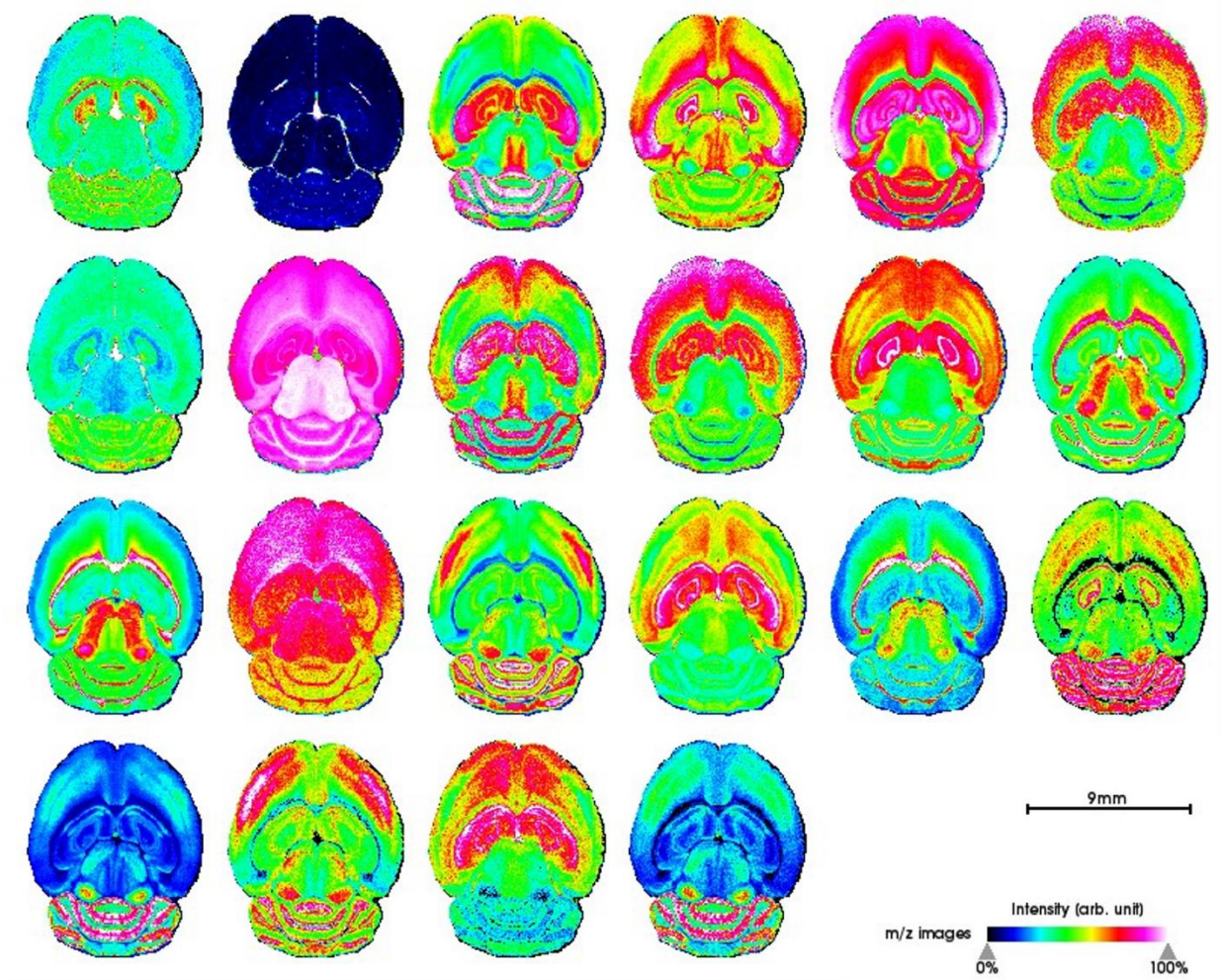


Figure S1: Root mean square normalized ion images for used for principal component analysis. From top left to bottom right *m/z* values are as follows: 524.381, 703.580, 731.611, 732.558, 734.674, 756.5571, 758.575, 760.588, 769.565, 772.529, 782.572, 786.605, 788.620, 798.543, 806.573, 810.604, 826.577, 832.586, 834.604, 844.528, 848.562, and 872.559.

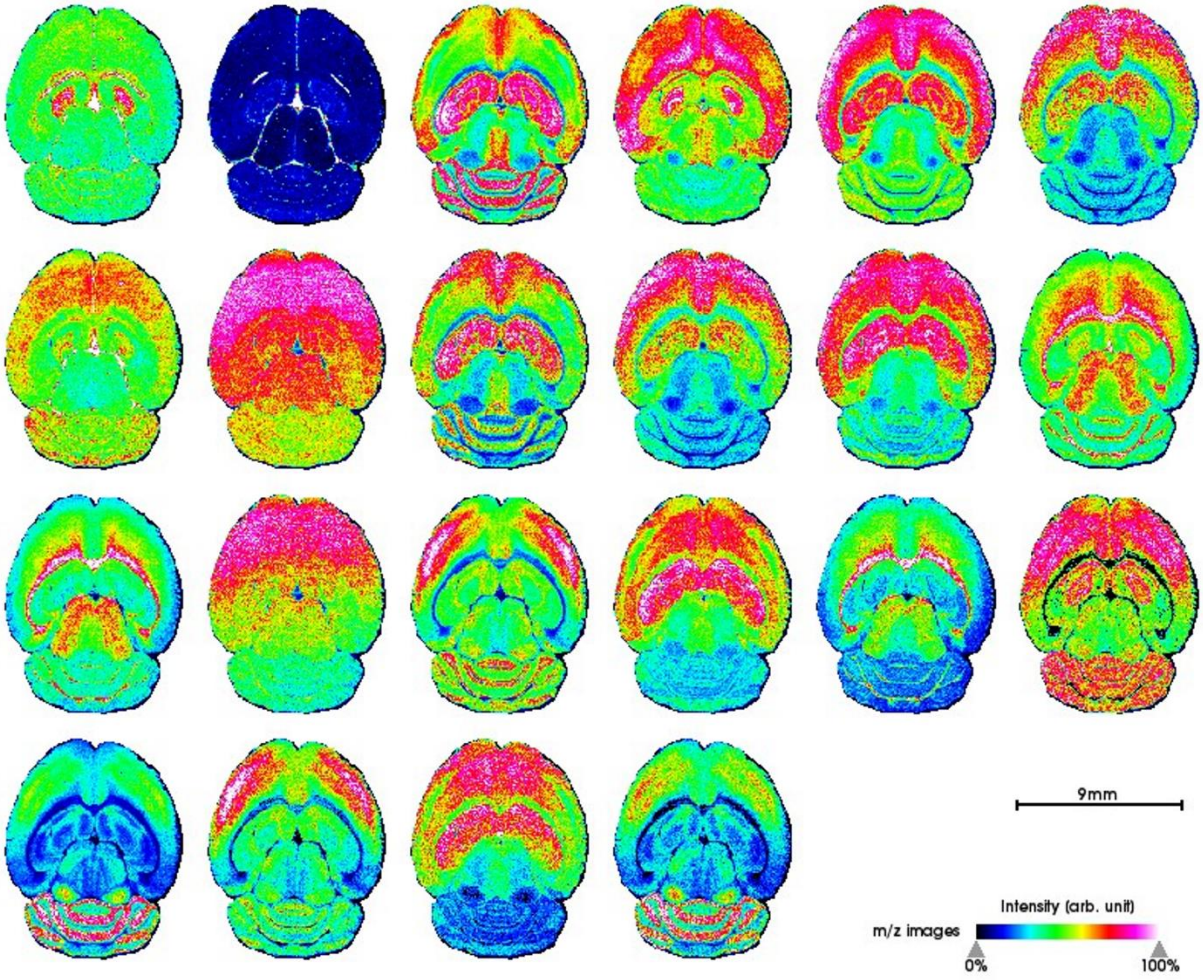


Figure S2: Ion images for used for principal component analysis. From top left to bottom right *m/z* values are as follows: 524.381, 703.580, 731.611, 732.558, 734.674, 756.5571, 758.575, 760.588, 769.565, 772.529, 782.572, 786.605, 788.620, 798.543, 806.573, 810.604, 826.577, 832.586, 834.604, 844.528, 848.562, and 872.559.

Table S1: Positive Ion Phospholipid Identification by High Mass Accuracy

Experimentally measured	Calculated[a]	ppm	Assignment
524.3812	524.3711	19.26116828	PC(18:0/OH)
703.5759	703.5748	1.563444285	SM(d18:1/16:0)
731.6106	731.6061	6.150850847	SM(d18:1/18:0)
734.5737	734.5694	5.853769569	PC(16:0/16:0)
756.5571	756.5514	7.534187367	PC(16:0/16:0)+Na
758.5755	758.5694	8.041452766	PC(34:2)
760.5884	760.5851	4.338764985	PC(34:1)
769.5654	769.5620	4.418097567	SM(d18:1/18:0)+K
772.5295	772.5253	5.436715147	PC(32:0)+K
782.5715	782.5670	5.750306364	PC(34:1)+Na
	782.5694	2.683468073	PC(36:4)
786.6054	786.6007	5.975077317	PC(36:2)
788.6197	788.6164	4.184543968	PC(36:1)
798.5434	798.5410	3.005481246	PC(34:1)+K
806.5728	806.5670	7.190971116	PC(36:3)+Na
	806.5694	4.215384318	PC(38:6)
810.6042	810.5983	7.278574357	PC(36:1)+Na
	810.6007	4.317785563	PC(38:4)
826.5767	826.5723	5.323188304	PC(36:1)+K
832.5857	832.5827	3.603245659	PC(38:4)+Na
834.6041	834.6007	4.073804395	PC(40:6)
844.5279	844.5253	3.078652587	PC(38:6)+K

848.5617	848.5566	6.010206037	PC(38:4)+K
872.5591	872.5566	2.865143648	PC(40:6)+

a) Calculated using "The LIPID MAPS® Lipidomics Gateway, https://www.lipidmaps.org/"

Non-normalized Principal Component Analysis

PCA was calculated with Python module sklearn. SVD_solver was set to randomized with iterated_power of 10000 and 5 components.

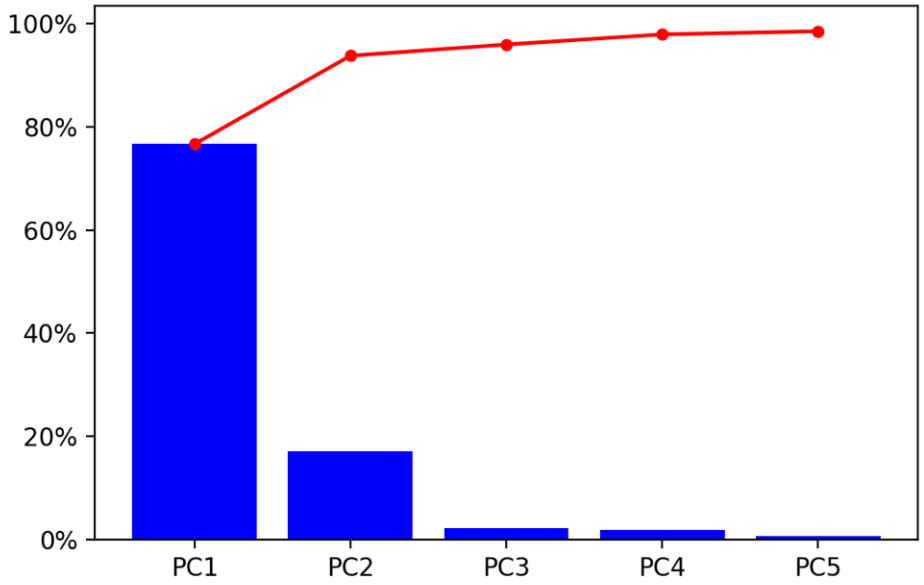


Figure S3: Pareto plot of the explained variance for non-normalized PCA. Explained variance for PC 1-5 are as follows: 76.7%, 17.1%, 2.18%, 1.96%, and 0.606%. The cumulative sum of the variance explained are as follows: 76.6%, 93.8%, 96.0, 97.9%, and 98.5%.

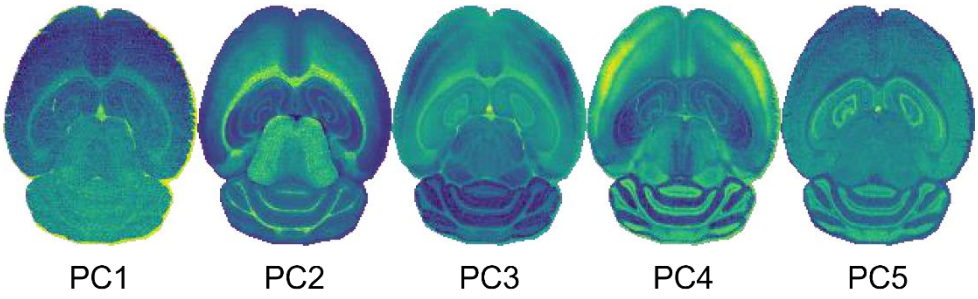


Figure S4: Spatial expression images for PC 1-5. Biological images similar to what was found in the RMS pre-processed PCA spatial-expression images can be seen.

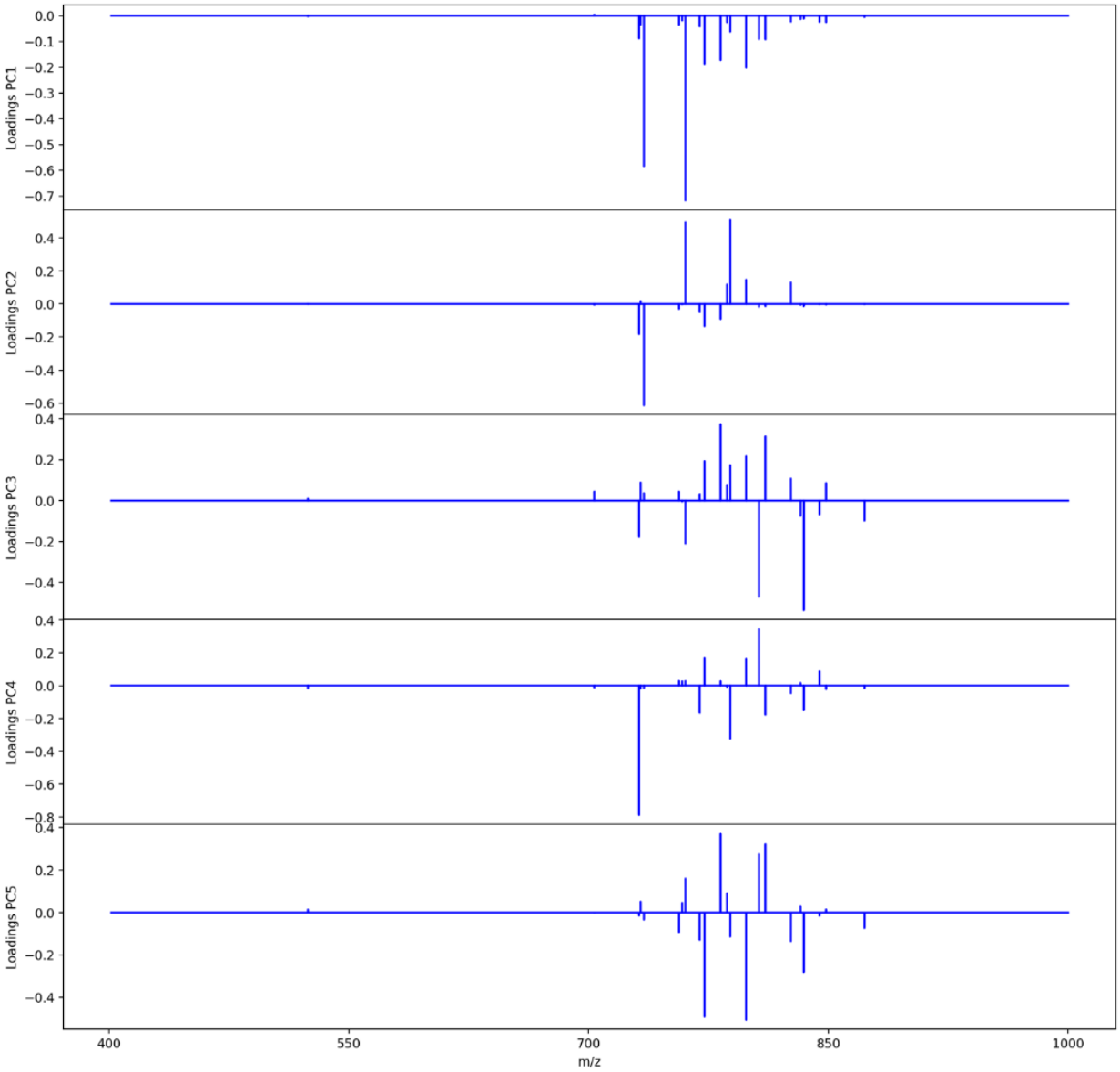


Figure S5: Pseudo-spectra for PC 1-5 from top to bottom.

Normalization Methods for Binary Fingerprint Conversion

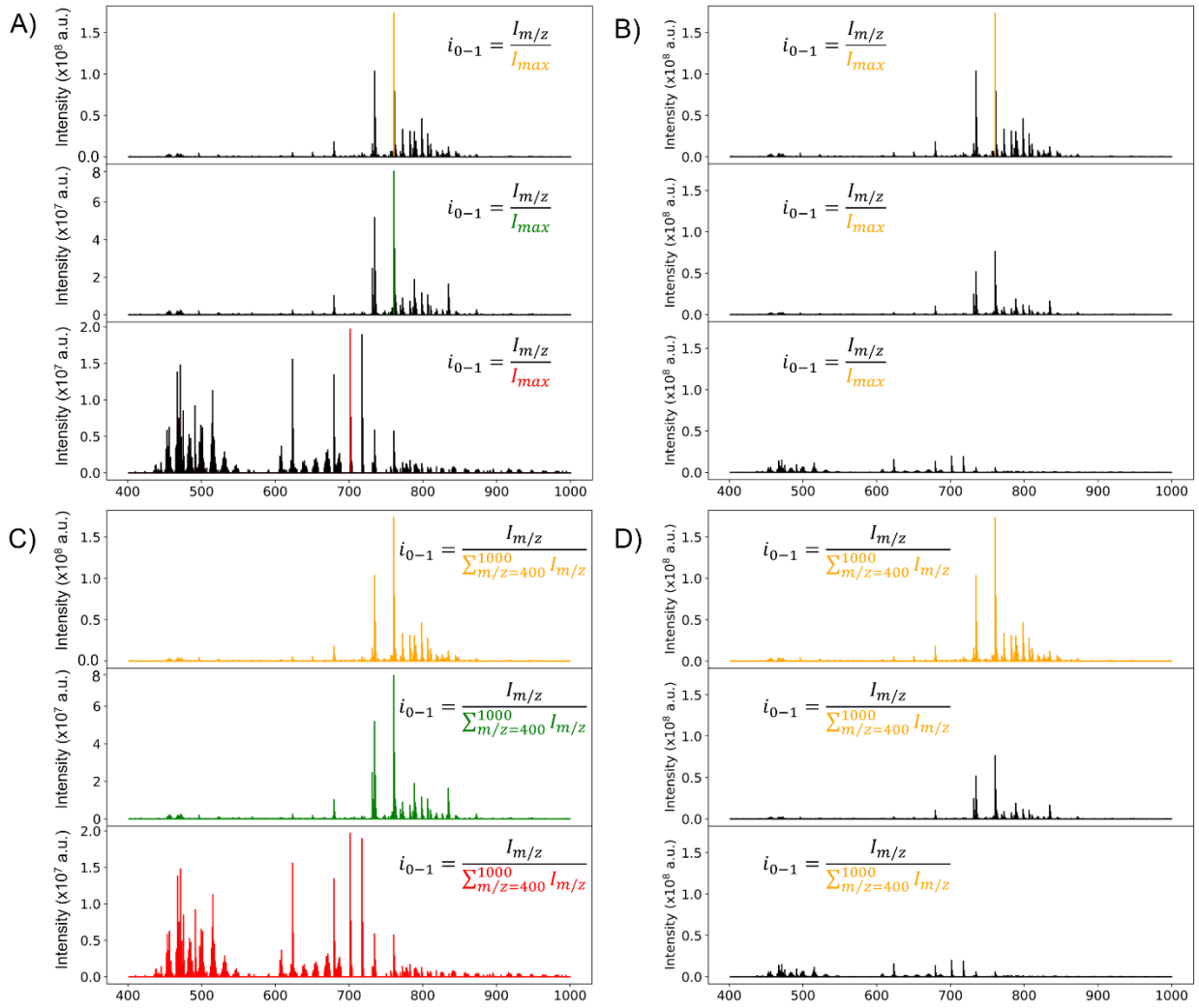


Figure S6: Each panel contains the same mass spectra from three unique pixels in the imaging mass spectrometry dataset that are normalized using A) local normalization, B) global, C) localTIC normalization, and D) globalTIC normalization. The colored peak(s) in each spectrum highlight the peak(s) used for normalization. $I_{m/z}$ is used to denote the raw intensity, $i_{0\text{-}1}$ is used to denote the resulting normalized intensity on a 0-1 scale, I_{max} is to denote the largest intensity within the mass spectrum of the corresponding color, and $\sum$ indicates the mass range of summed ion intensities.

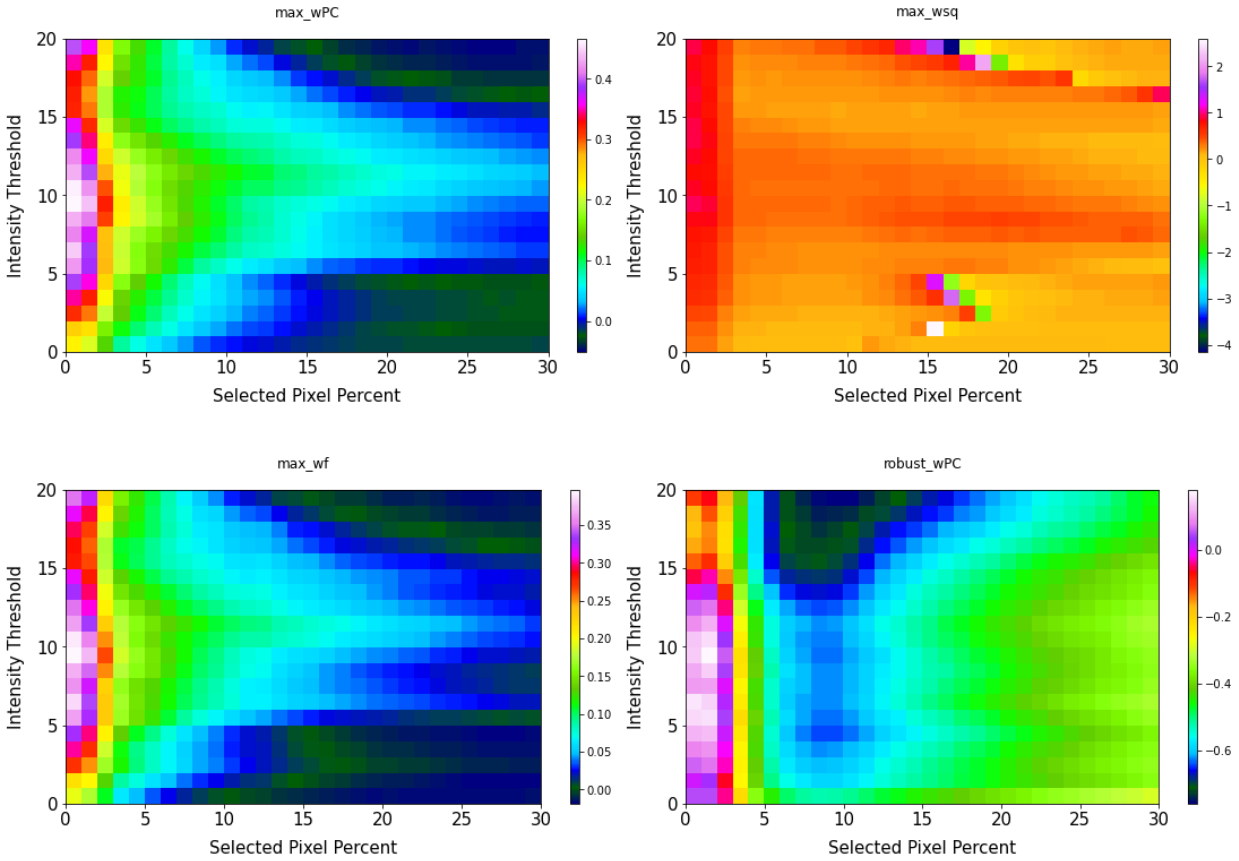
E-index Plots

PC 1 was omitted from the E-index calculations from non-normalized PCA, since it corresponds to non-tissue regions. Negative E values would indicate that the mid region has greater similarity, meaning the normalization method could not properly represent the spectra and the extended similarity calculations failed.

E-index could not be calculated for globalTIC normalized data as the intensities were suppressed too low to be above the threshold and coincide across multiple spectra.

global normalization from non-normalized PCA results

The E-index fails to show any relevant trends that could point to an optimal set of parameters. Both robust and max functions with the squared weight show flat lines with spiked in positive and negative directions. The spikes do not correspond to optimal parameters.



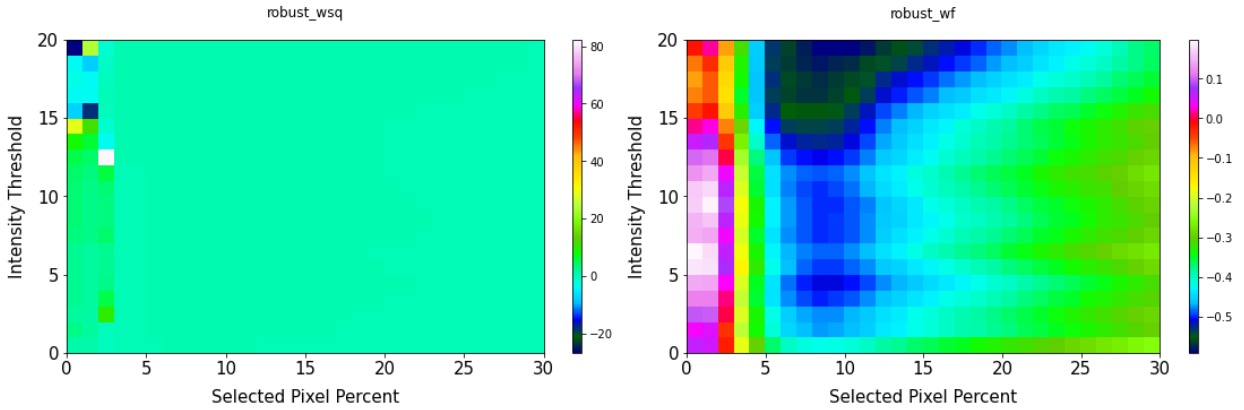
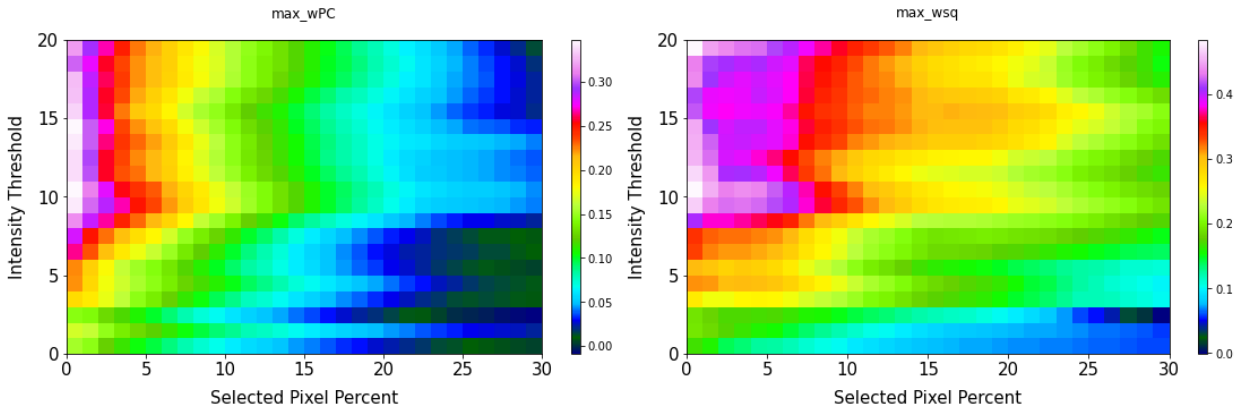


Figure S7: E-index plots for global normalization from non-normalized PCA results.

Local normalization from non-normalized PCA results

The E-index fails to show any relevant trends that could point to an optimal set of parameters. The max function with the squared weight show flat lines with spiked in positive and negative directions. The spikes do not correspond to optimal parameters. A gradual decrease in E-index values does point to local normalization be a better method of correlating the spectra.



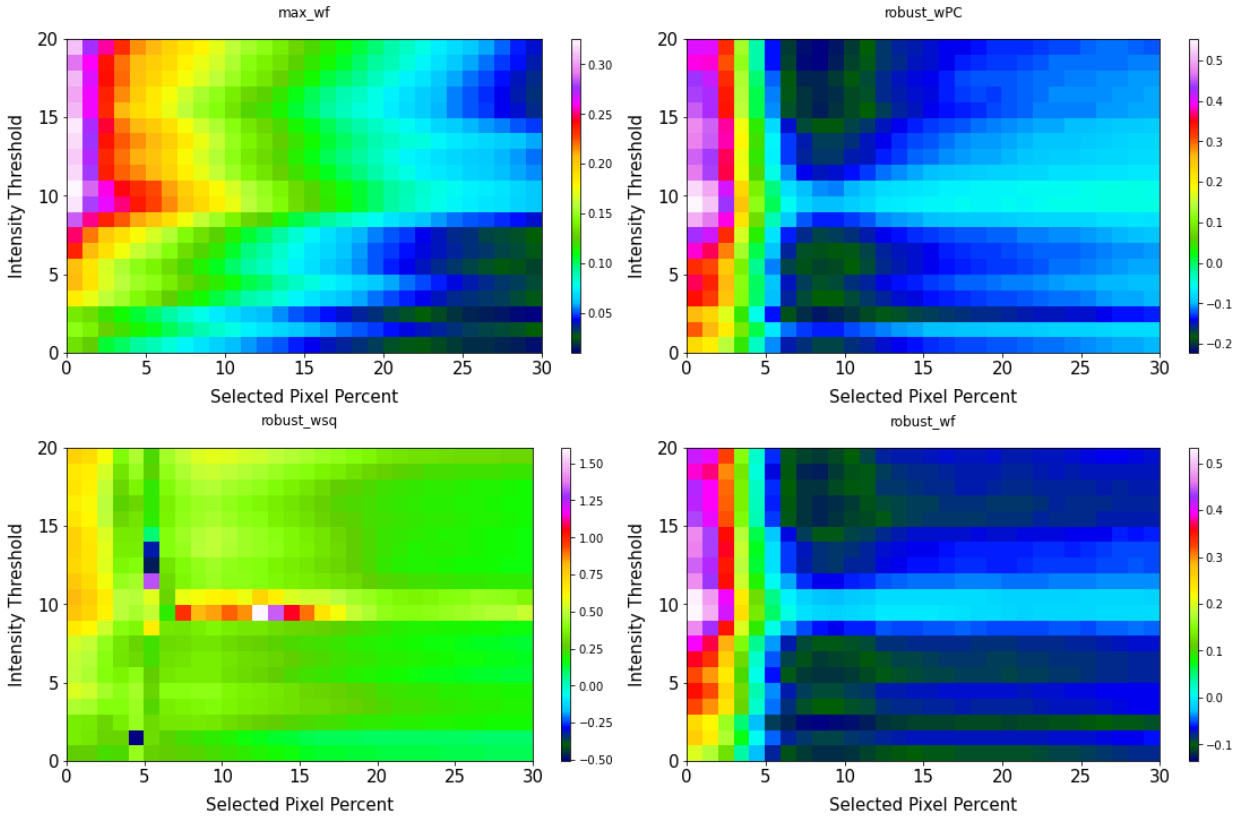
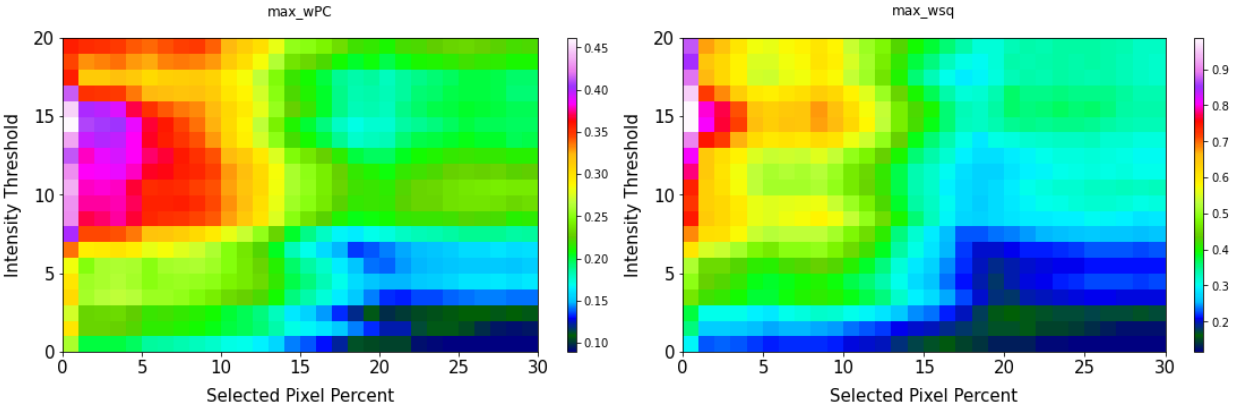


Figure S8: E-index plots for local normalization from non-normalized PCA results.

Global normalization from root mean squared normalized PCA results

Here we see the expected trend of E-values where we see greater values of E in the first 10% of selected pixels and lower values for larger amounts of pixels.



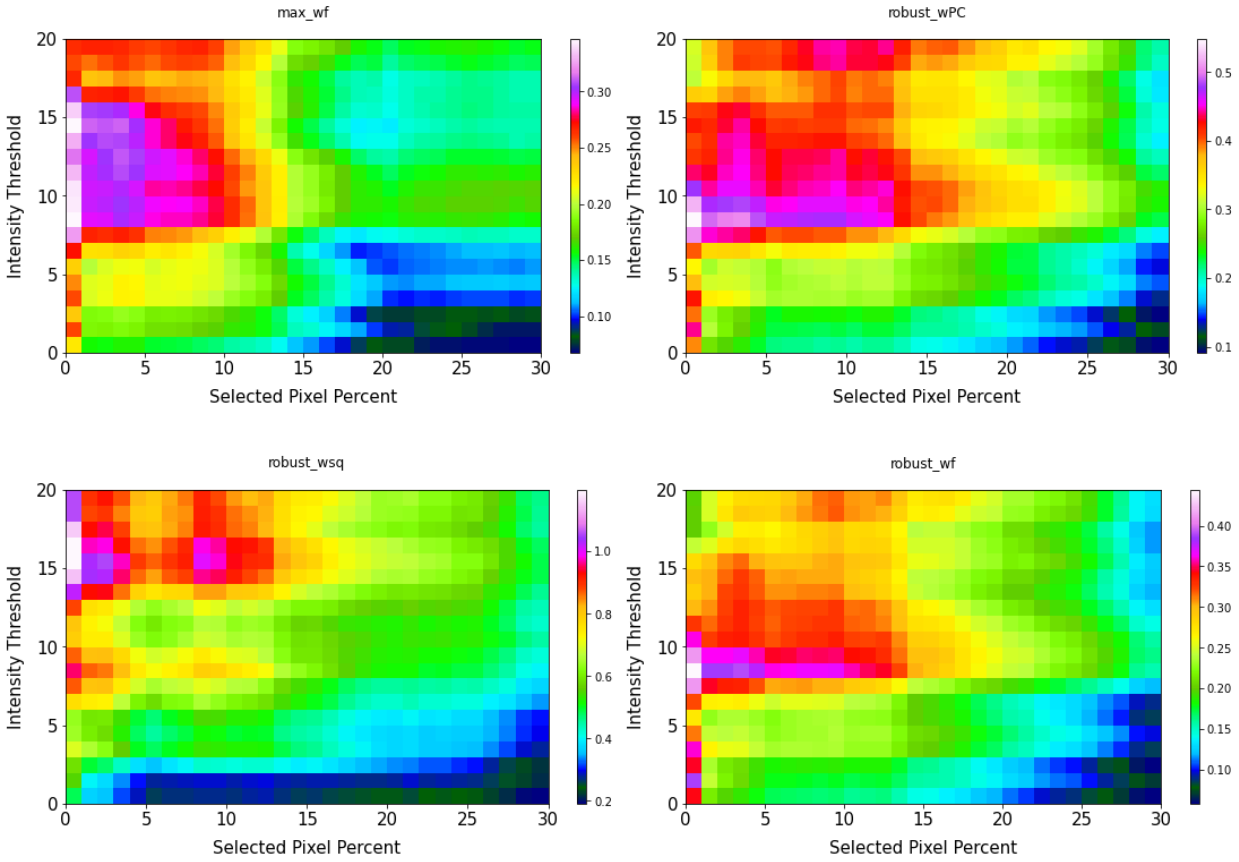


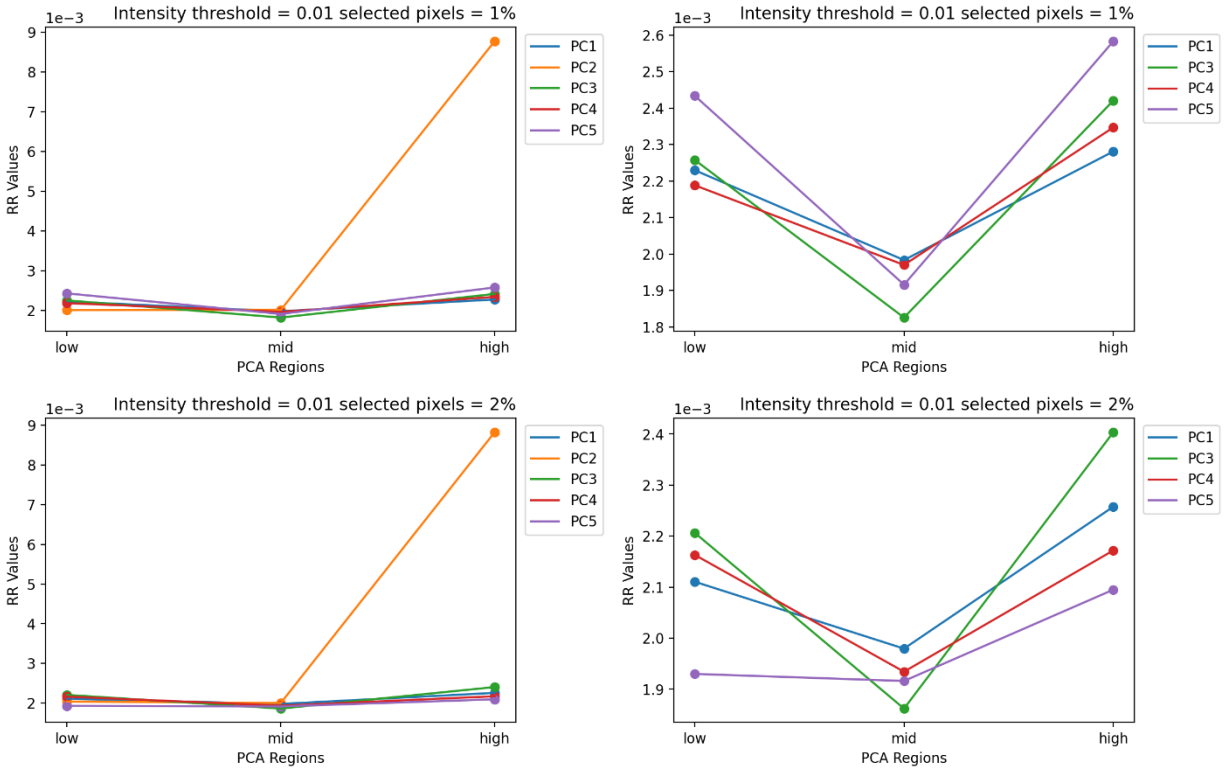
Figure S9: E-index plots for global normalization from root mean squared normalized PCA results.

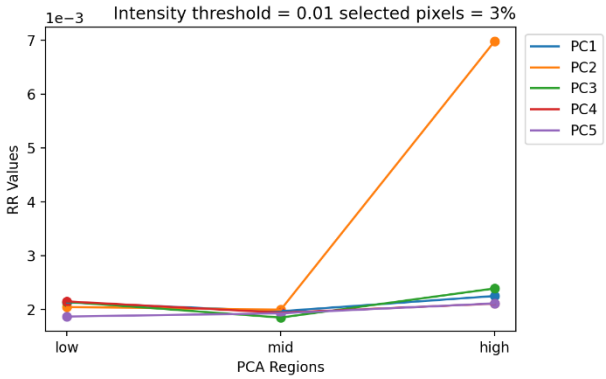
Russel-Rao 2-D V Plots

2-D V plots for local normalization with root mean squared normalized results

RMS normalized PCA was used for pixel selection. Left column has the 2D V plots with PC 2. Right column has the 2D V plots without PC 2. Distinct “V” shape can be seen up to ~20% for intensity threshold 0.01. However, the “V” shape begins to diminish after 14%. This corresponds to the decrease in E values that looks to find these large distinctions in similarity between the regions.

For intensity threshold 0.10 the V shape is not as apparent but still present for PC 3 and disappears for other PCs sooner.

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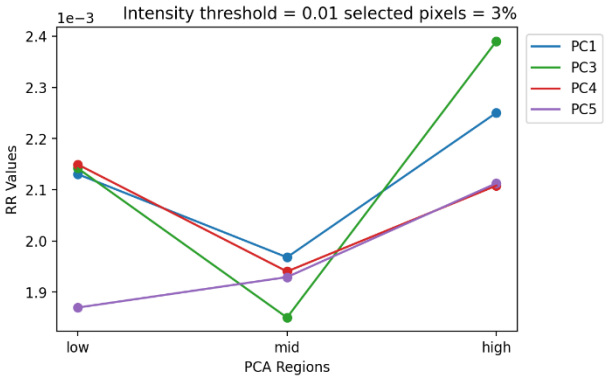
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5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RR Values
PCA Regions
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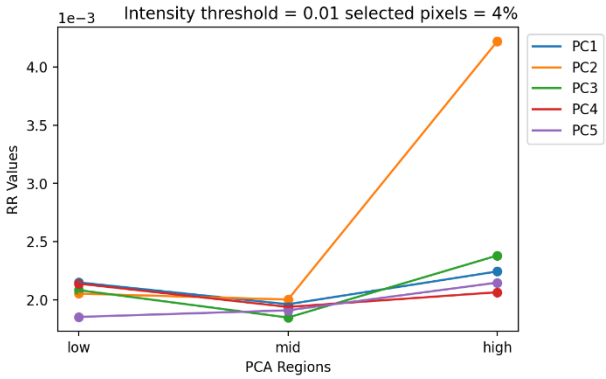
mid
high
PC1
PC3
PC4
PC5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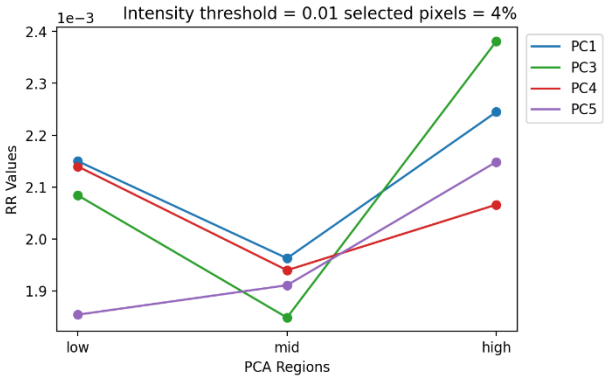
PC2
PC3
PC4
PC5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RR Values
PCA Regions
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mid
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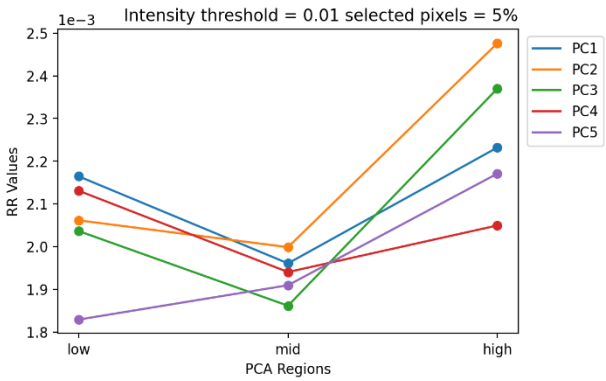
PC1
PC3
PC4
PC5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PCA Regions
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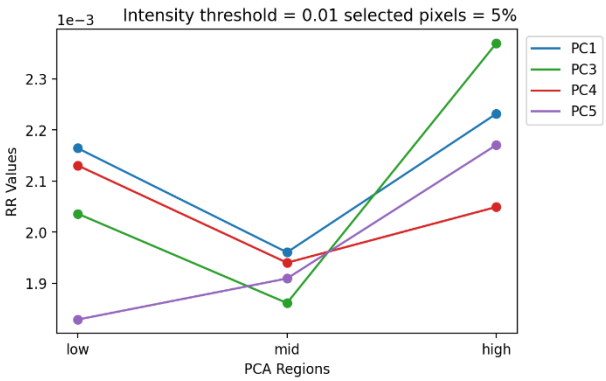
PC1
PC2
PC3
PC4
PC5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RR Values
PCA Regions
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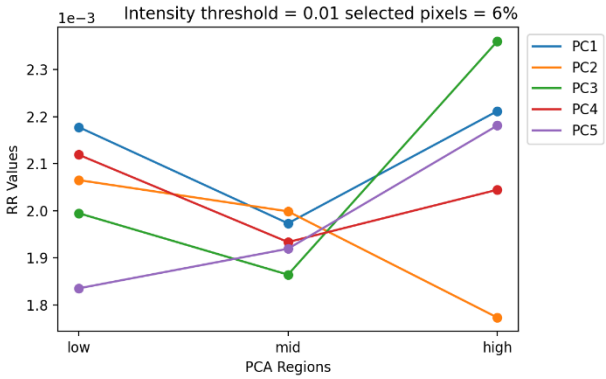
high
PC1
PC3
PC4
PC5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PC4
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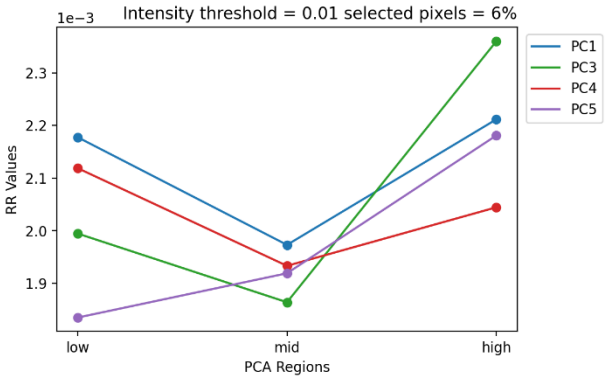
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PC4
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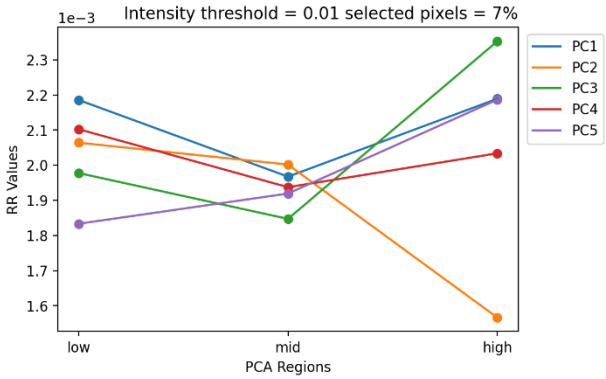
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PC2
PC3
PC4
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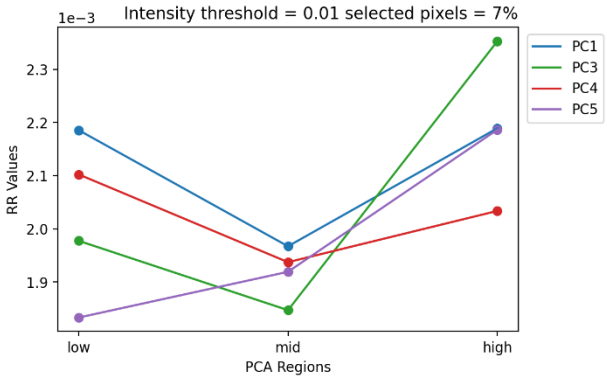
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RR Values
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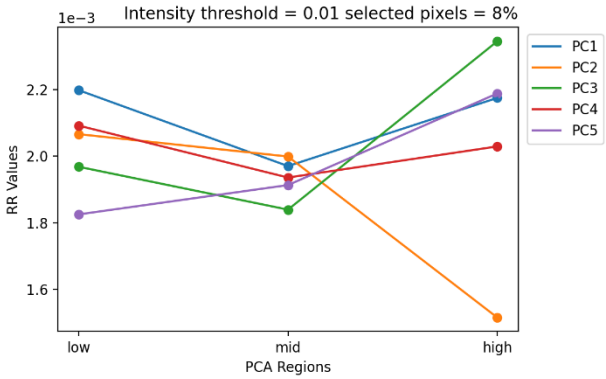
PC3
PC4
PC5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PCA Regions
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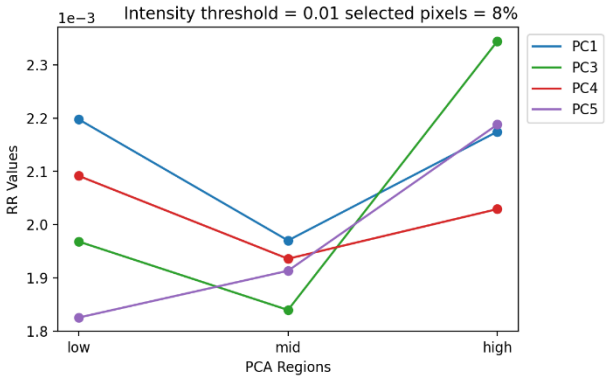
PC2
PC3
PC4
PC5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RR Values
PCA Regions
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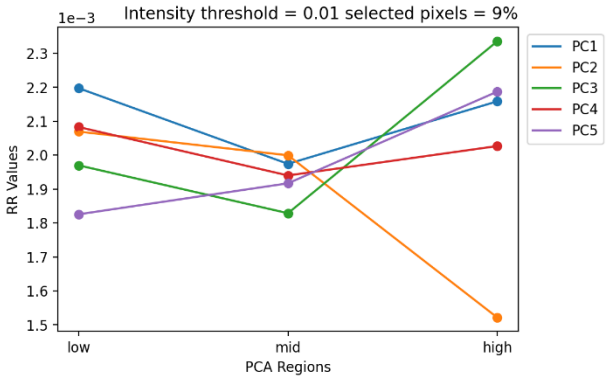
PC1
PC3
PC4
PC5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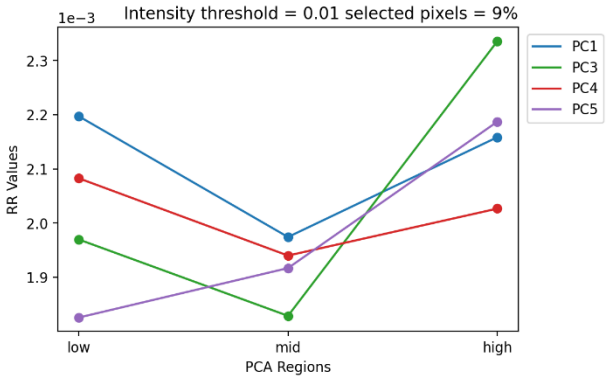
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RR Values
PCA Regions
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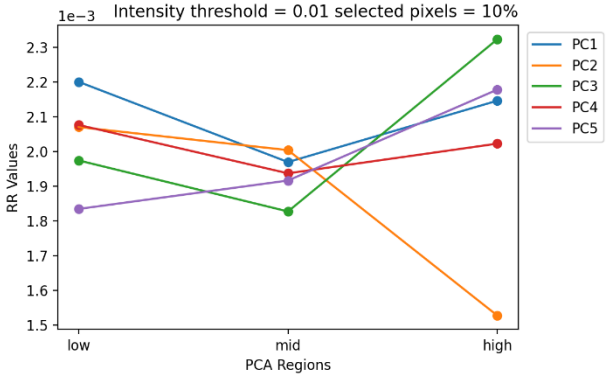
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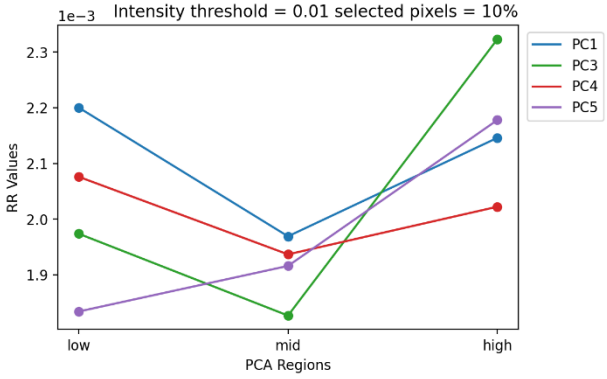
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PCA Regions
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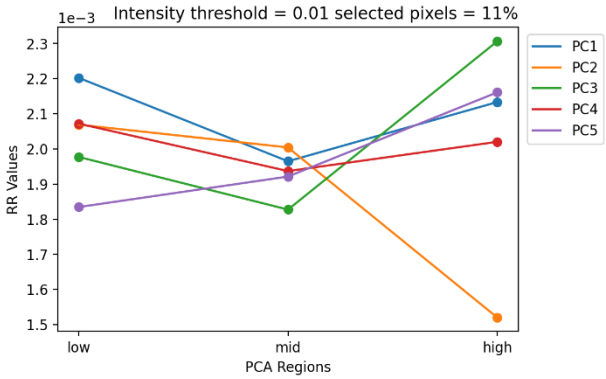
PC5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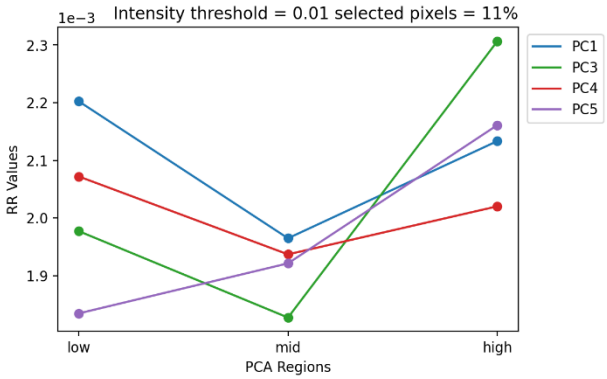
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RR Values
PCA Regions
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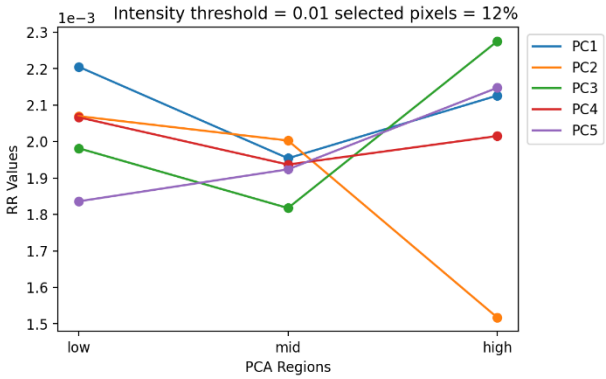
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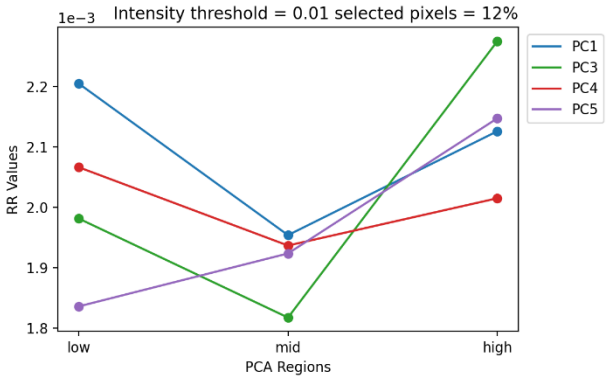
PC5


Intensity threshold = 0.01 selected pixels = 12%
1e−3
RR Values
PCA Regions
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PC1
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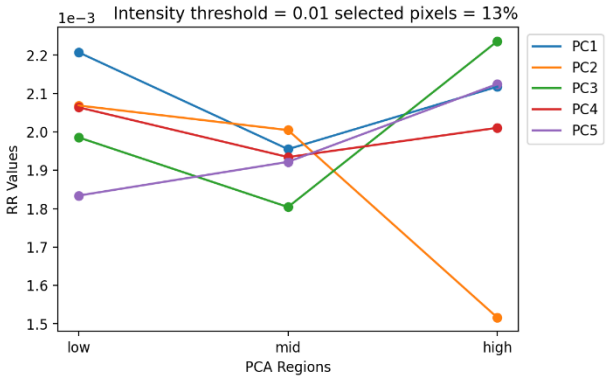
PC5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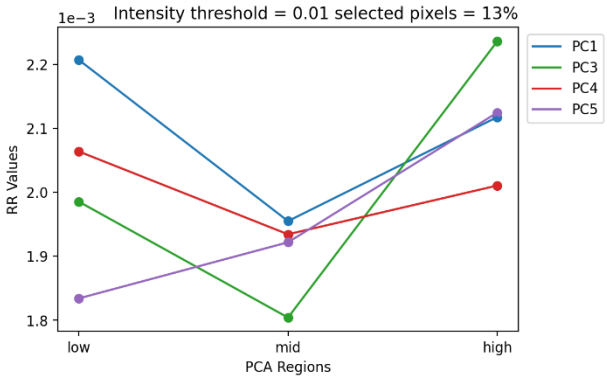
PC2
PC3
PC4
PC5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RR Values
PCA Regions
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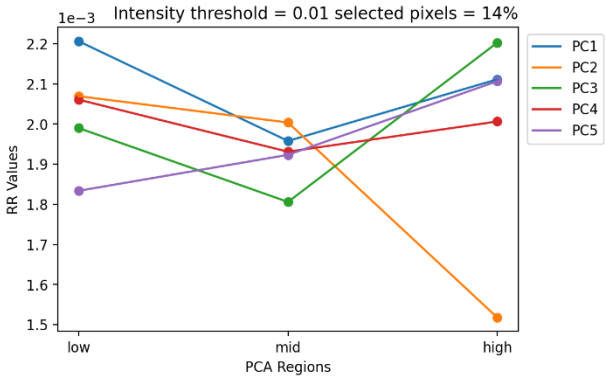
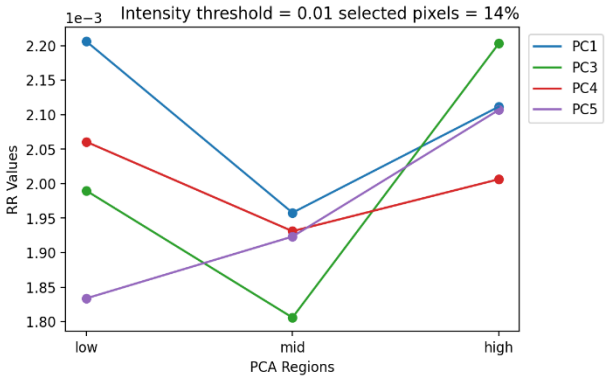
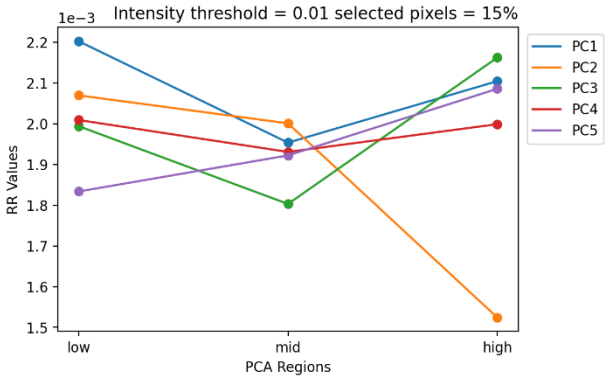
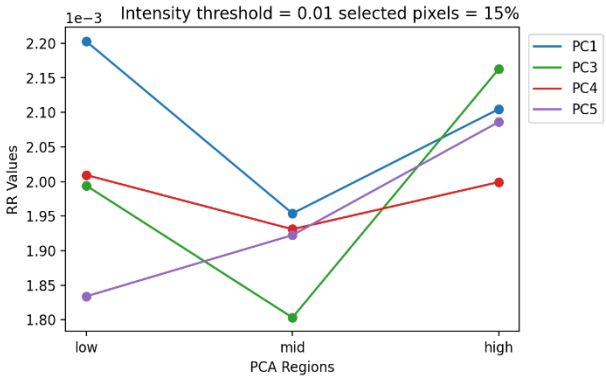
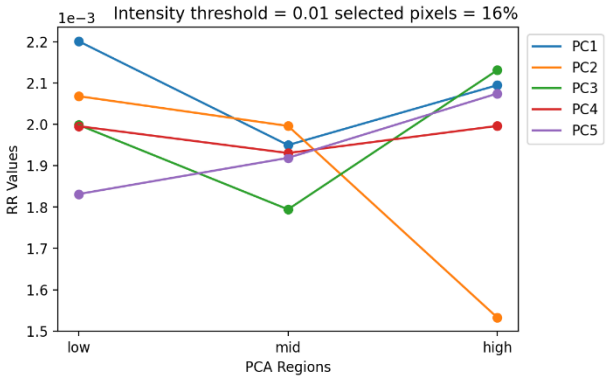
PC3
PC4
PC5

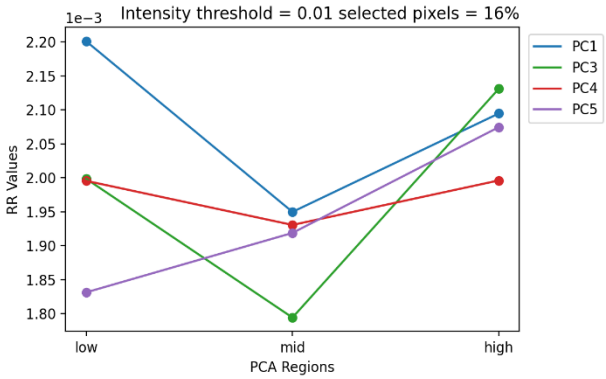

Intensity threshold = 0.01 selected pixels = 14%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

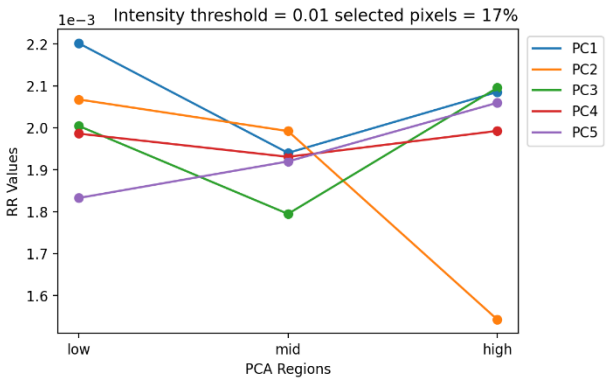

Intensity threshold = 0.01 selected pixels = 14%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

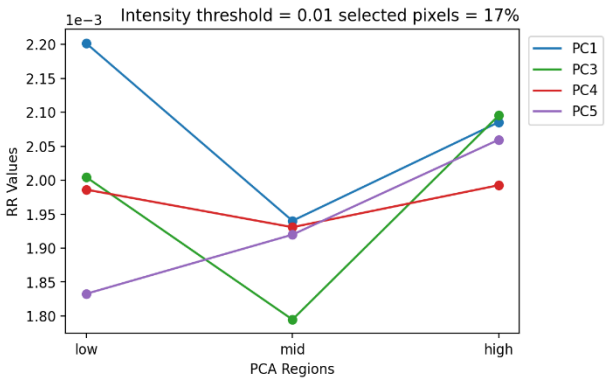

Intensity threshold = 0.01 selected pixels = 15%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

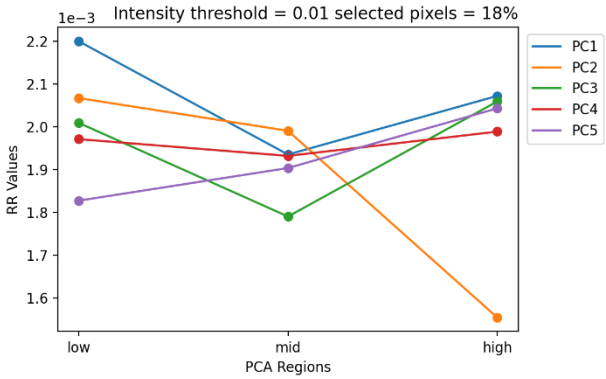

Intensity threshold = 0.01 selected pixels = 15%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

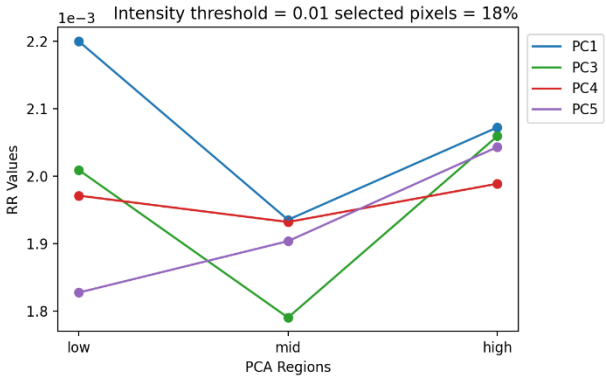

Intensity threshold = 0.01 selected pixels = 16%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

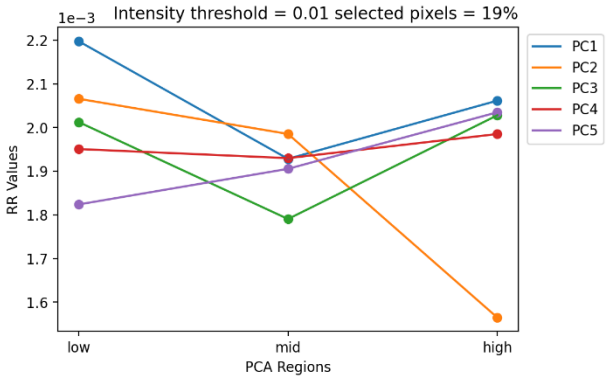

Intensity threshold = 0.01 selected pixels = 16%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

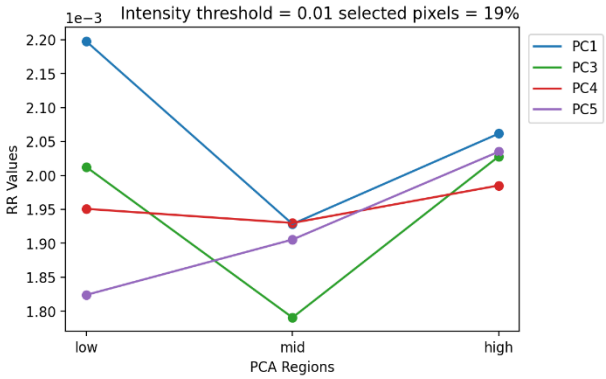

Intensity threshold = 0.01 selected pixels = 17%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

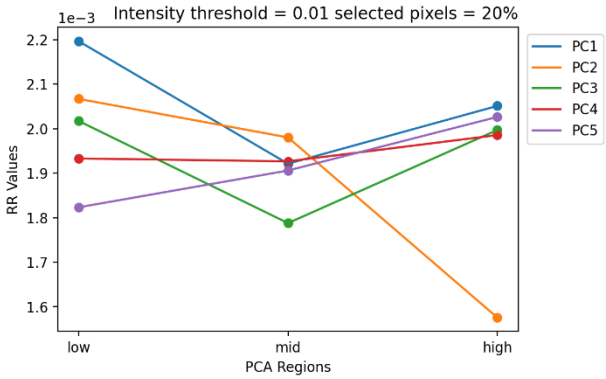

Intensity threshold = 0.01 selected pixels = 17%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

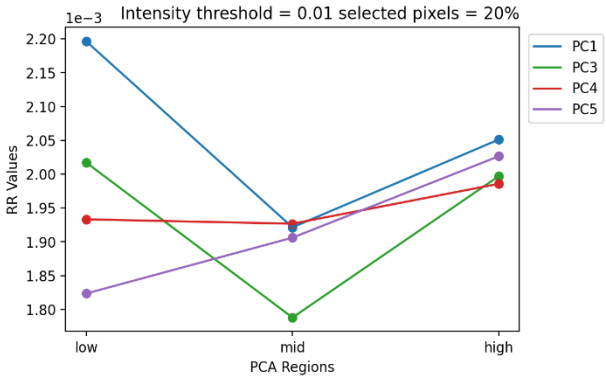

Intensity threshold = 0.01 selected pixels = 18%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

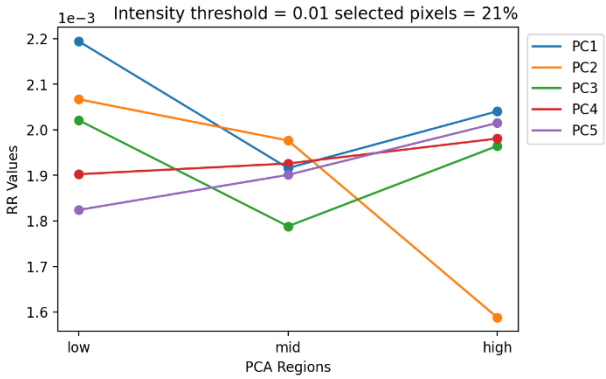

Intensity threshold = 0.01 selected pixels = 18%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

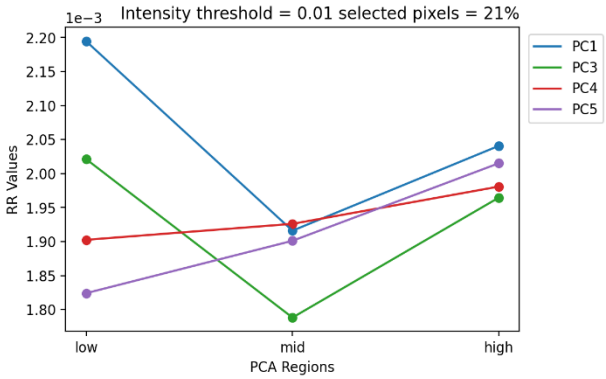

Intensity threshold = 0.01 selected pixels = 19%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

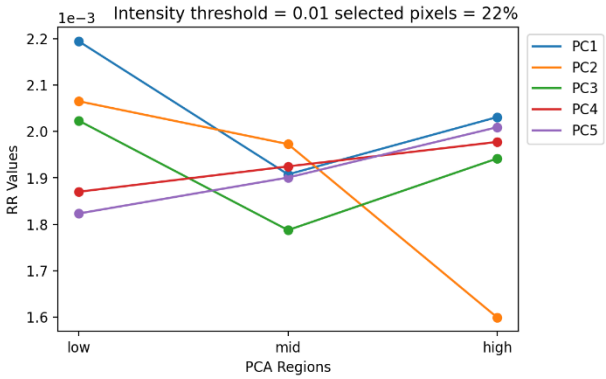

Intensity threshold = 0.01 selected pixels = 19%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

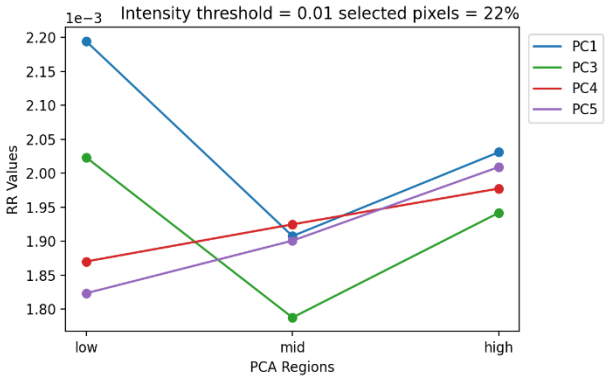

Intensity threshold = 0.01 selected pixels = 20%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

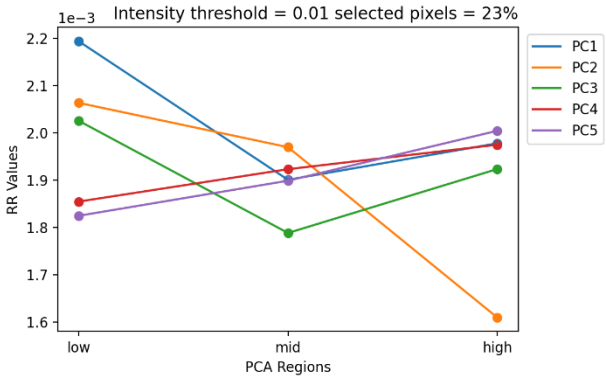

Intensity threshold = 0.01 selected pixels = 20%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

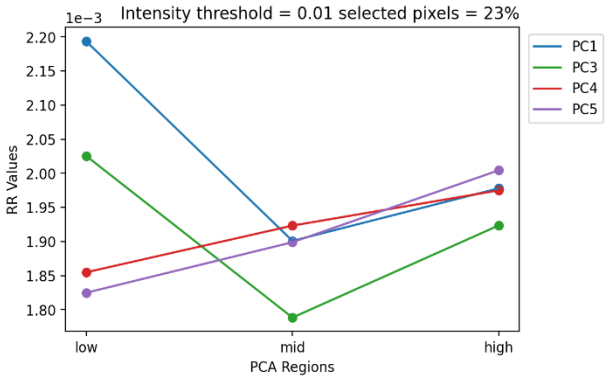

Intensity threshold = 0.01 selected pixels = 21%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

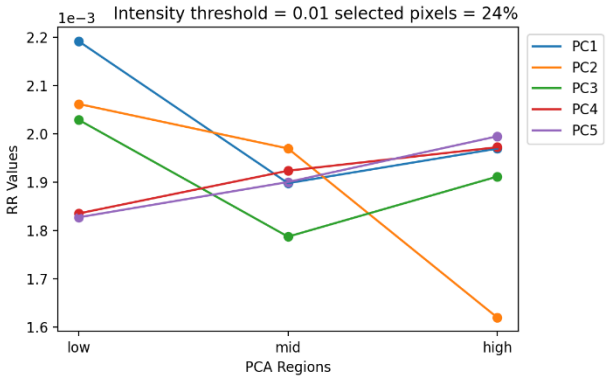

Intensity threshold = 0.01 selected pixels = 21%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

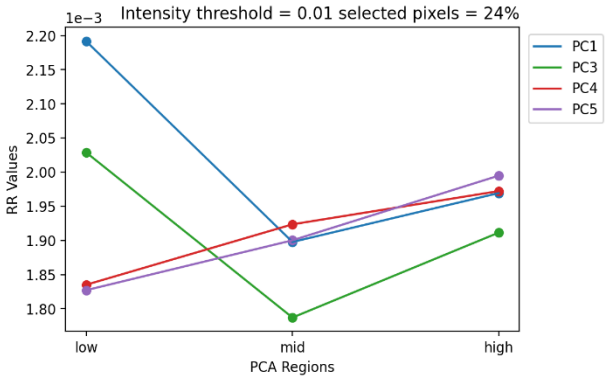

Intensity threshold = 0.01 selected pixels = 22%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

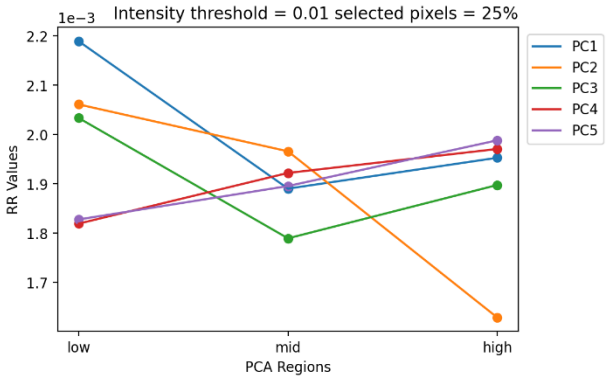

Intensity threshold = 0.01 selected pixels = 22%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

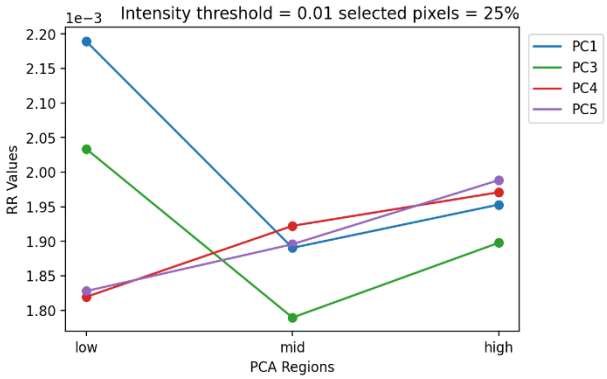

Intensity threshold = 0.01 selected pixels = 23%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

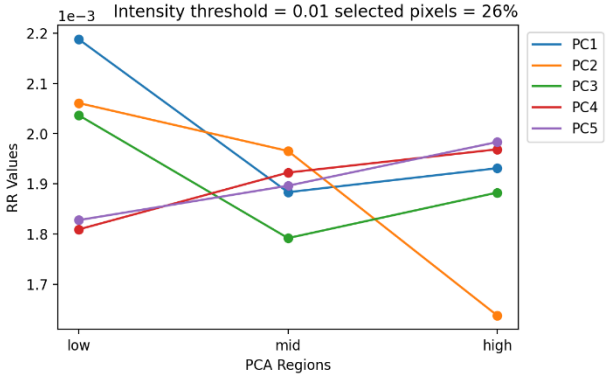

Intensity threshold = 0.01 selected pixels = 23%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

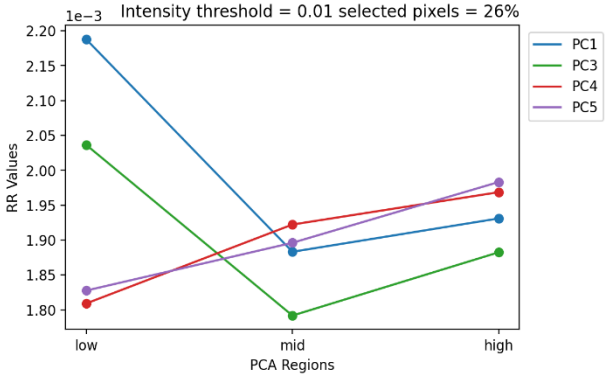

Intensity threshold = 0.01 selected pixels = 24%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 24%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 25%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 25%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 26%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 26%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

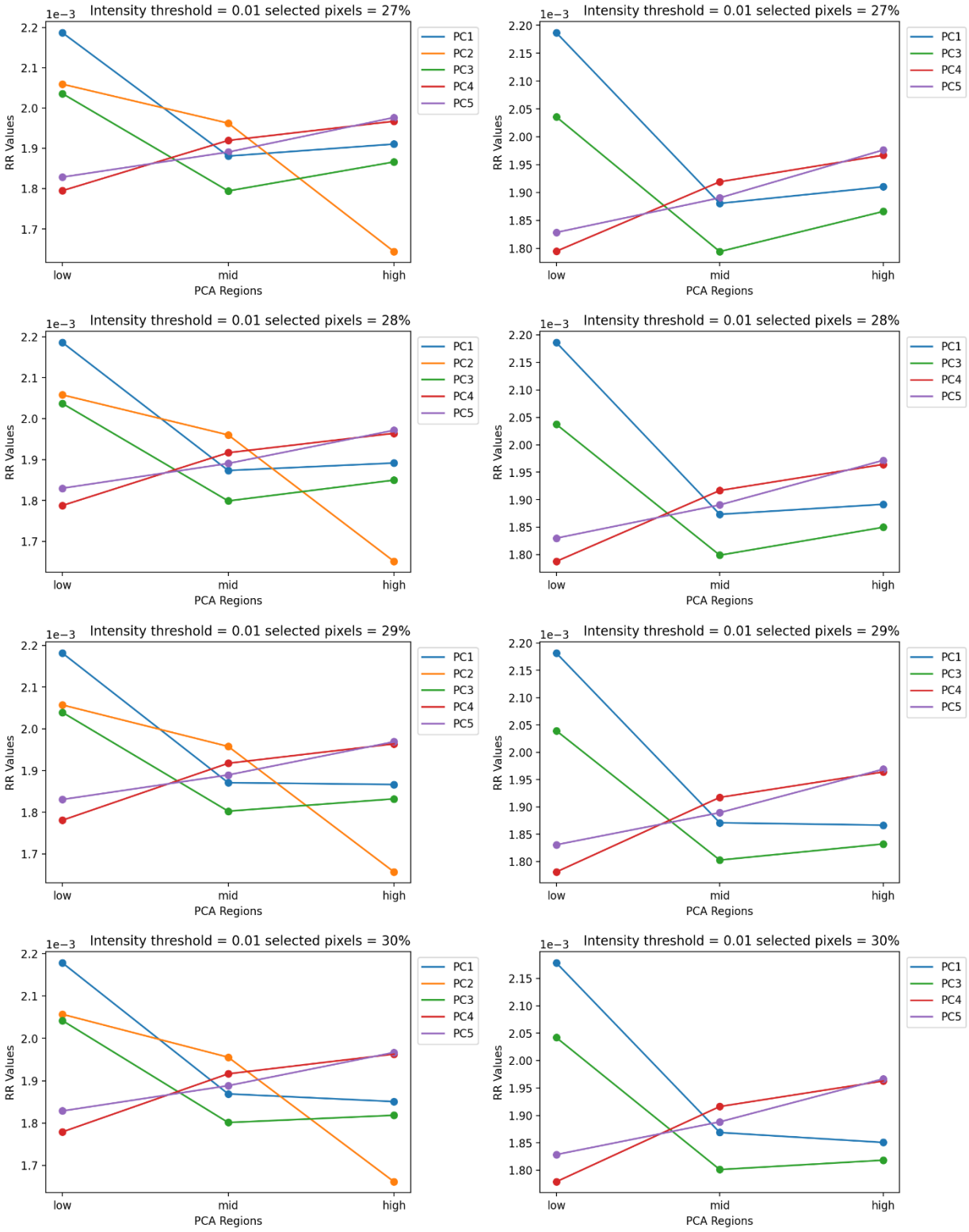
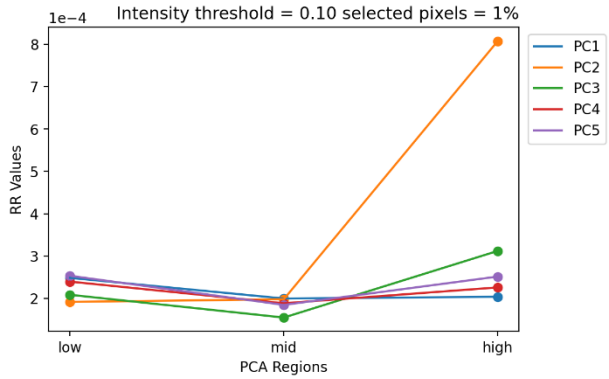
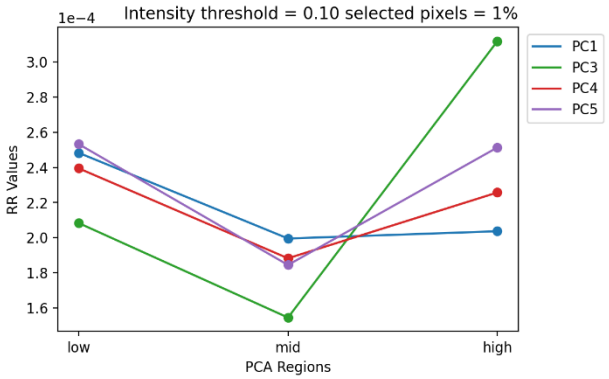
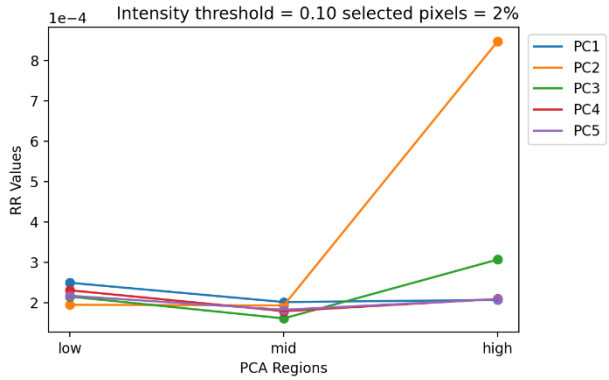
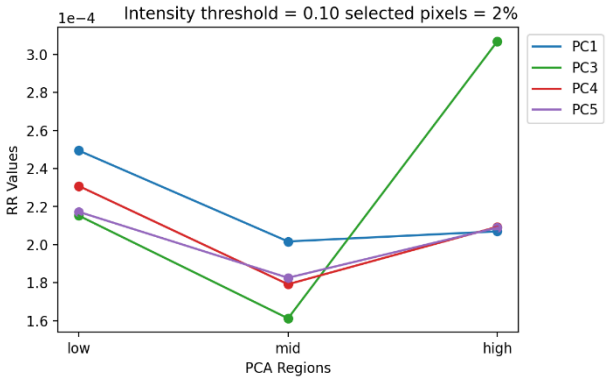


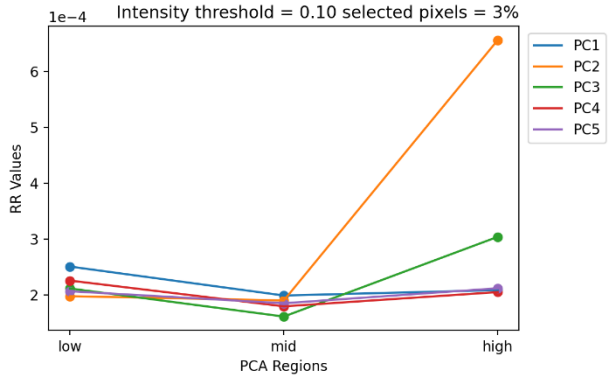
Figure S10: 2-D V plots for local normalization with root mean squared normalized results for intensity threshold 0.01.

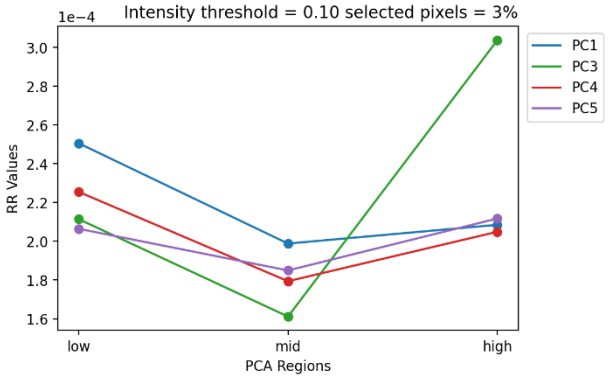

Intensity threshold = 0.10 selected pixels = 1%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

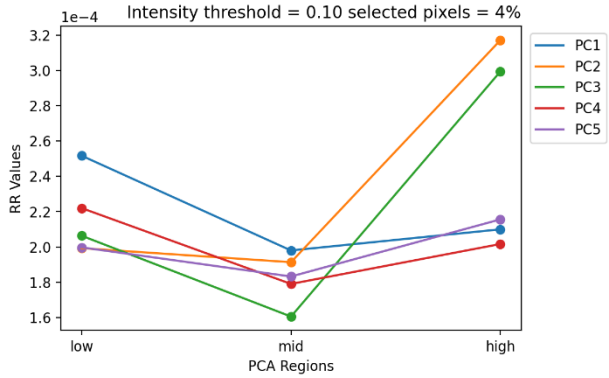

Intensity threshold = 0.10 selected pixels = 1%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

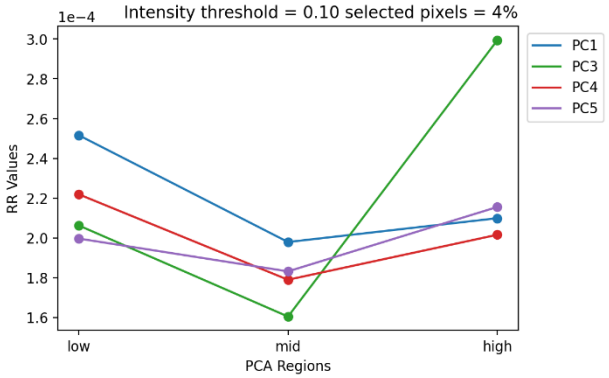

Intensity threshold = 0.10 selected pixels = 2%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

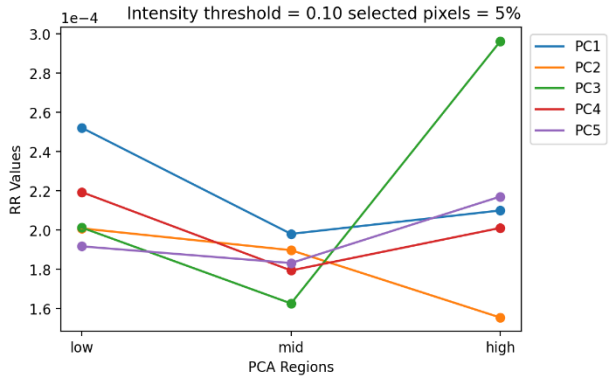

Intensity threshold = 0.10 selected pixels = 2%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

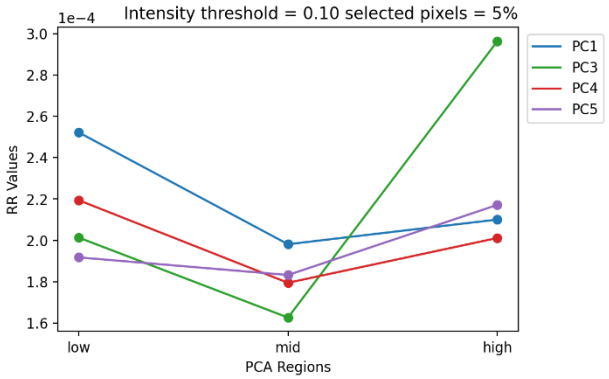

Intensity threshold = 0.10 selected pixels = 3%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

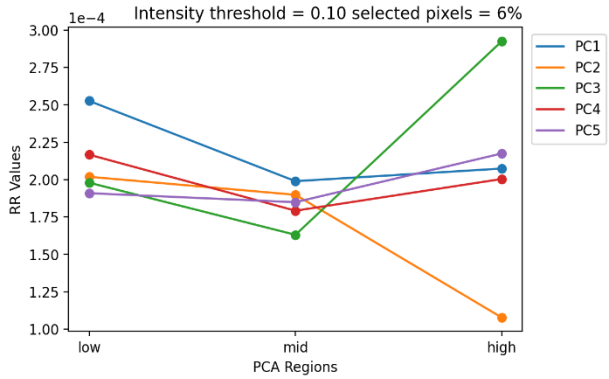

Intensity threshold = 0.10 selected pixels = 3%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

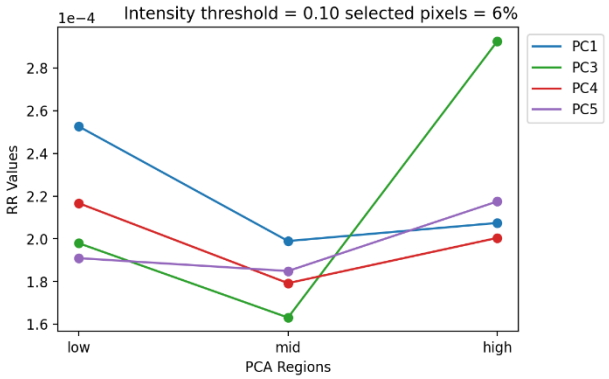

Intensity threshold = 0.10 selected pixels = 4%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

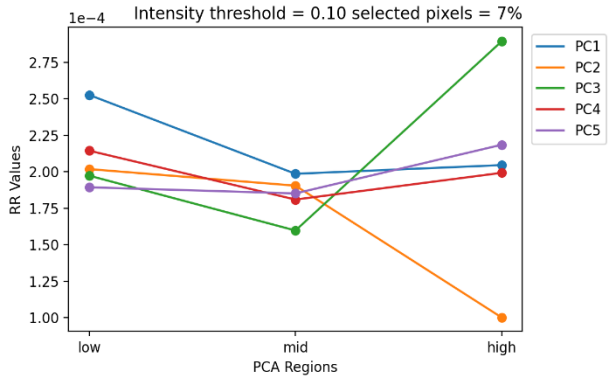

Intensity threshold = 0.10 selected pixels = 4%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

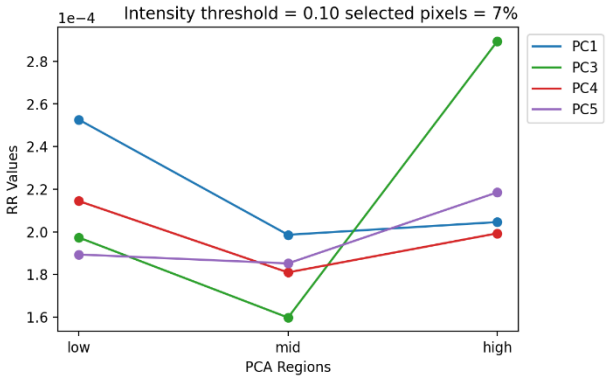

Intensity threshold = 0.10 selected pixels = 5%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

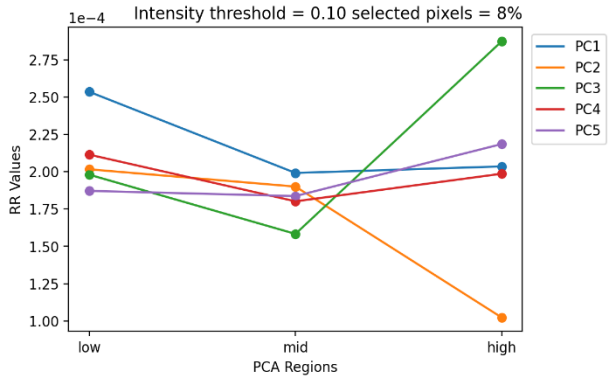

Intensity threshold = 0.10 selected pixels = 5%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

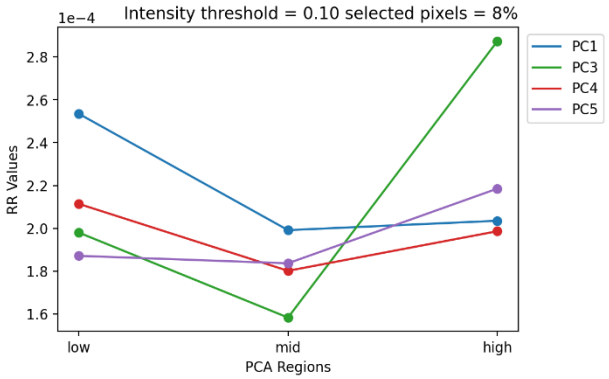

Intensity threshold = 0.10 selected pixels = 6%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

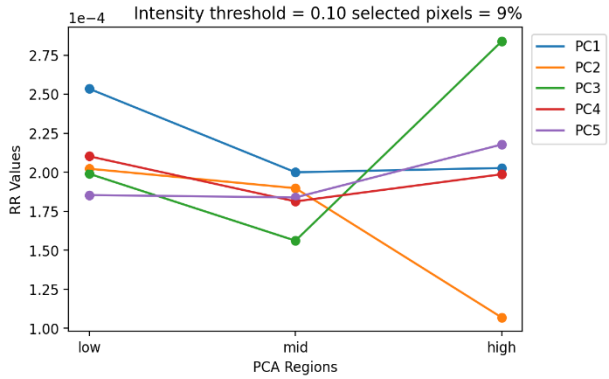

Intensity threshold = 0.10 selected pixels = 6%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

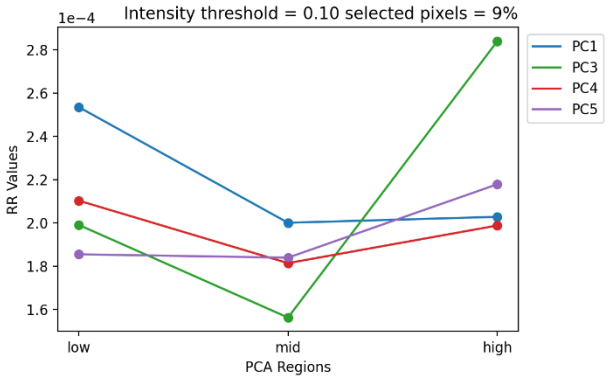

Intensity threshold = 0.10 selected pixels = 7%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

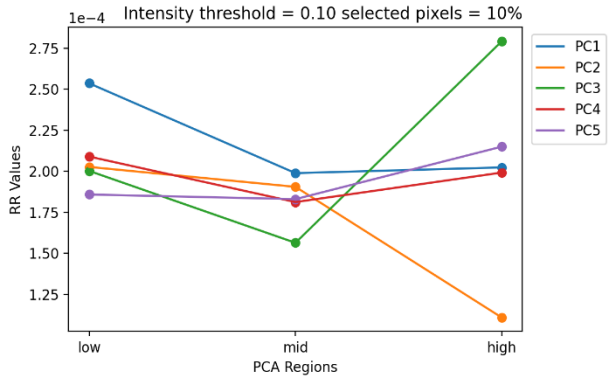

Intensity threshold = 0.10 selected pixels = 7%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

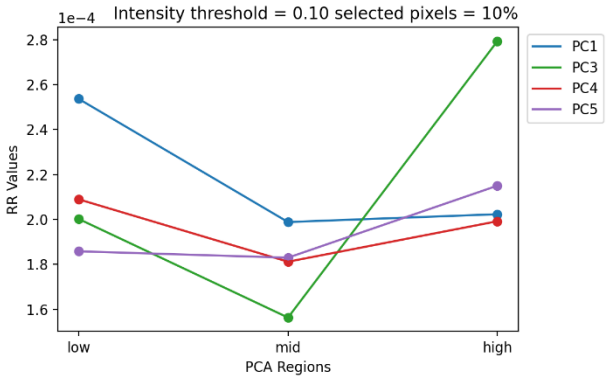

Intensity threshold = 0.10 selected pixels = 8%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

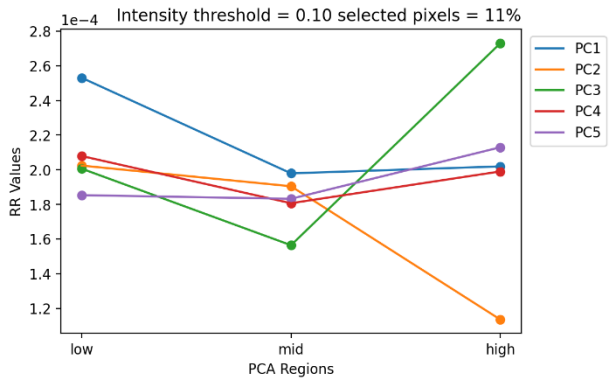

Intensity threshold = 0.10 selected pixels = 8%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

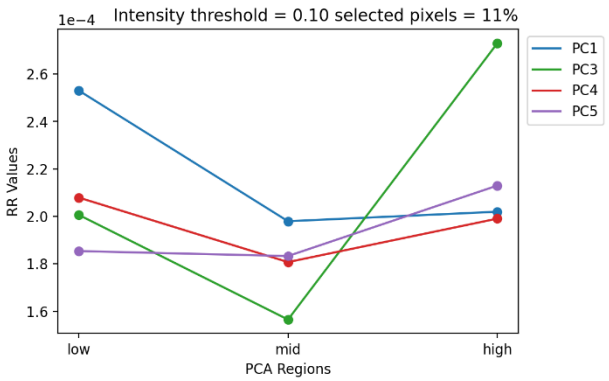

Intensity threshold = 0.10 selected pixels = 9%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

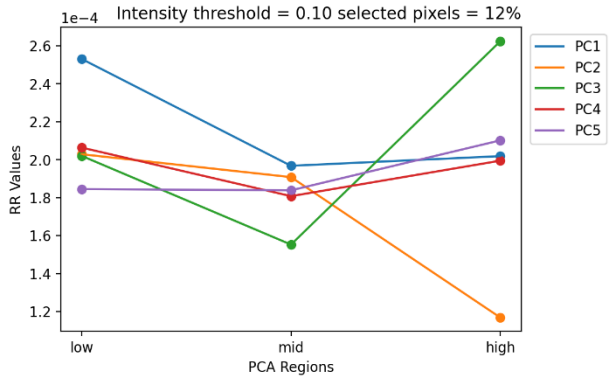

Intensity threshold = 0.10 selected pixels = 9%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

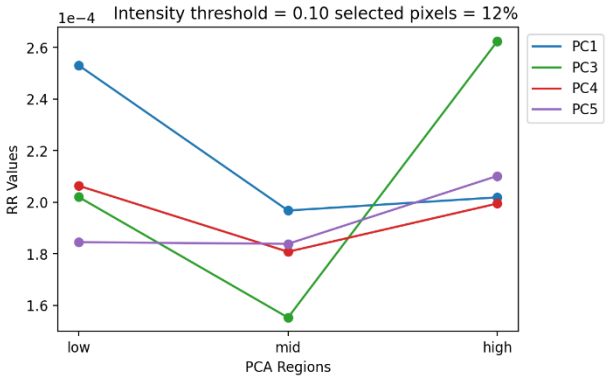

Intensity threshold = 0.10 selected pixels = 10%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

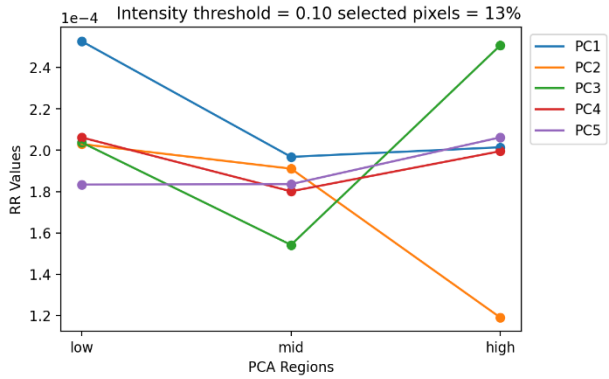

Intensity threshold = 0.10 selected pixels = 10%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

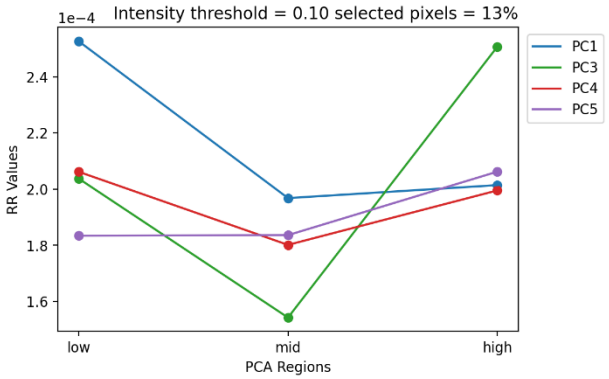

Intensity threshold = 0.10 selected pixels = 11%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

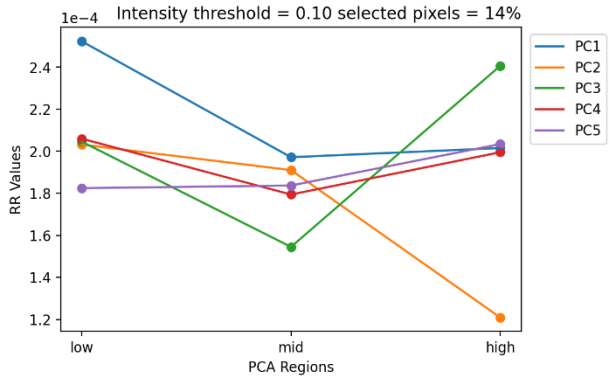

Intensity threshold = 0.10 selected pixels = 11%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

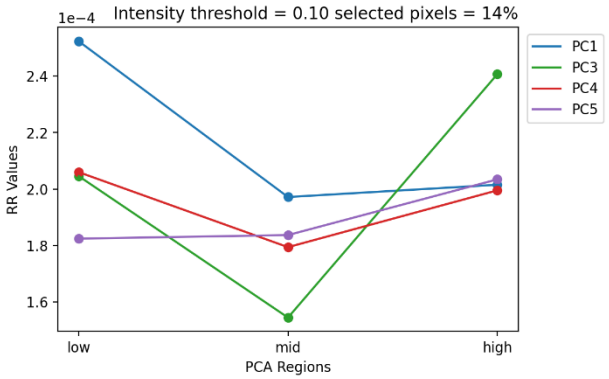

Intensity threshold = 0.10 selected pixels = 12%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

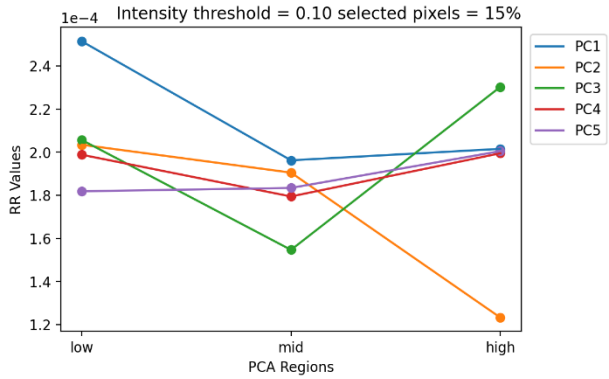

Intensity threshold = 0.10 selected pixels = 12%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

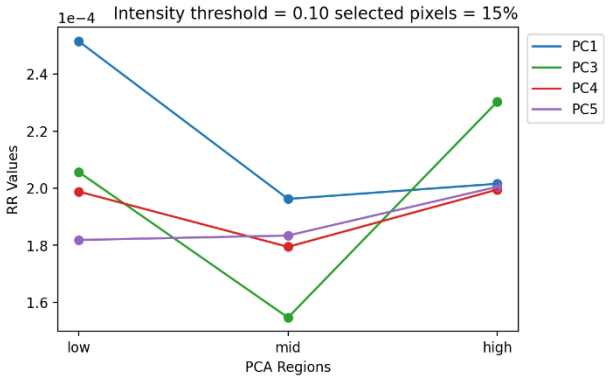

Intensity threshold = 0.10 selected pixels = 13%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

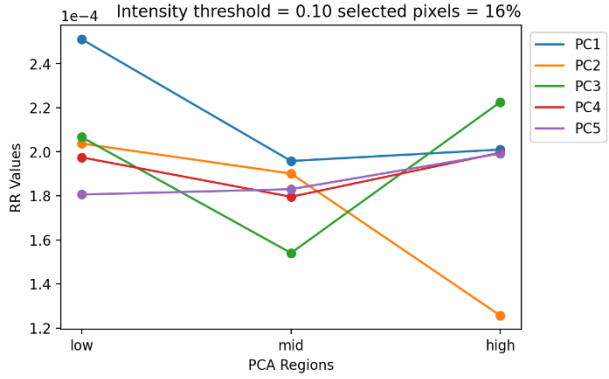

Intensity threshold = 0.10 selected pixels = 13%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

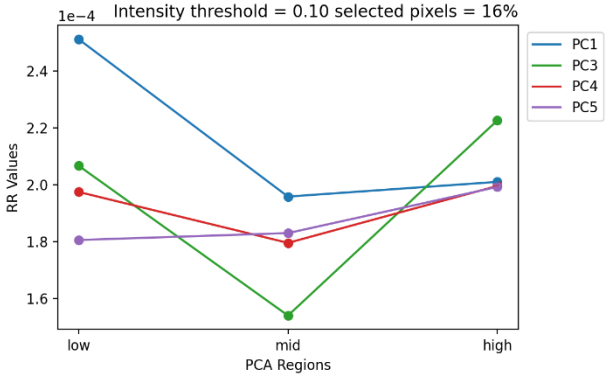

Intensity threshold = 0.10 selected pixels = 14%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

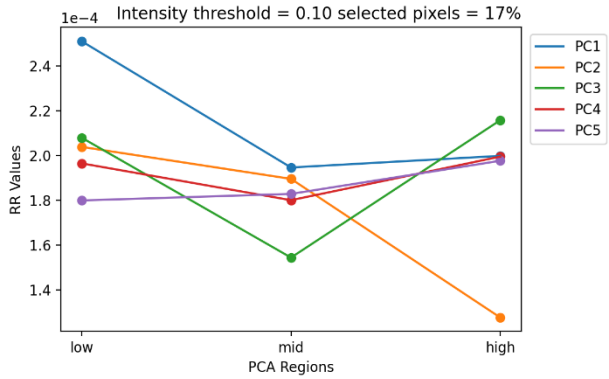

Intensity threshold = 0.10 selected pixels = 14%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

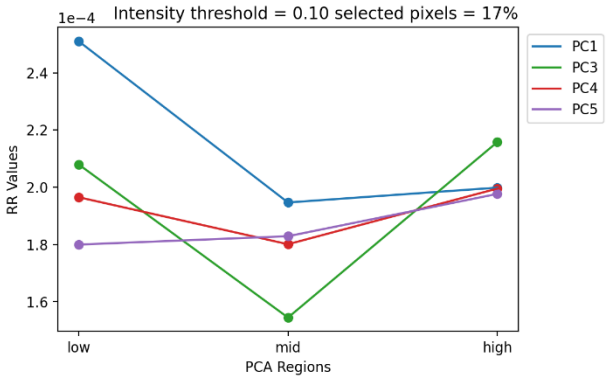

Intensity threshold = 0.10 selected pixels = 15%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

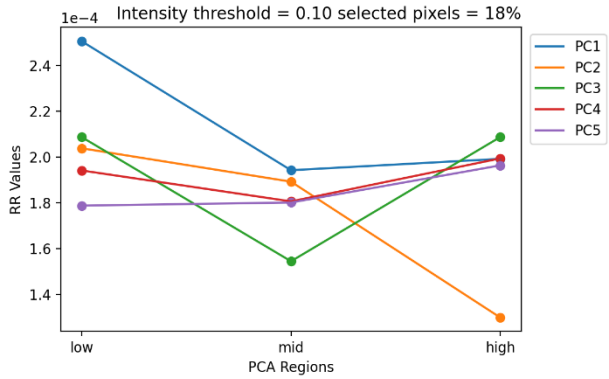

Intensity threshold = 0.10 selected pixels = 15%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

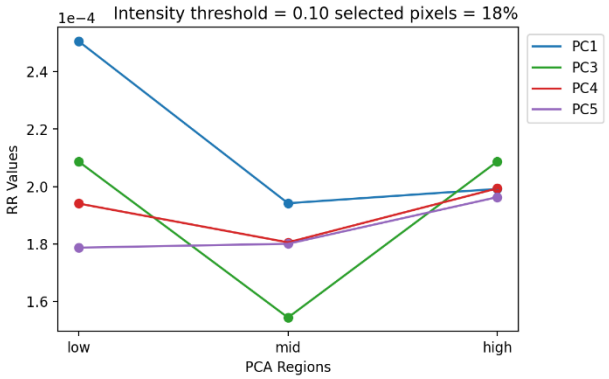

Intensity threshold = 0.10 selected pixels = 16%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

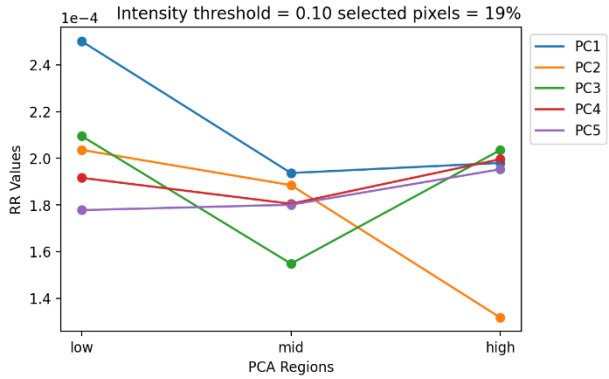

Intensity threshold = 0.10 selected pixels = 16%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

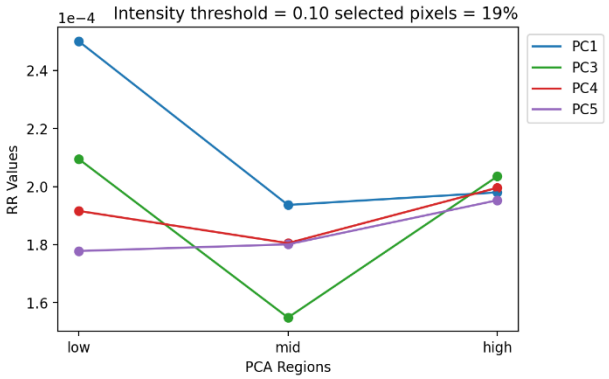

Intensity threshold = 0.10 selected pixels = 17%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

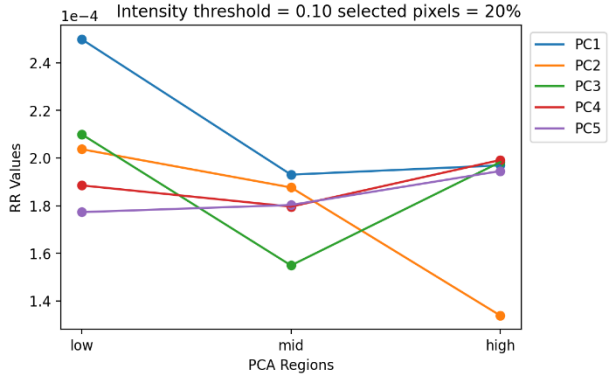

Intensity threshold = 0.10 selected pixels = 17%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

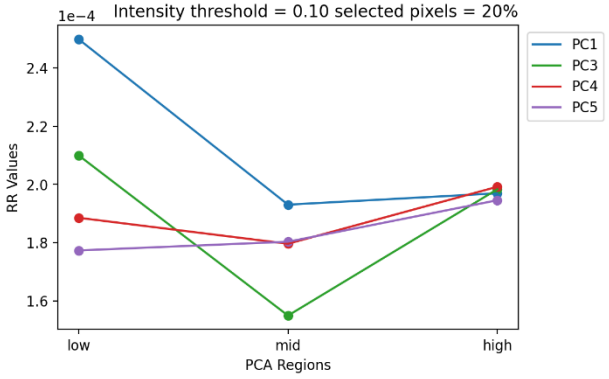

Intensity threshold = 0.10 selected pixels = 18%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

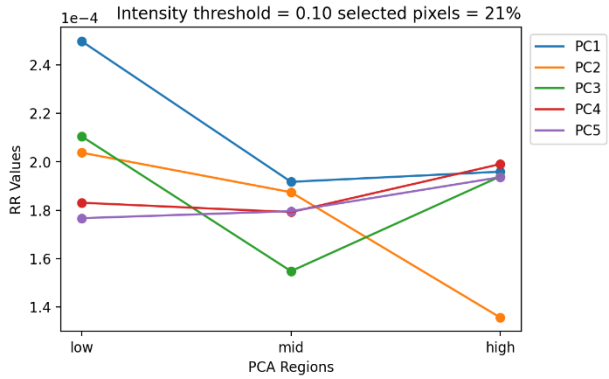

Intensity threshold = 0.10 selected pixels = 18%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

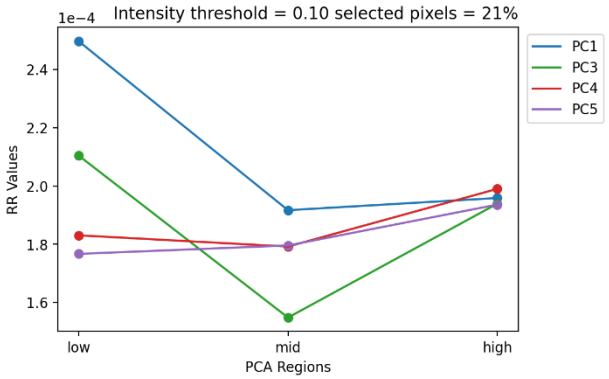

Intensity threshold = 0.10 selected pixels = 19%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

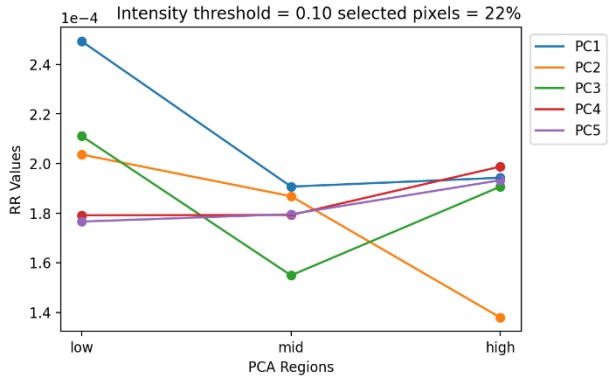

Intensity threshold = 0.10 selected pixels = 19%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

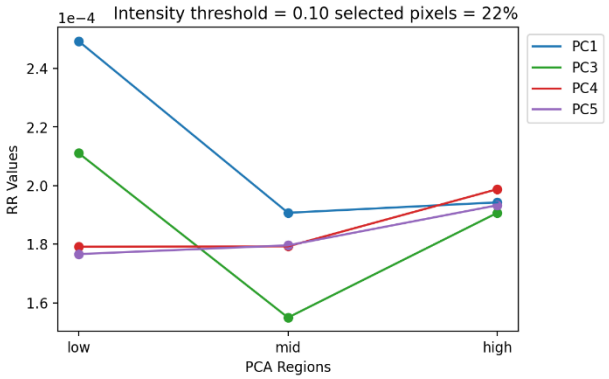

Intensity threshold = 0.10 selected pixels = 20%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

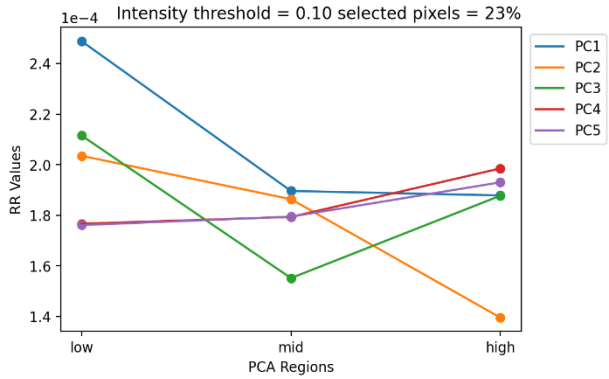

Intensity threshold = 0.10 selected pixels = 20%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

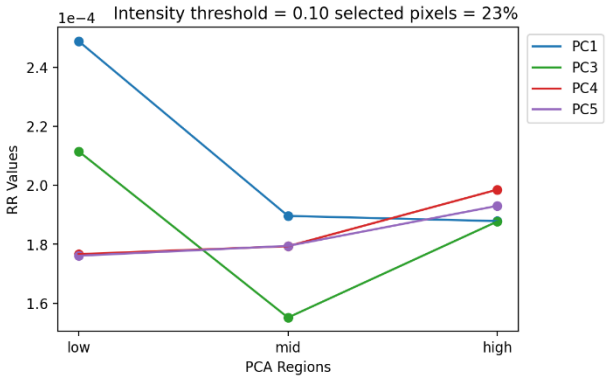

Intensity threshold = 0.10 selected pixels = 21%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

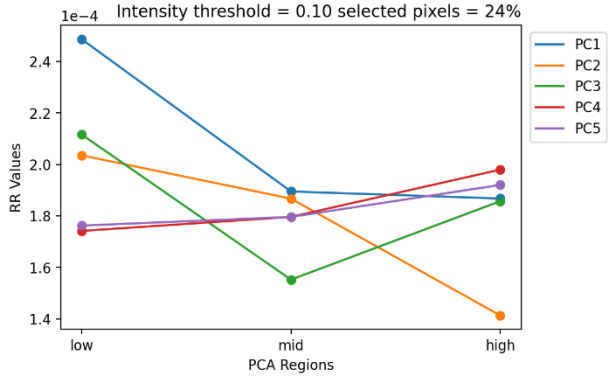

Intensity threshold = 0.10 selected pixels = 21%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

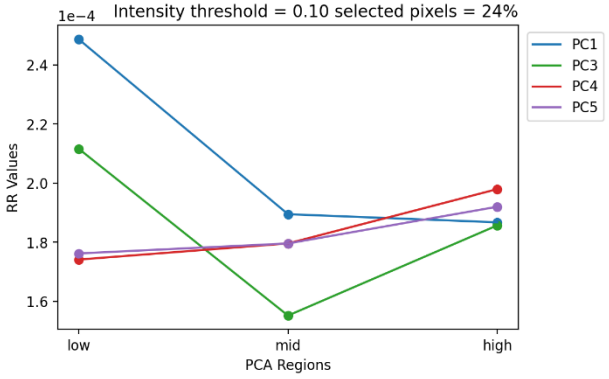

Intensity threshold = 0.10 selected pixels = 22%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

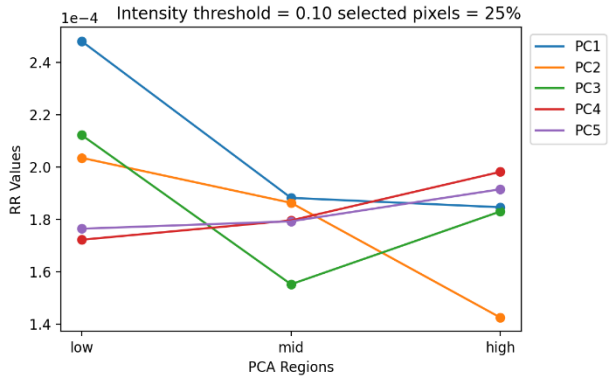

Intensity threshold = 0.10 selected pixels = 22%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

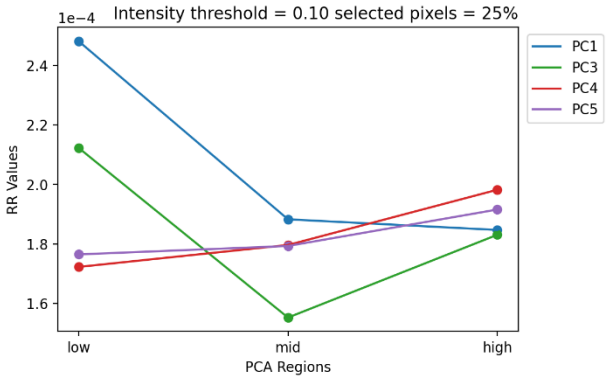

Intensity threshold = 0.10 selected pixels = 23%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

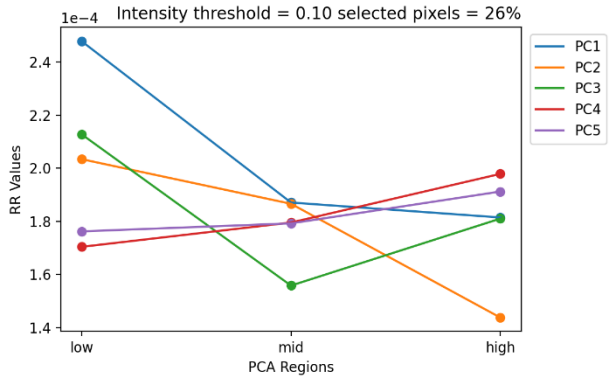

Intensity threshold = 0.10 selected pixels = 23%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

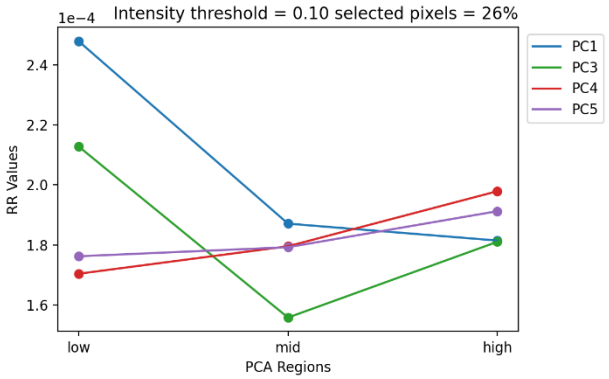

Intensity threshold = 0.10 selected pixels = 24%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

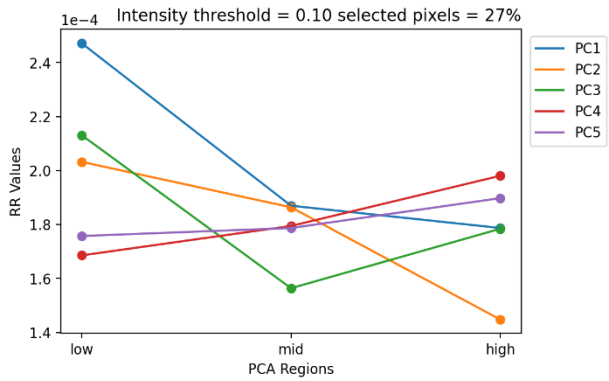

Intensity threshold = 0.10 selected pixels = 24%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

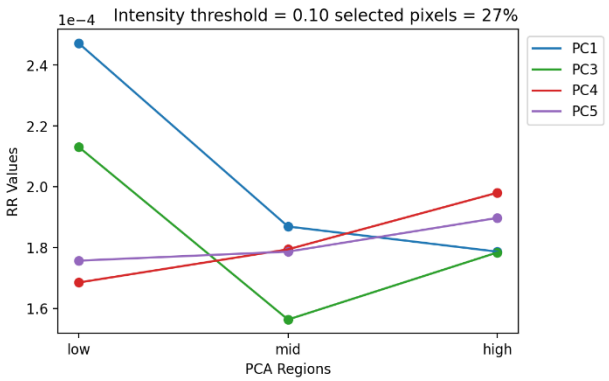

Intensity threshold = 0.10 selected pixels = 25%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

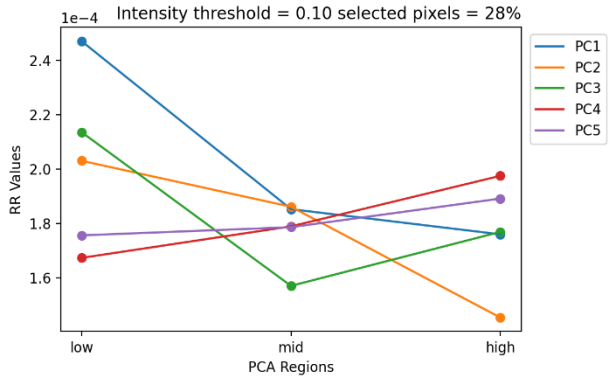

Intensity threshold = 0.10 selected pixels = 25%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

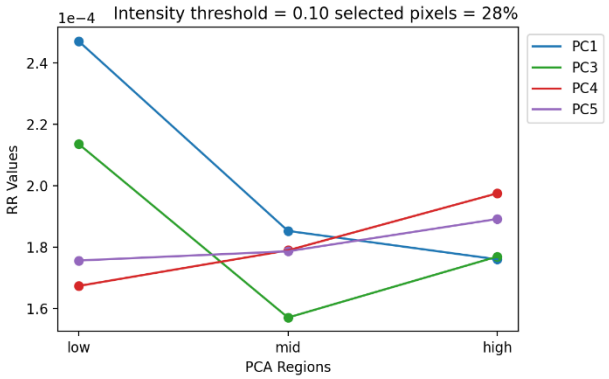

Intensity threshold = 0.10 selected pixels = 26%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 26%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 27%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 27%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 28%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 28%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

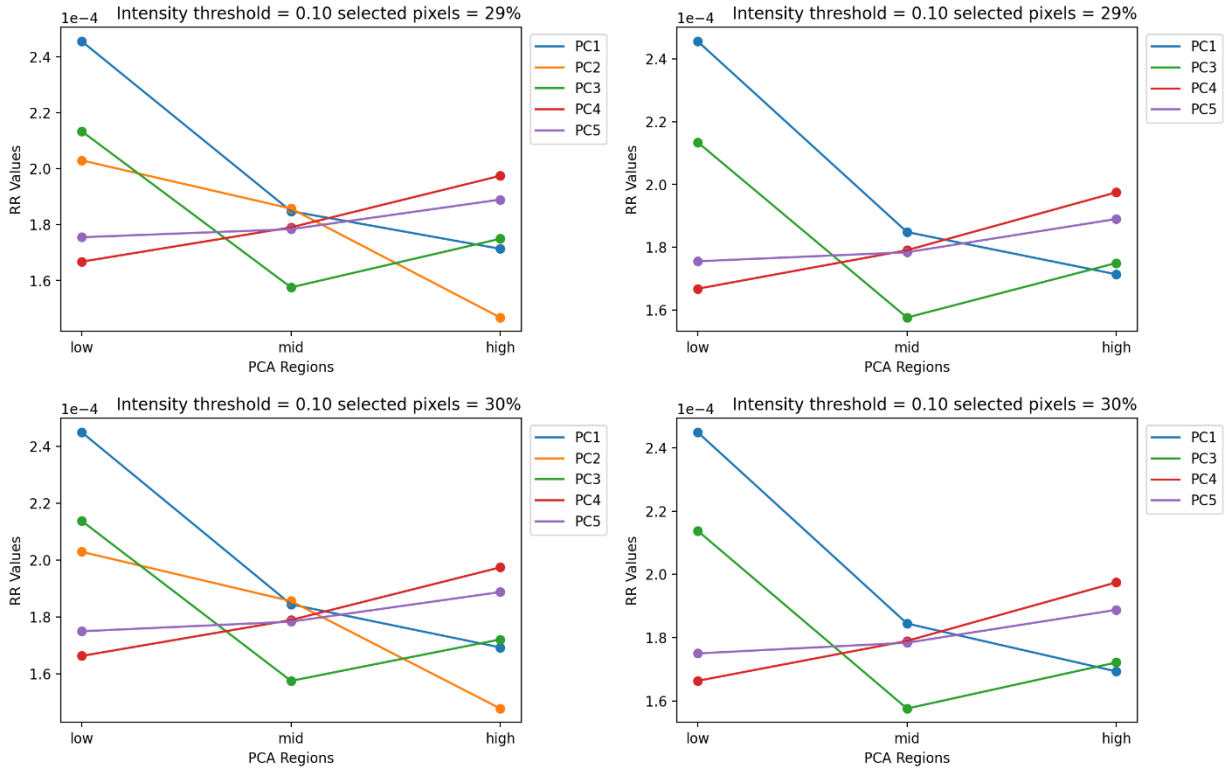
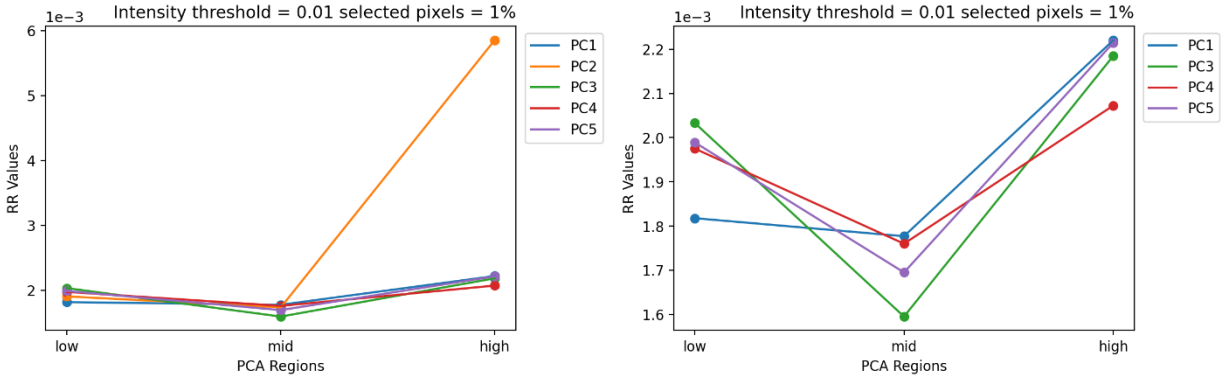
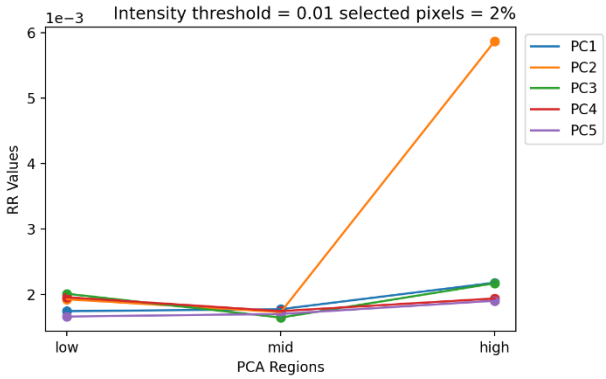


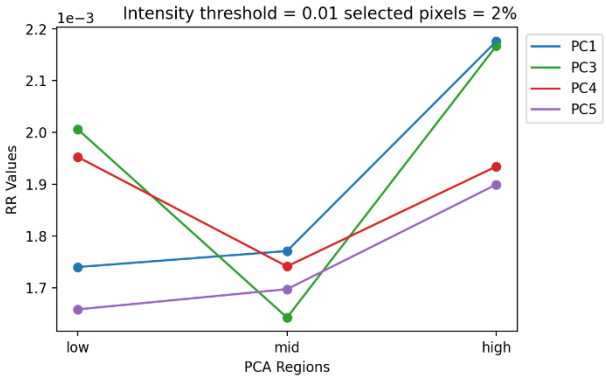
Figure S11: 2-D V plots for local normalization with root mean squared normalized results for intensity threshold 0.10.

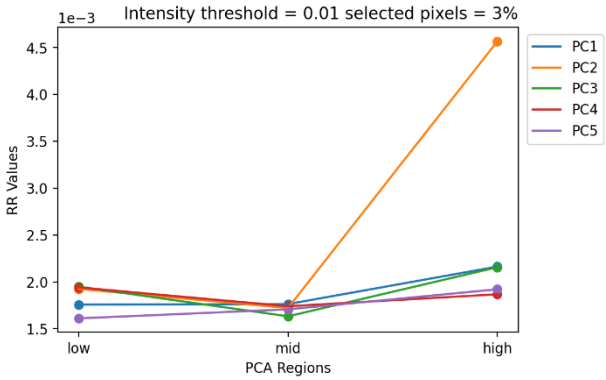
2-D V plots for global normalization with root mean squared normalized results

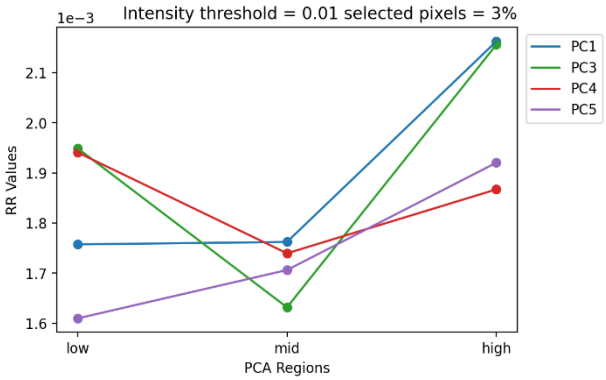
RMS pre-processing for PCA was used for pixel selection. Global normalization with RMS pre-processing gives comparable distinctions of similarity to the RMS normalized intensities with local normalization. The local method was still determined to be better since the V shape of PC 1 is present throughout the first 10% of selected pixels which makes up the strongest correlated pixels.

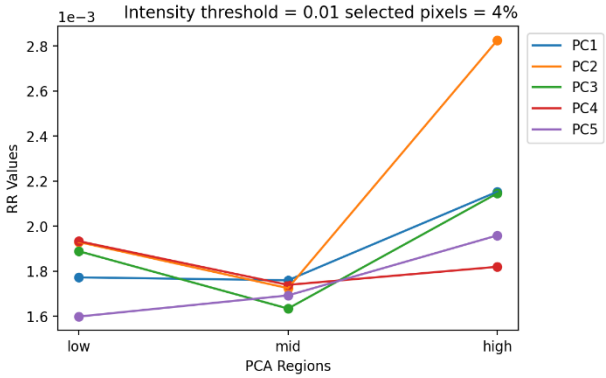


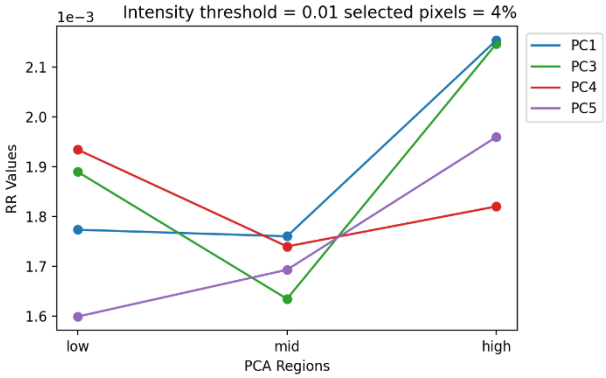

Intensity threshold = 0.01 selected pixels = 2%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

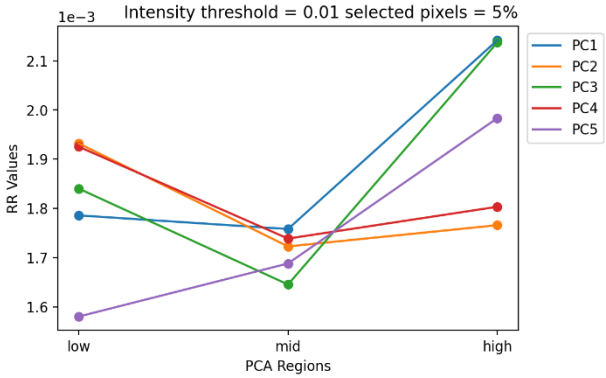

Intensity threshold = 0.01 selected pixels = 2%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

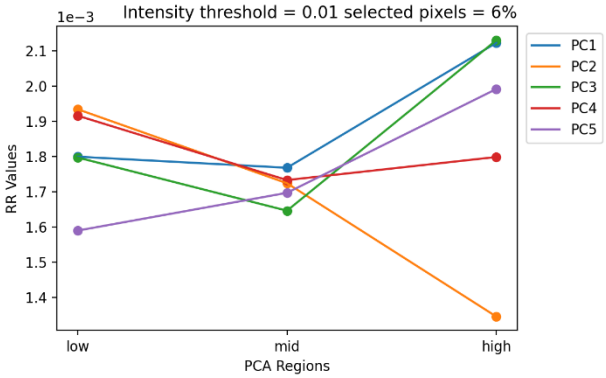

Intensity threshold = 0.01 selected pixels = 3%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

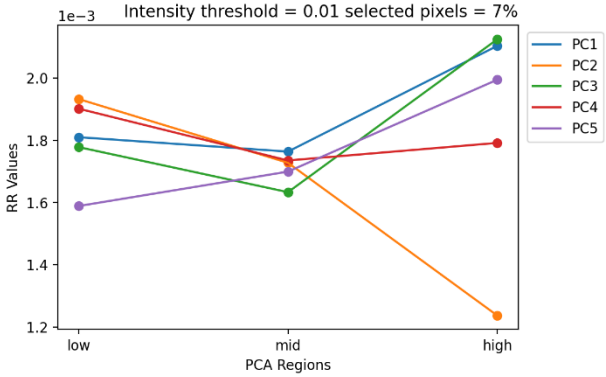

Intensity threshold = 0.01 selected pixels = 3%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

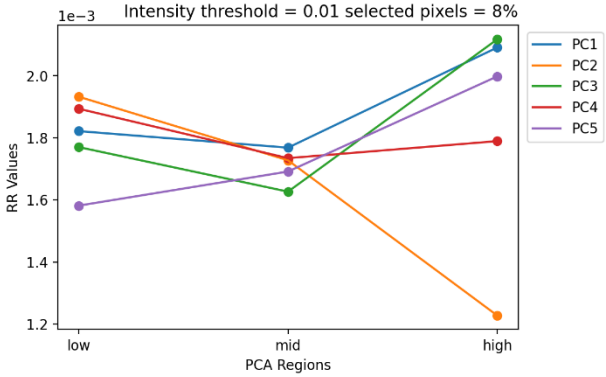

Intensity threshold = 0.01 selected pixels = 4%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

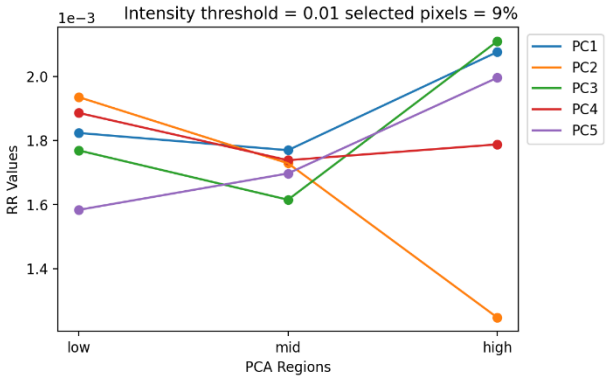

Intensity threshold = 0.01 selected pixels = 4%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

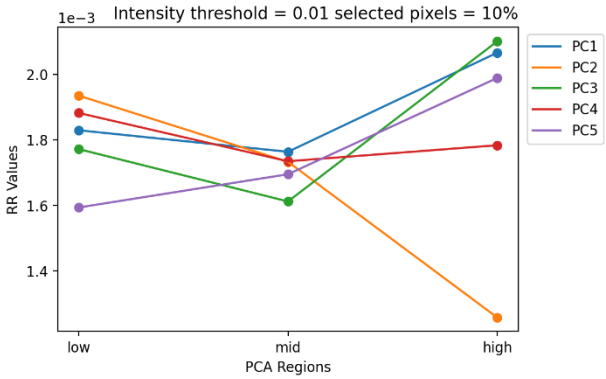

Intensity threshold = 0.01 selected pixels = 5%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

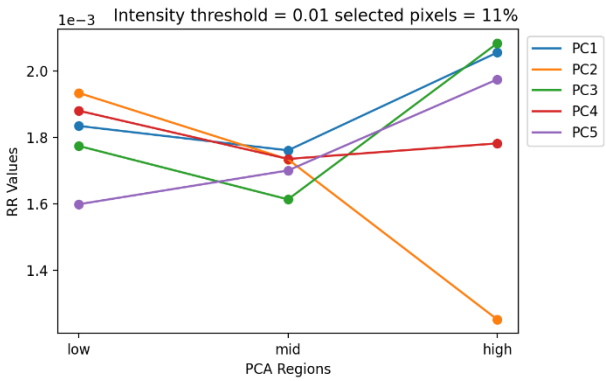

Intensity threshold = 0.01 selected pixels = 6%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

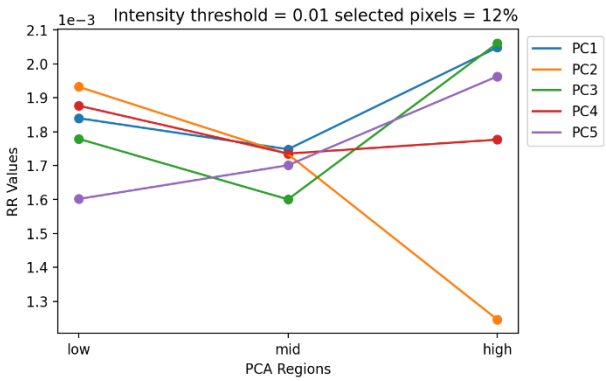

Intensity threshold = 0.01 selected pixels = 7%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

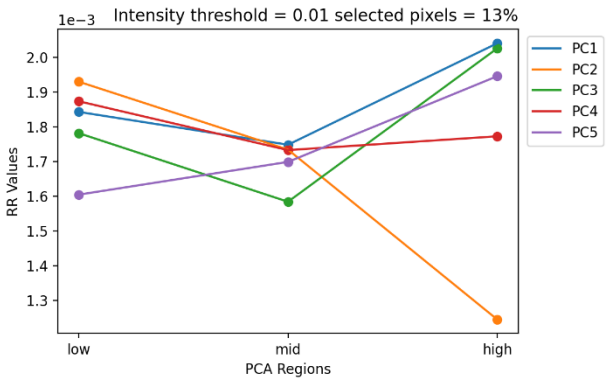

Intensity threshold = 0.01 selected pixels = 8%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

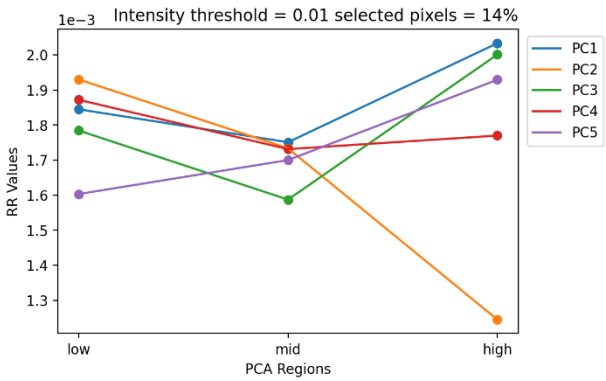

Intensity threshold = 0.01 selected pixels = 9%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

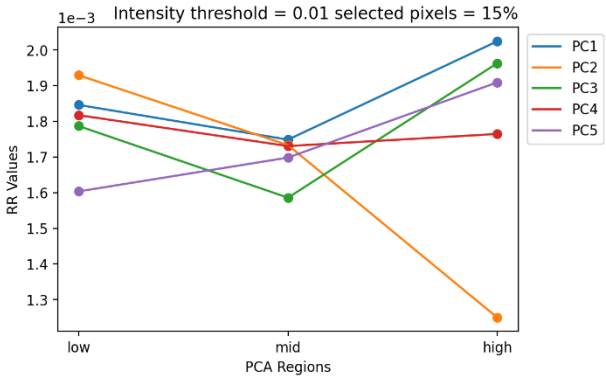

Intensity threshold = 0.01 selected pixels = 10%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

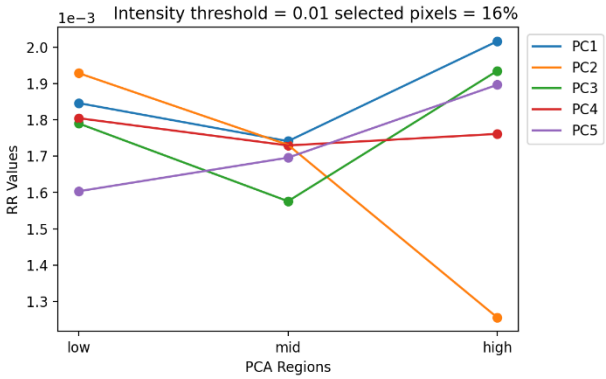

Intensity threshold = 0.01 selected pixels = 11%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

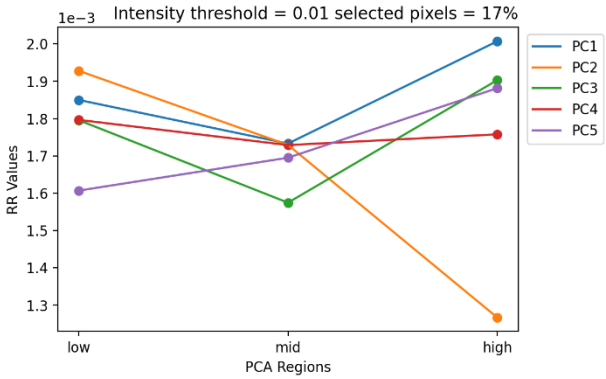

Intensity threshold = 0.01 selected pixels = 12%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

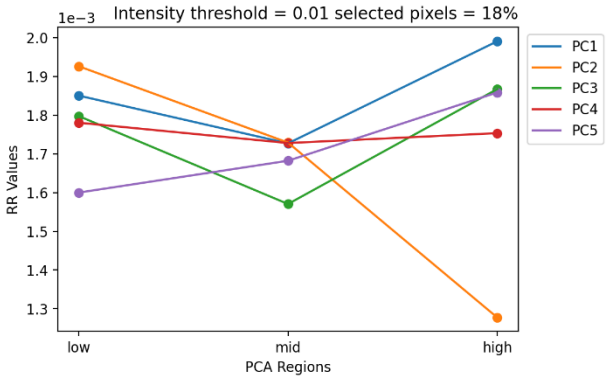

Intensity threshold = 0.01 selected pixels = 13%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

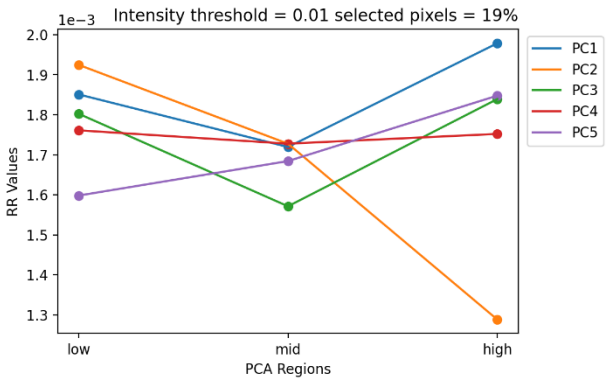

Intensity threshold = 0.01 selected pixels = 14%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

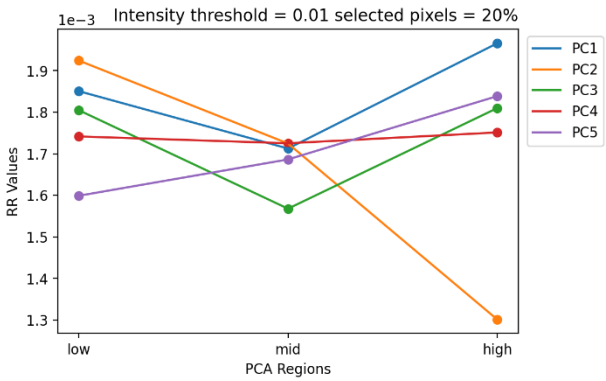

Intensity threshold = 0.01 selected pixels = 15%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

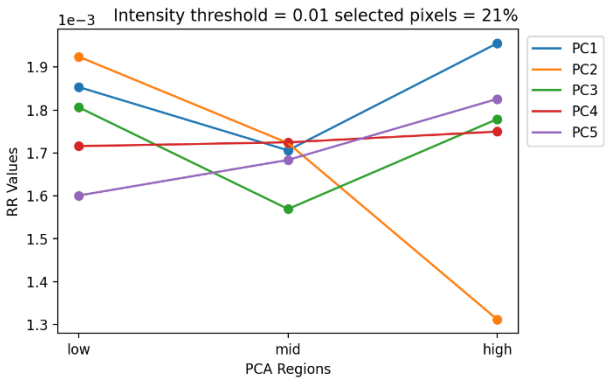

Intensity threshold = 0.01 selected pixels = 16%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

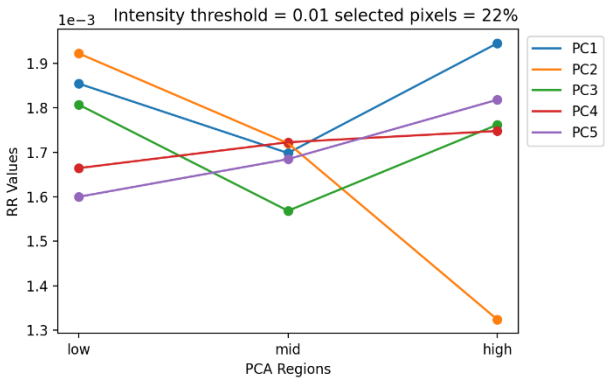

Intensity threshold = 0.01 selected pixels = 17%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 18%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 19%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 20%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 21%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 22%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

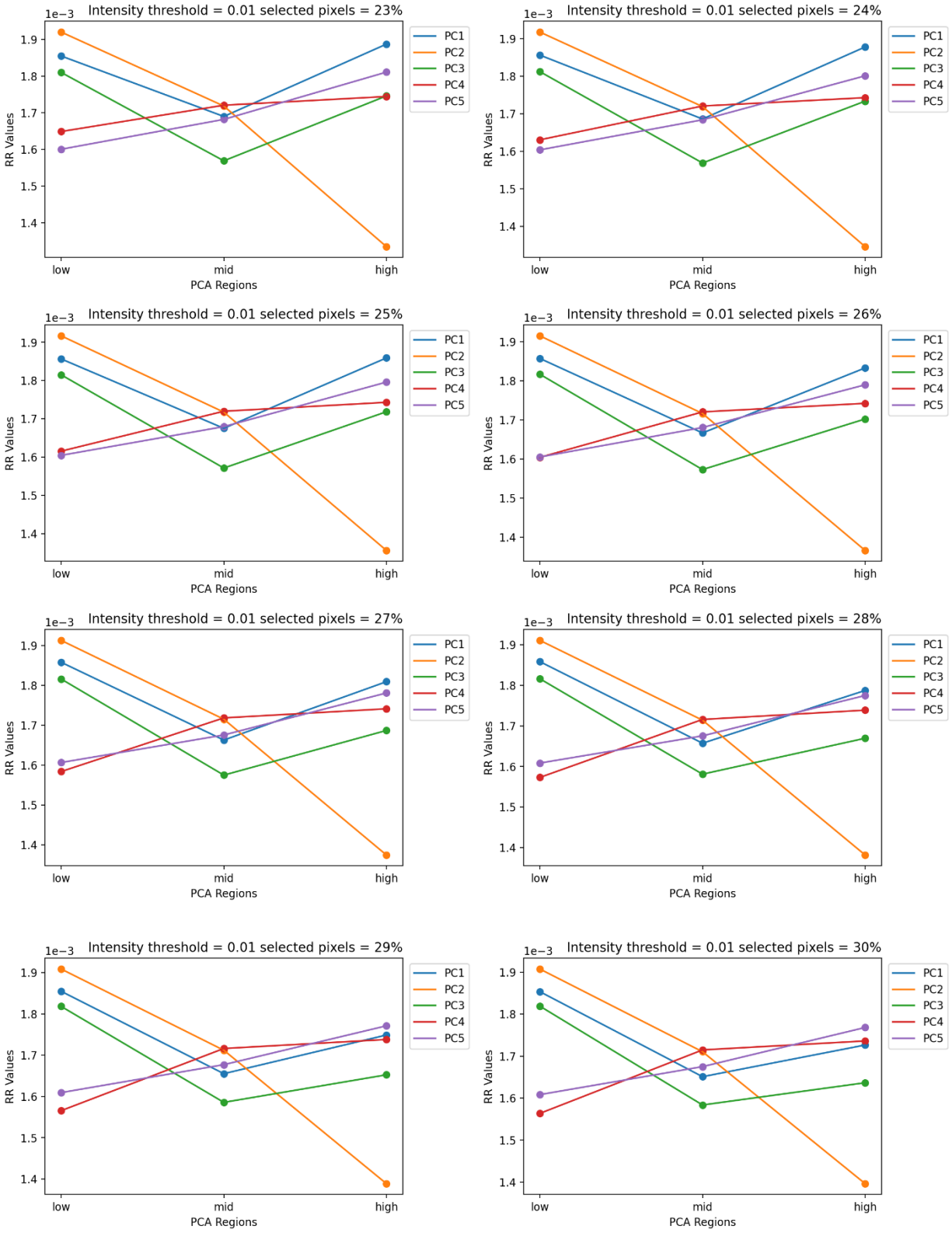
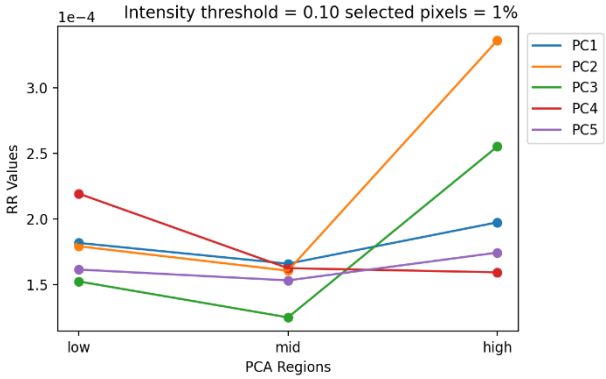
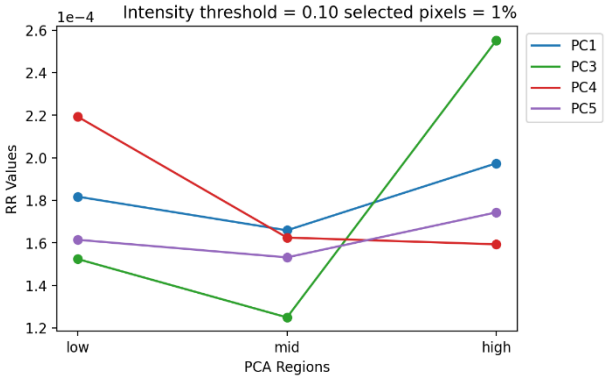
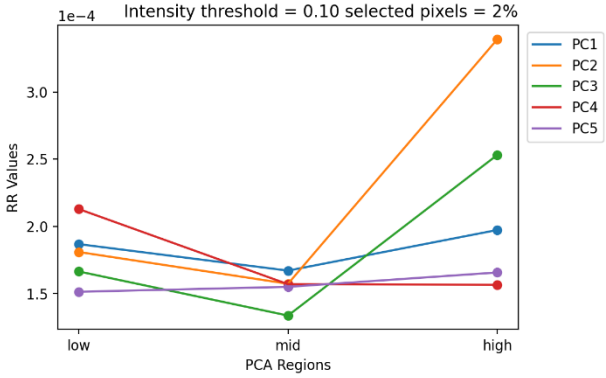
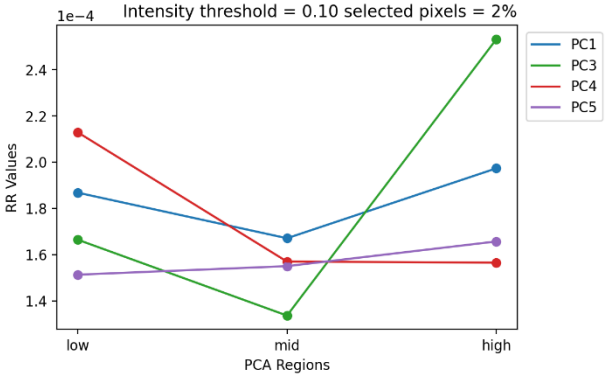


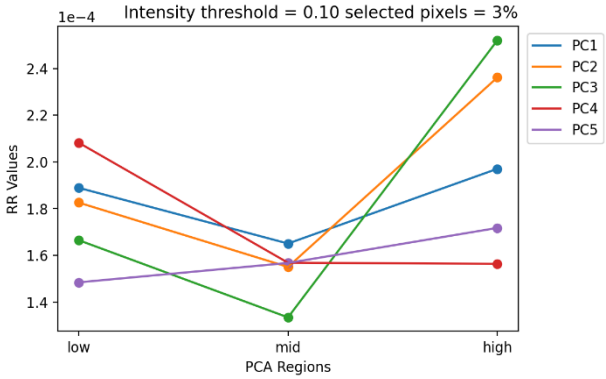
Figure S12: 2-D V plots for global normalization with root mean squared normalized results for intensity threshold 0.01.

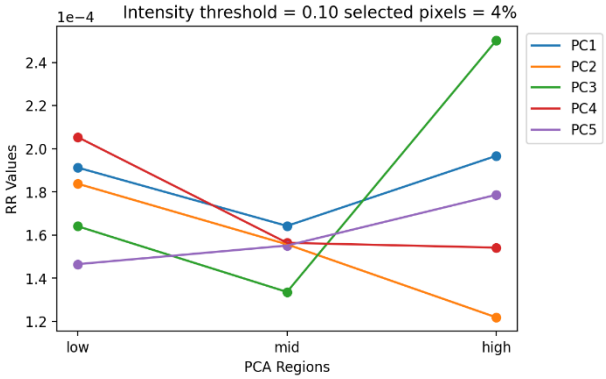

Intensity threshold = 0.10 selected pixels = 1%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

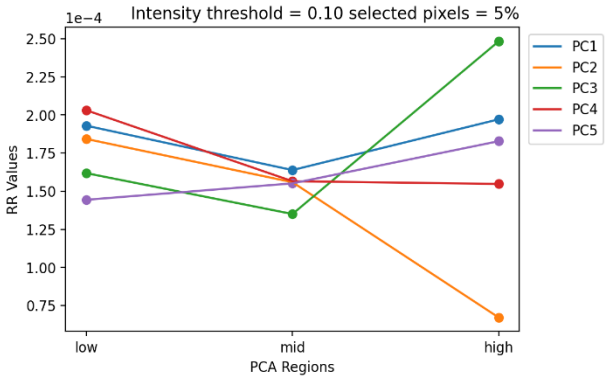

Intensity threshold = 0.10 selected pixels = 1%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

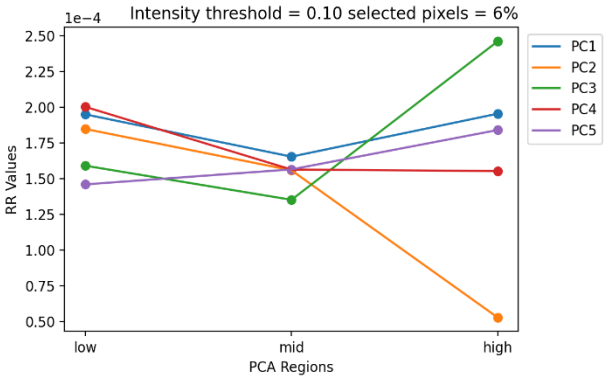

Intensity threshold = 0.10 selected pixels = 2%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

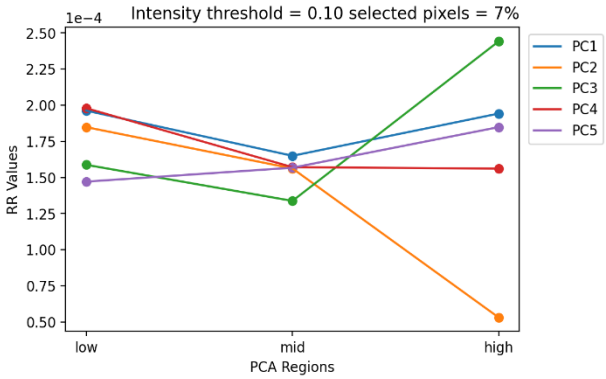

Intensity threshold = 0.10 selected pixels = 2%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC3
PC4
PC5

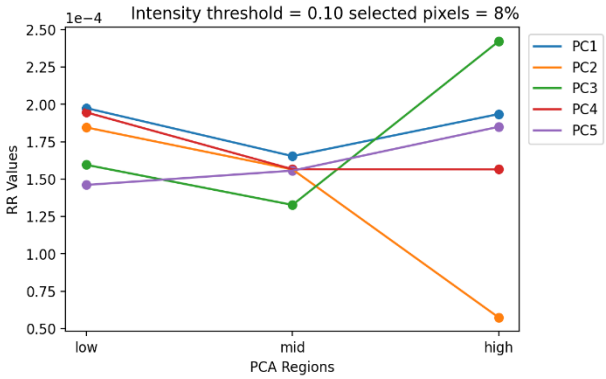

Intensity threshold = 0.10 selected pixels = 3%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

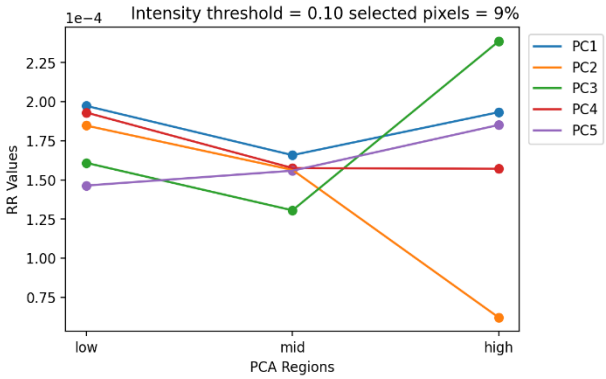

Intensity threshold = 0.10 selected pixels = 4%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

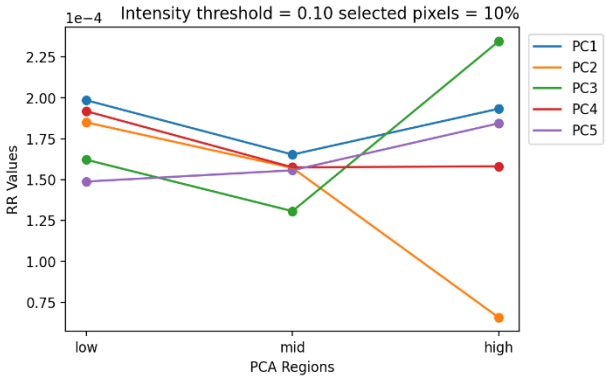

Intensity threshold = 0.10 selected pixels = 5%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

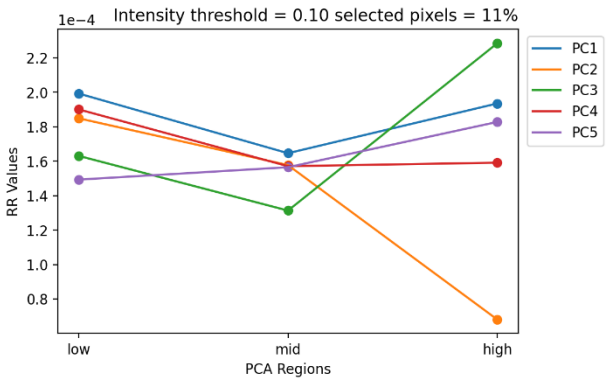

Intensity threshold = 0.10 selected pixels = 6%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

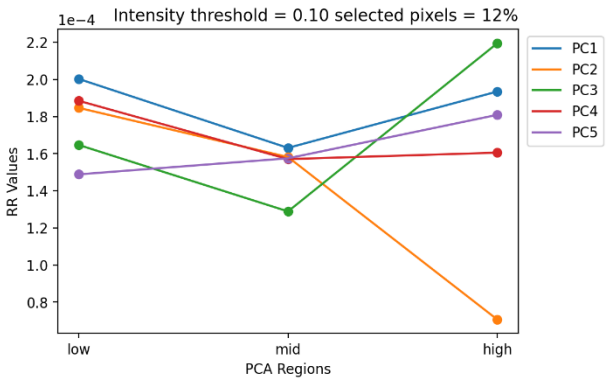

Intensity threshold = 0.10 selected pixels = 7%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

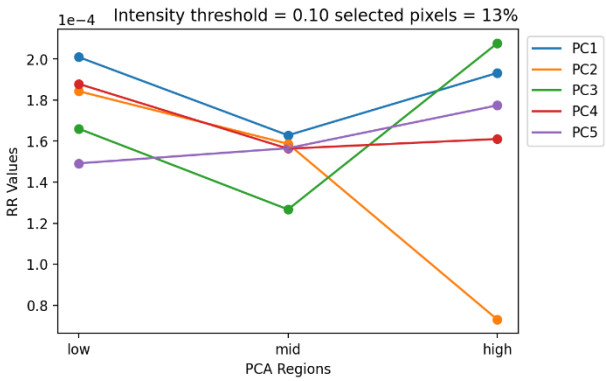

Intensity threshold = 0.10 selected pixels = 8%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

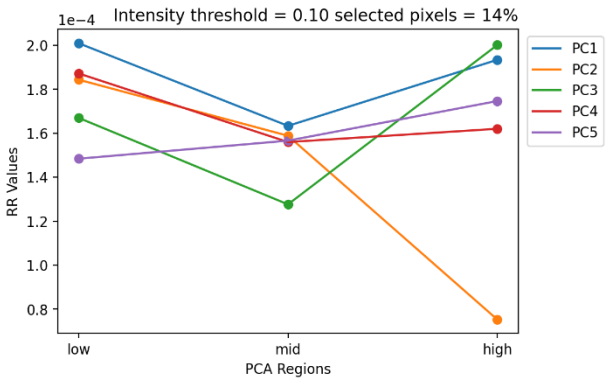

Intensity threshold = 0.10 selected pixels = 9%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

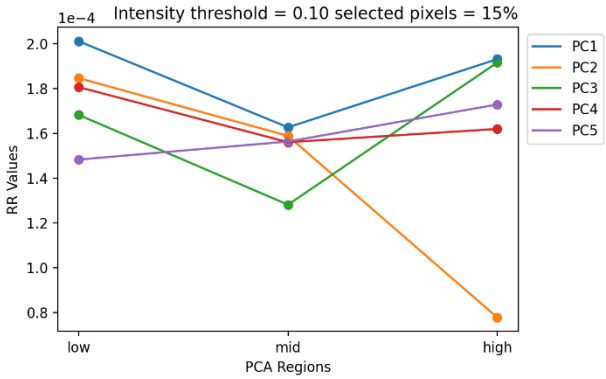

Intensity threshold = 0.10 selected pixels = 10%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

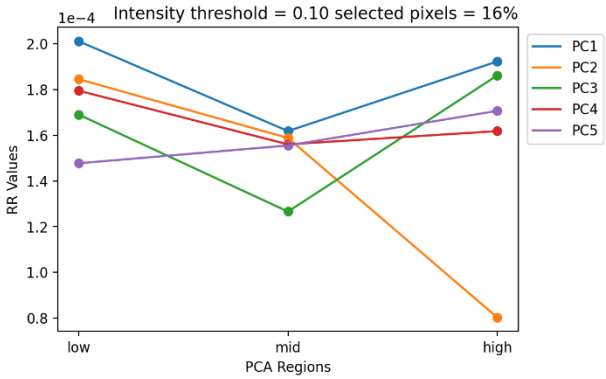

Intensity threshold = 0.10 selected pixels = 11%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

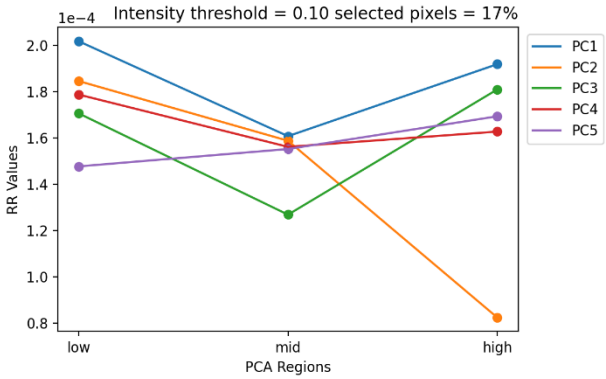

Intensity threshold = 0.10 selected pixels = 12%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

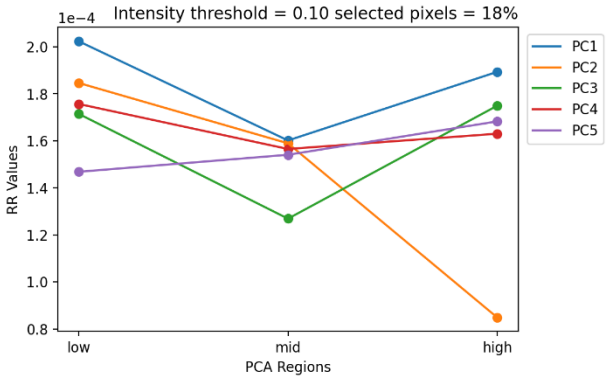

Intensity threshold = 0.10 selected pixels = 13%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

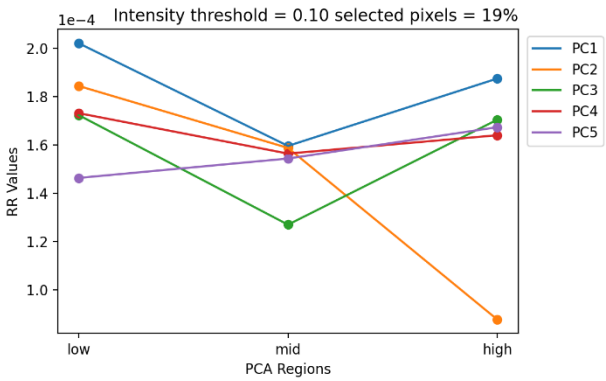

Intensity threshold = 0.10 selected pixels = 14%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

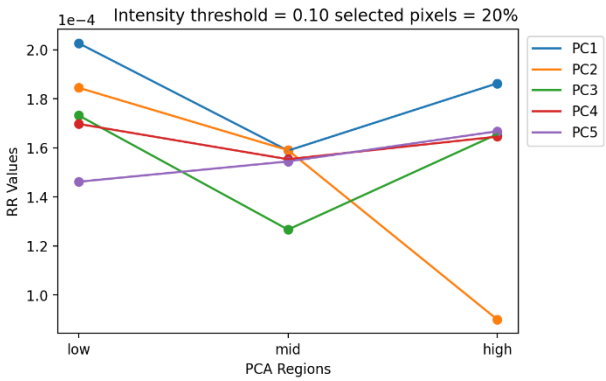

Intensity threshold = 0.10 selected pixels = 15%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

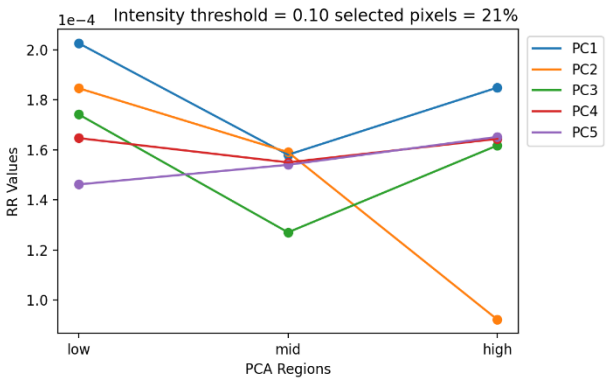

Intensity threshold = 0.10 selected pixels = 16%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

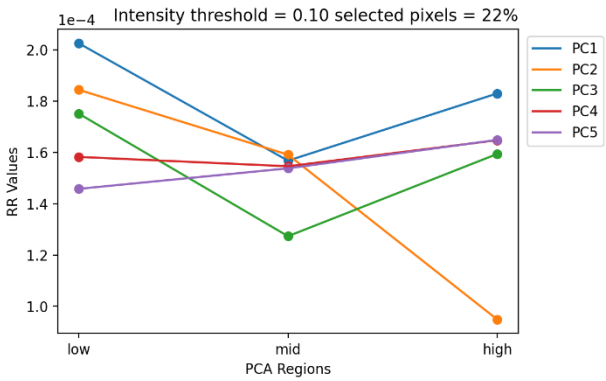

Intensity threshold = 0.10 selected pixels = 17%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 18%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 19%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 20%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 21%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 22%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

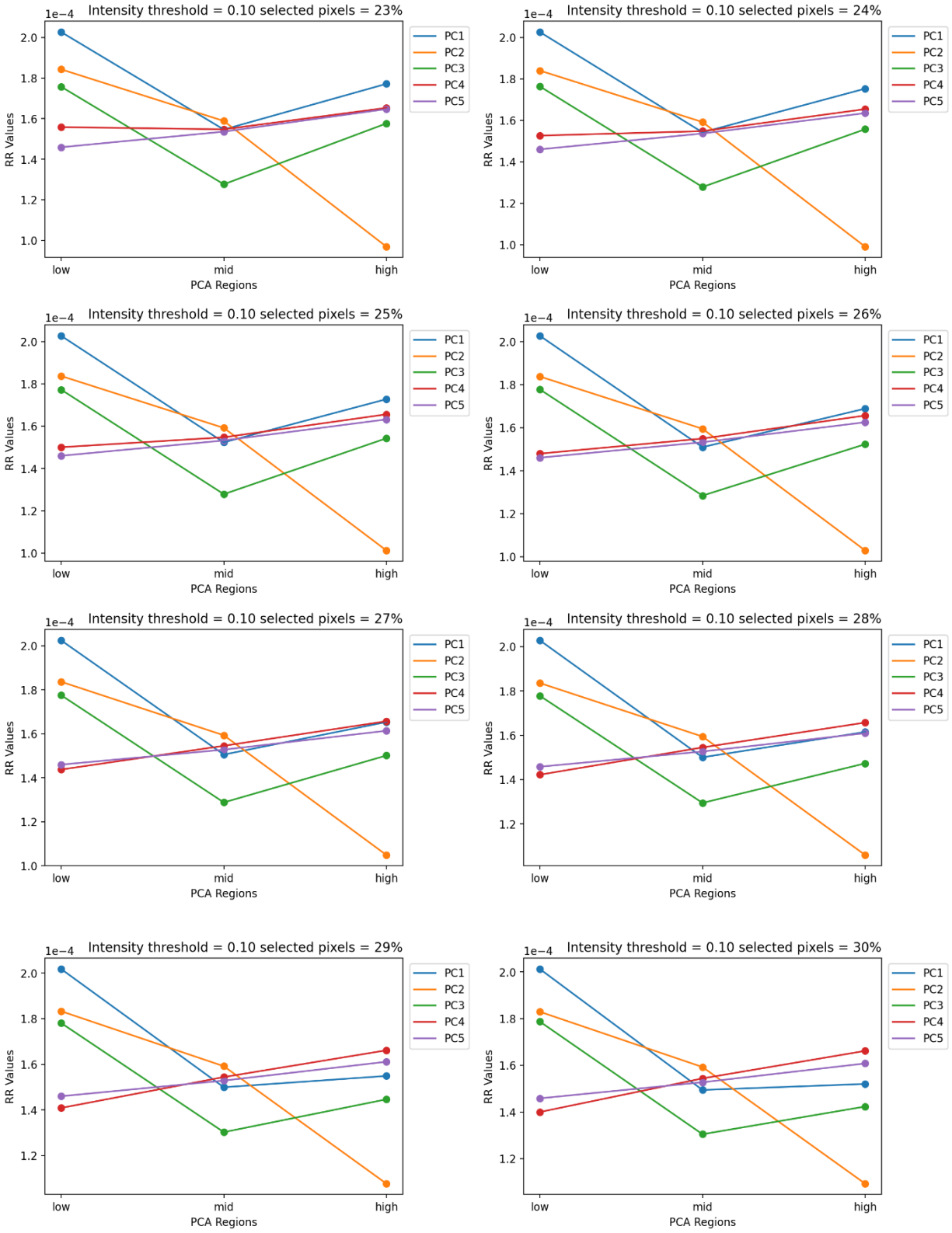
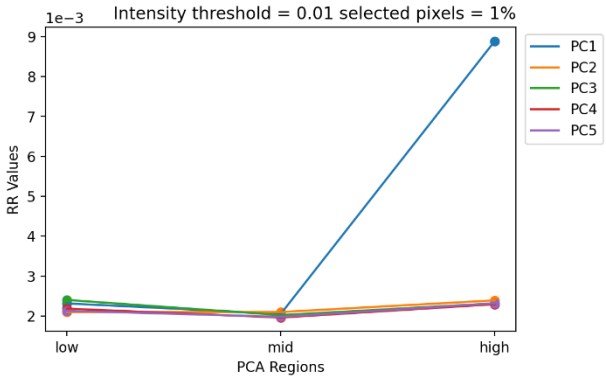
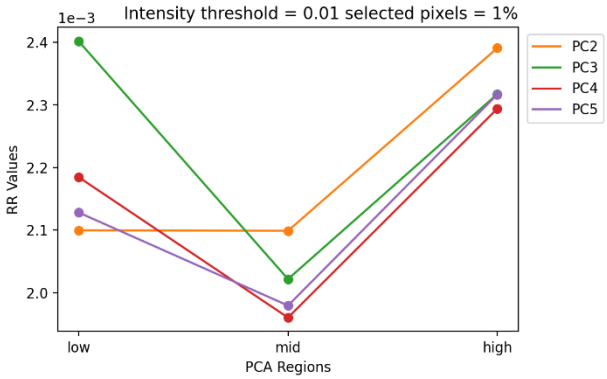


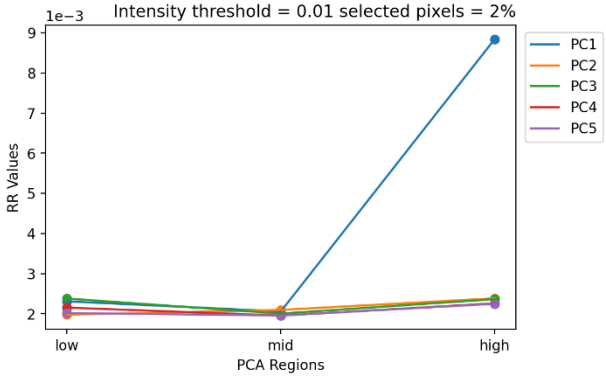
Figure S13: 2-D V plots for global normalization with root mean squared normalized results for intensity threshold 0.10.

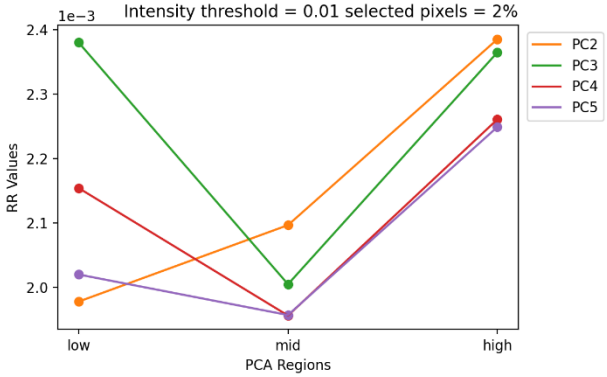
2-D V plots for local normalization with non-normalized PCA results

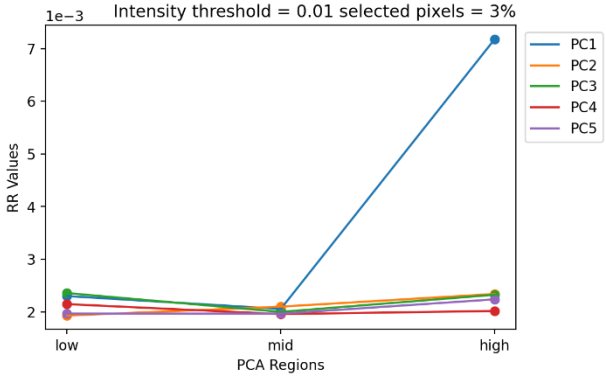
Non-normalized PCA results were used for pixel selection. Local normalization without RMS pre-processing at first showed promising results with good V shapes in the first few percentages of pixels selected. However for intensity threshold 0.01 and 0.10, after 5% the V shape completely disappears for all PCs.

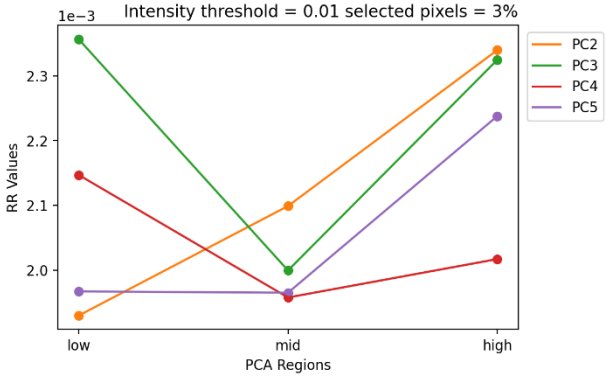


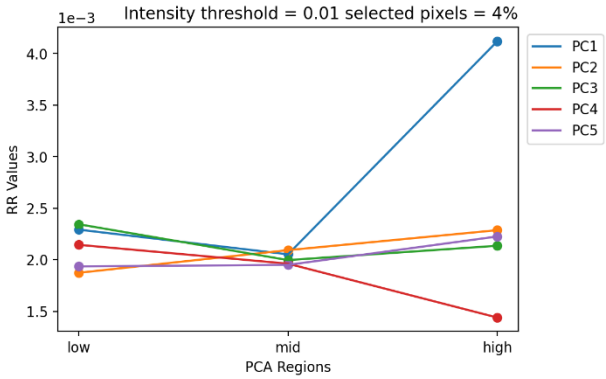


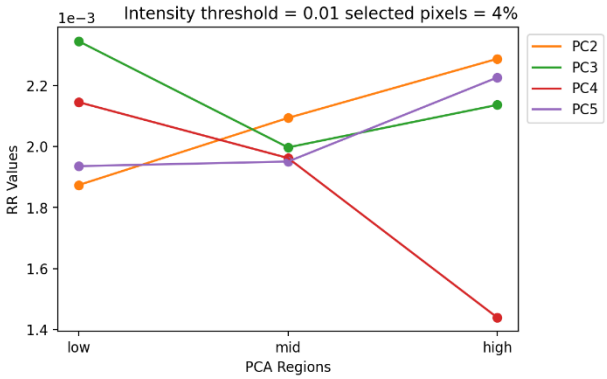


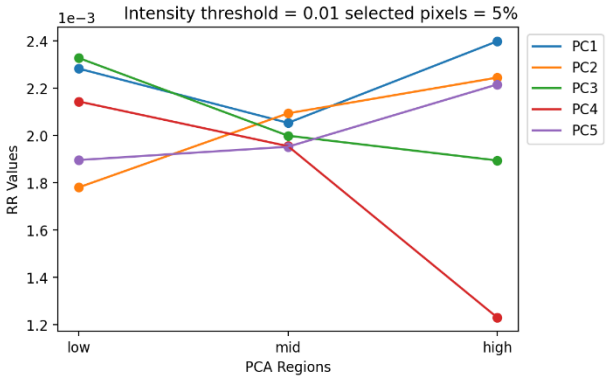


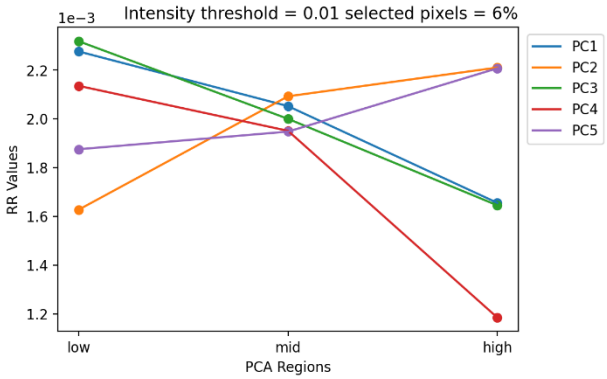


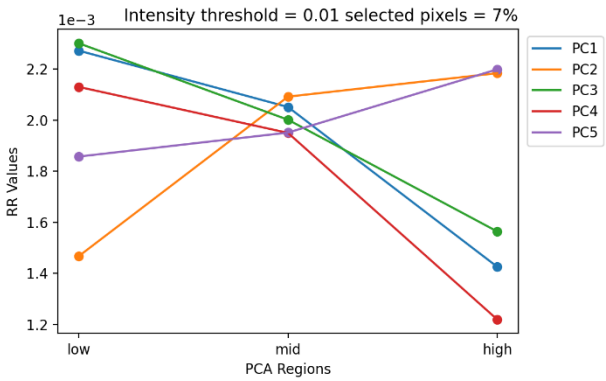


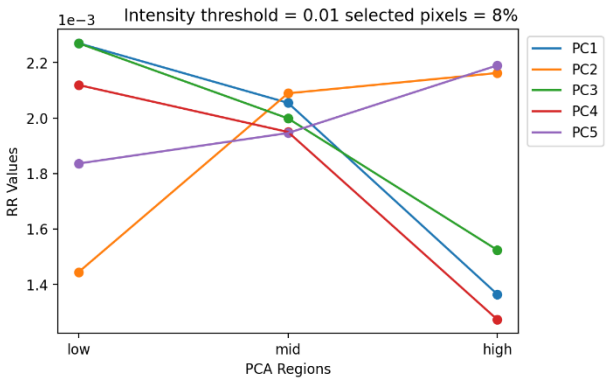

Intensity threshold = 0.01 selected pixels = 4%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

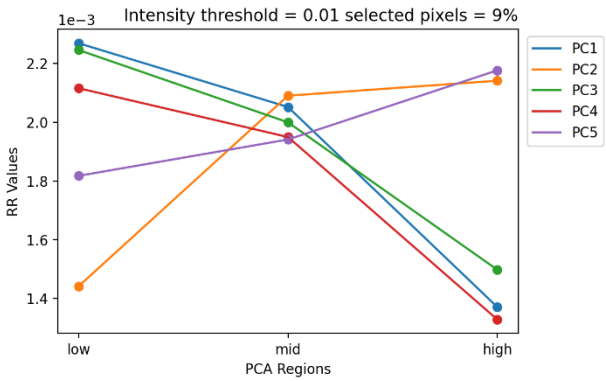

Intensity threshold = 0.01 selected pixels = 4%
1e−3
RR Values
PCA Regions
low
mid
high
PC2
PC3
PC4
PC5

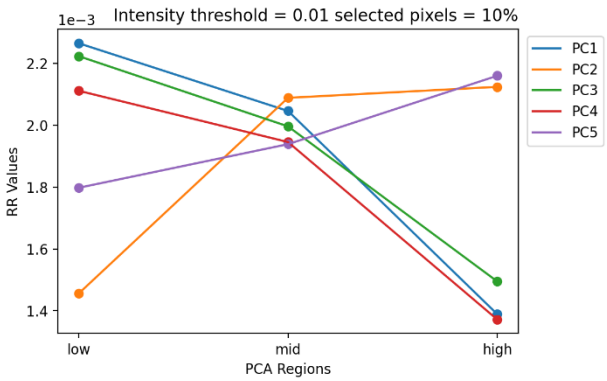

Intensity threshold = 0.01 selected pixels = 5%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

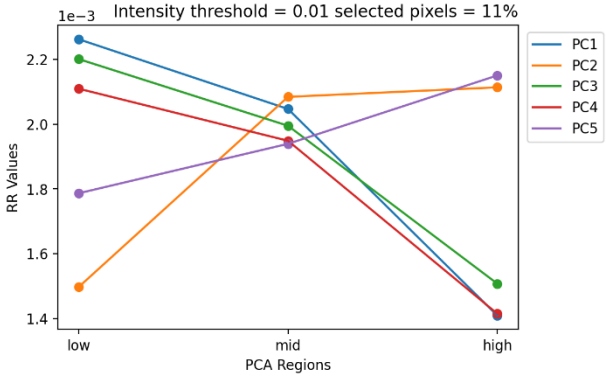

Intensity threshold = 0.01 selected pixels = 6%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

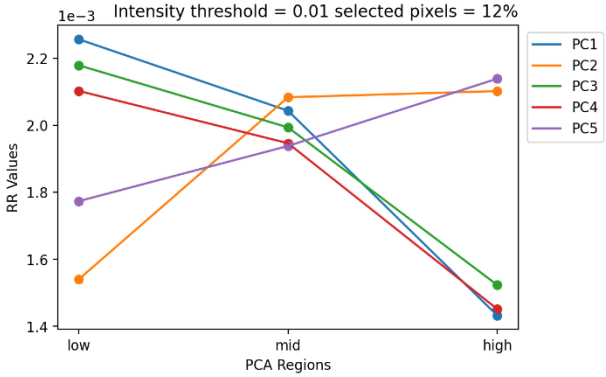

Intensity threshold = 0.01 selected pixels = 7%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

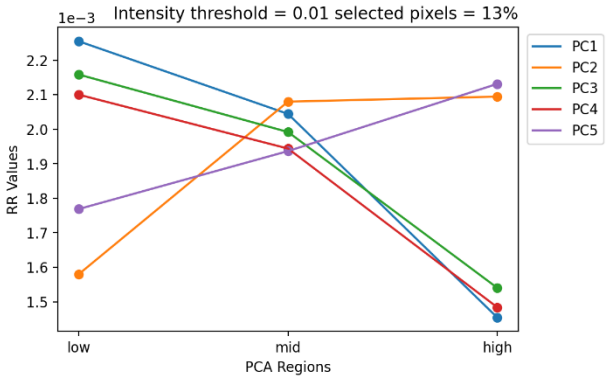

Intensity threshold = 0.01 selected pixels = 8%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

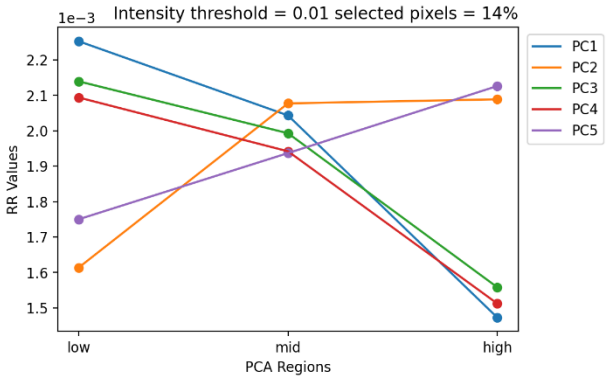

Intensity threshold = 0.01 selected pixels = 9%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

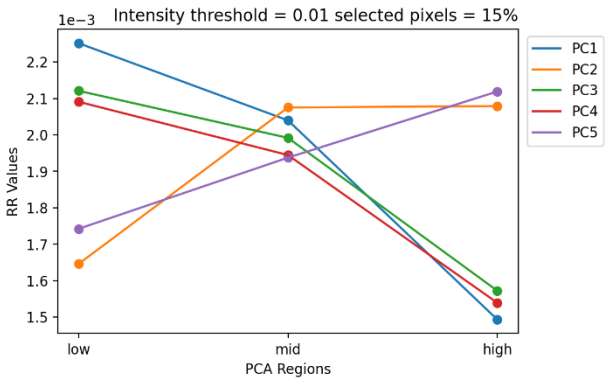

Intensity threshold = 0.01 selected pixels = 10%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

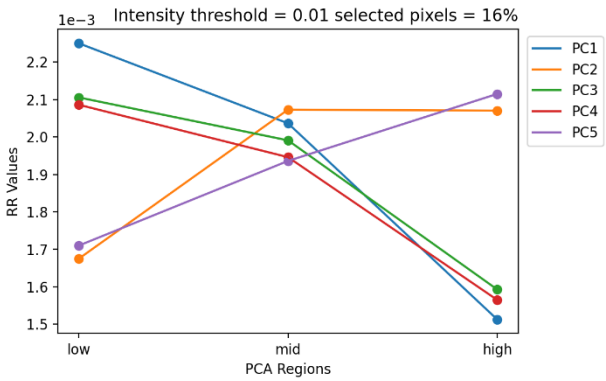

Intensity threshold = 0.01 selected pixels = 11%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

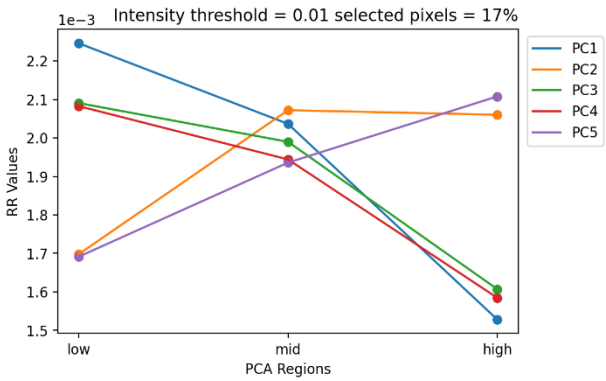

Intensity threshold = 0.01 selected pixels = 12%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

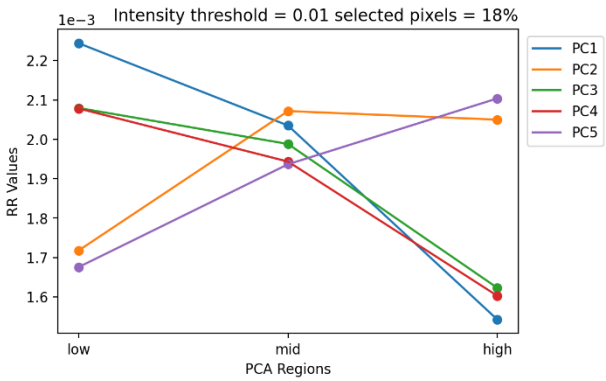

Intensity threshold = 0.01 selected pixels = 13%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

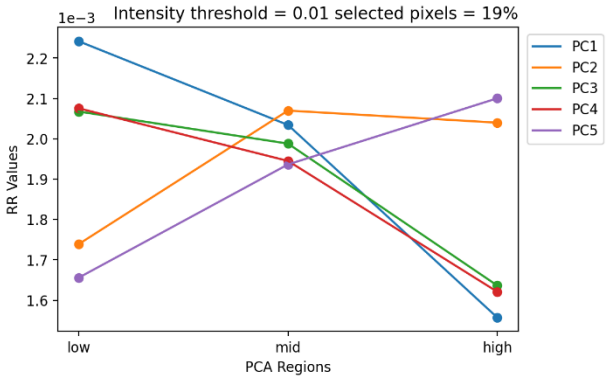

Intensity threshold = 0.01 selected pixels = 14%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

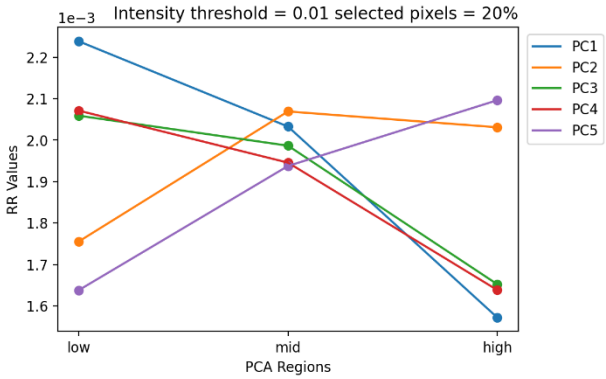

Intensity threshold = 0.01 selected pixels = 15%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

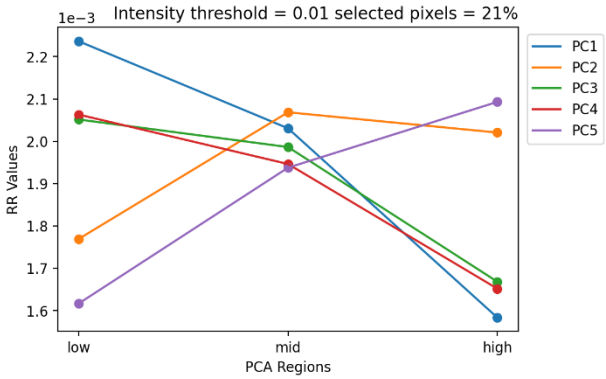

Intensity threshold = 0.01 selected pixels = 16%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

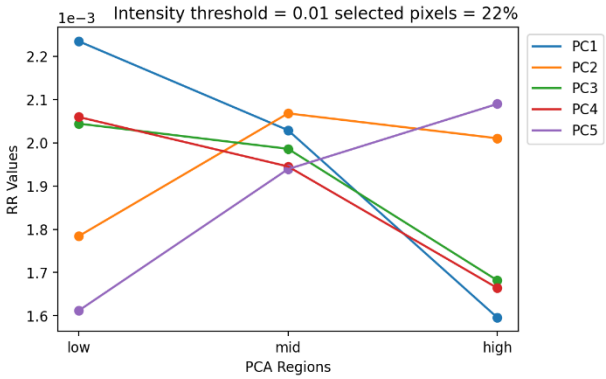

Intensity threshold = 0.01 selected pixels = 17%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

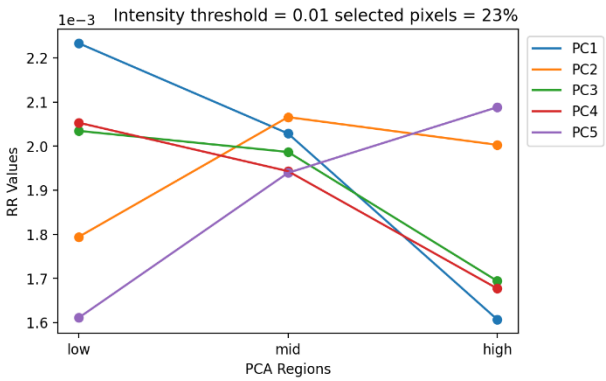

Intensity threshold = 0.01 selected pixels = 18%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

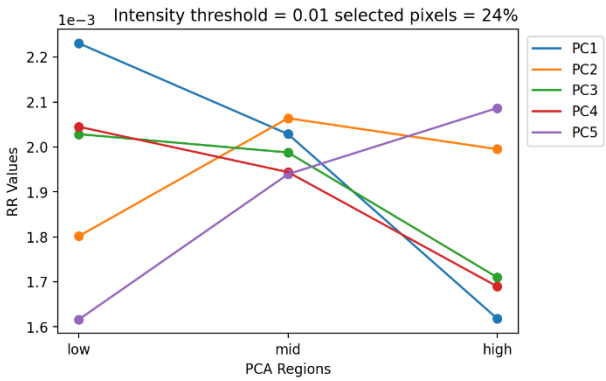

Intensity threshold = 0.01 selected pixels = 19%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 20%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 21%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 22%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 23%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 24%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

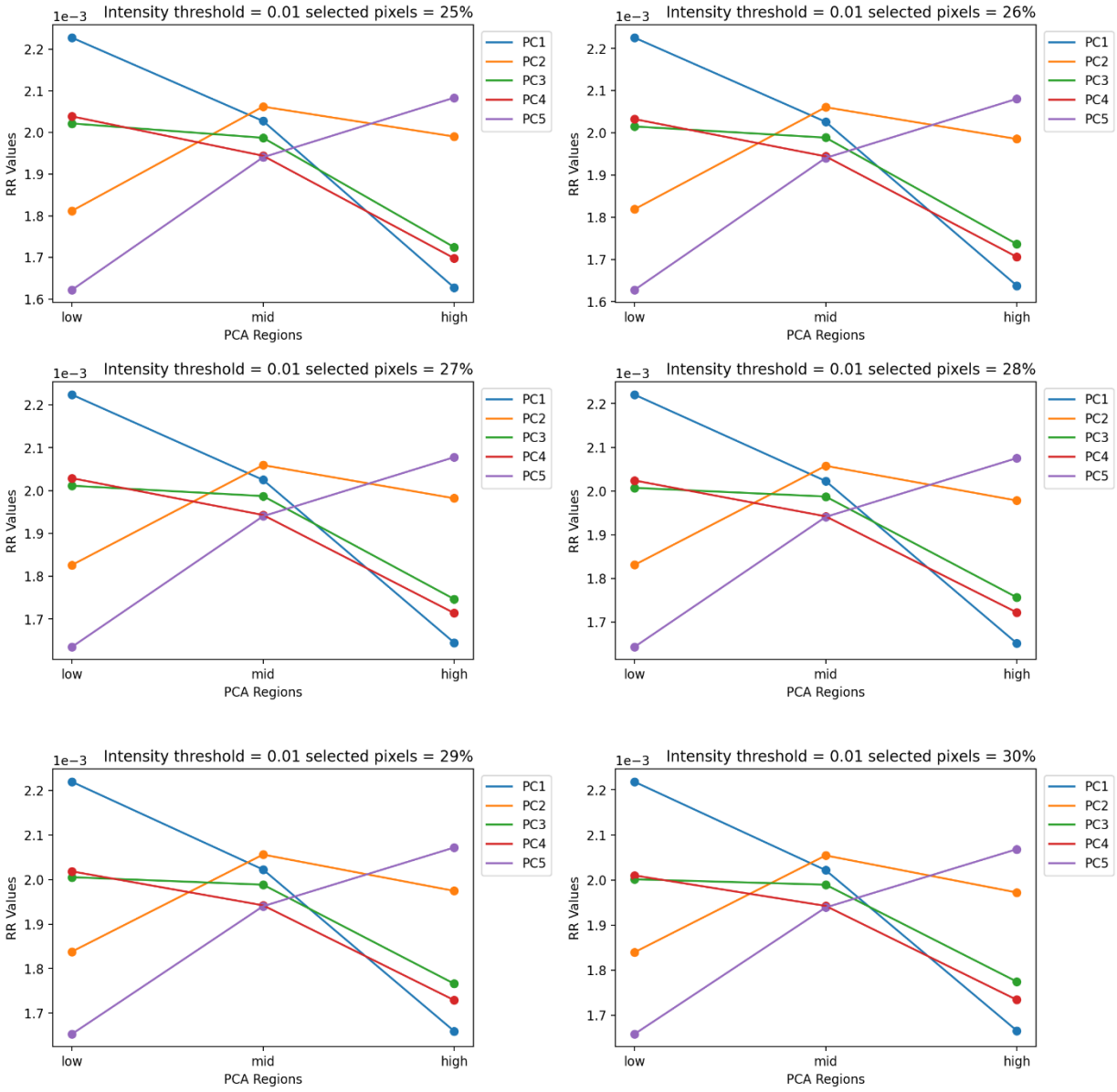
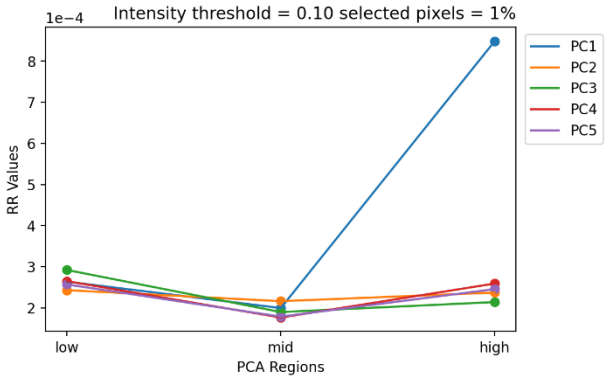
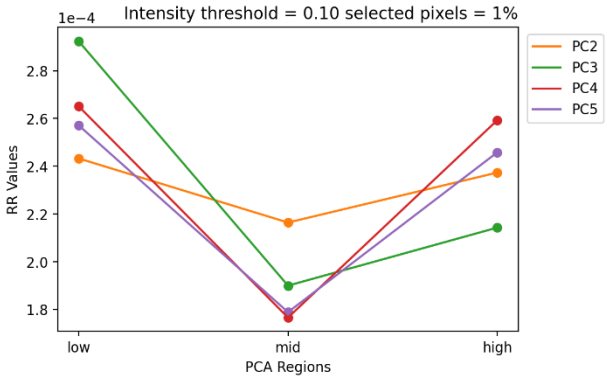
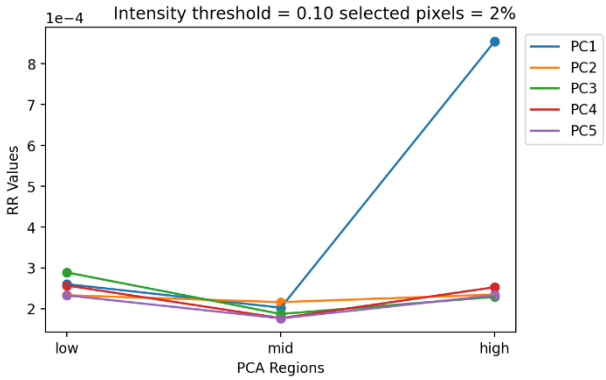
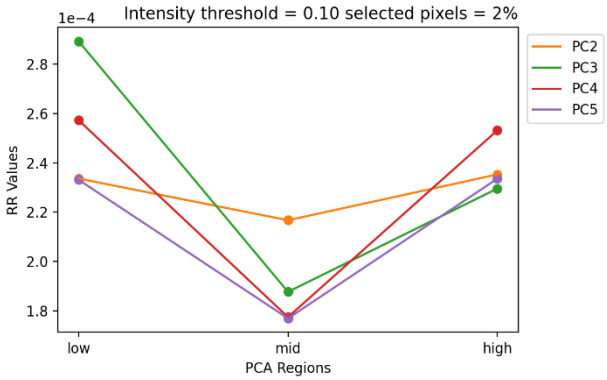


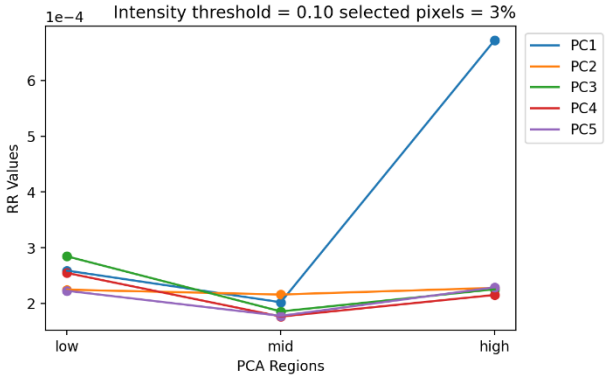
Figure S14: 2-D V plots for local normalization with non-normalized PCA results for intensity threshold 0.01.

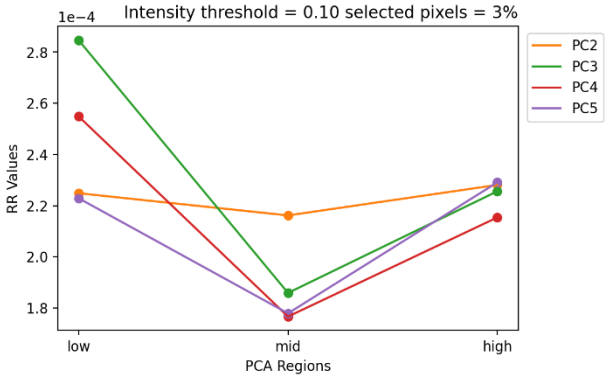

Intensity threshold = 0.10 selected pixels = 1%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

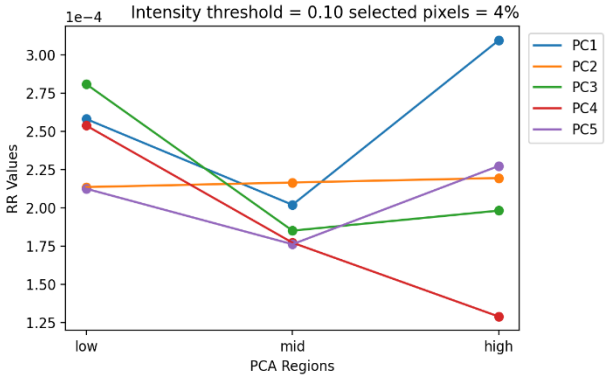

Intensity threshold = 0.10 selected pixels = 1%
1e−4
RR Values
PCA Regions
low
mid
high
PC2
PC3
PC4
PC5

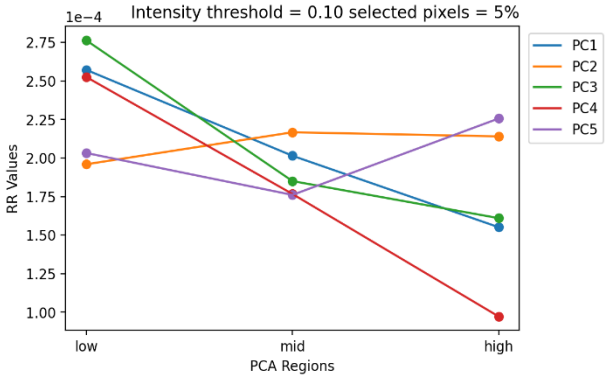

Intensity threshold = 0.10 selected pixels = 2%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

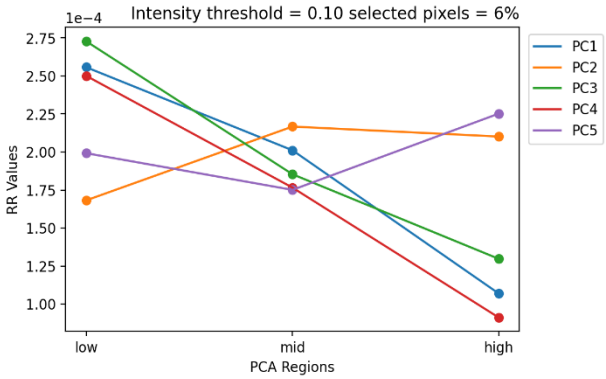

Intensity threshold = 0.10 selected pixels = 2%
1e−4
RR Values
PCA Regions
low
mid
high
PC2
PC3
PC4
PC5

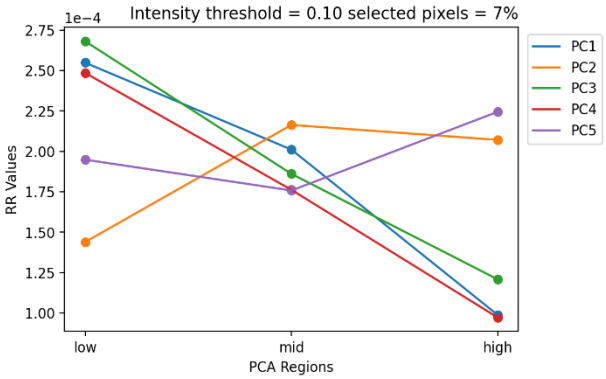

Intensity threshold = 0.10 selected pixels = 3%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

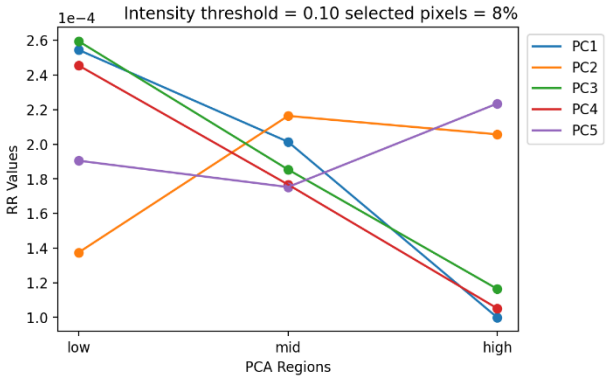

Intensity threshold = 0.10 selected pixels = 3%
1e−4
RR Values
PCA Regions
low
mid
high
PC2
PC3
PC4
PC5

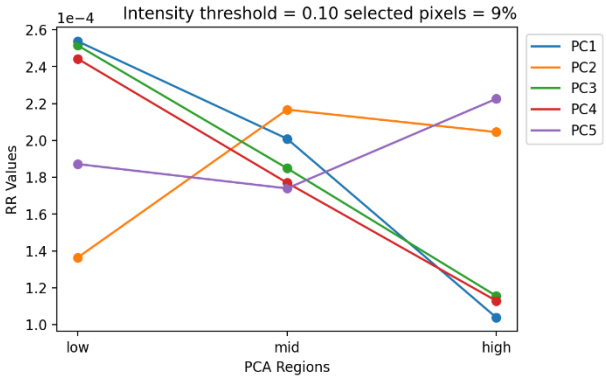

Intensity threshold = 0.10 selected pixels = 4%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

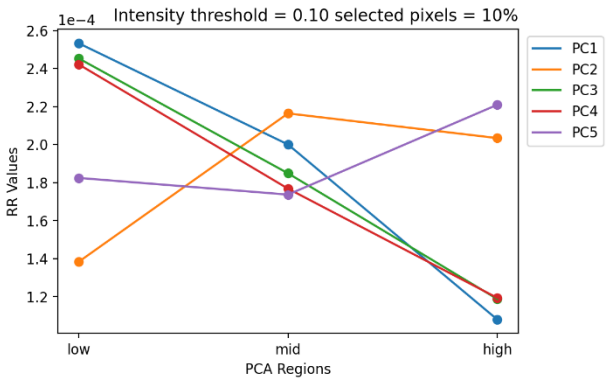

Intensity threshold = 0.10 selected pixels = 5%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

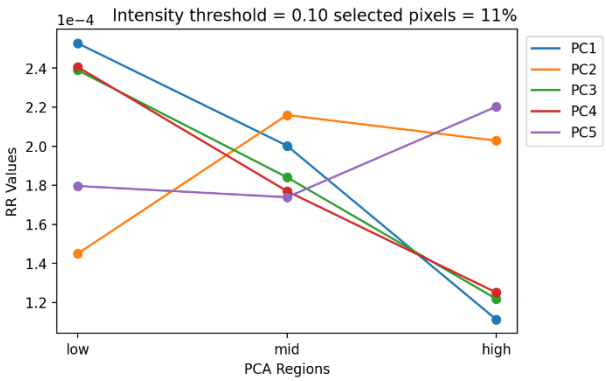

Intensity threshold = 0.10 selected pixels = 6%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

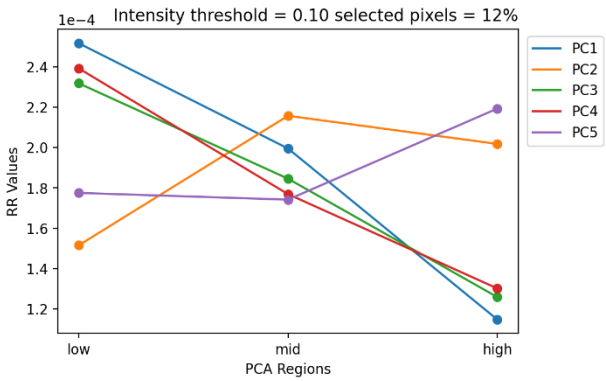

Intensity threshold = 0.10 selected pixels = 7%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

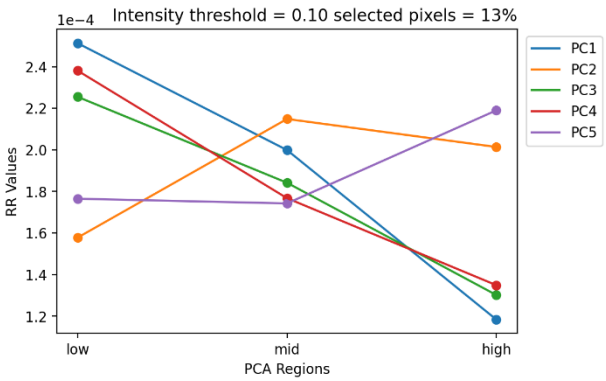

Intensity threshold = 0.10 selected pixels = 8%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

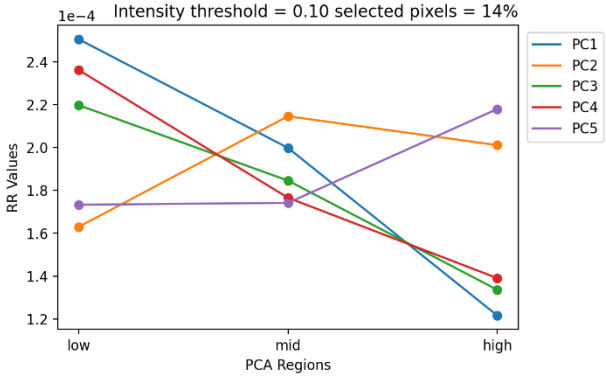

Intensity threshold = 0.10 selected pixels = 9%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

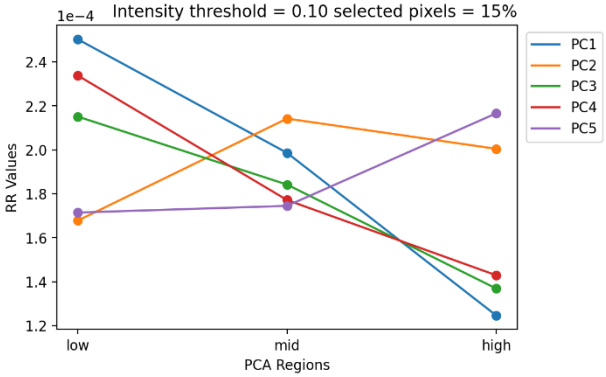

Intensity threshold = 0.10 selected pixels = 10%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

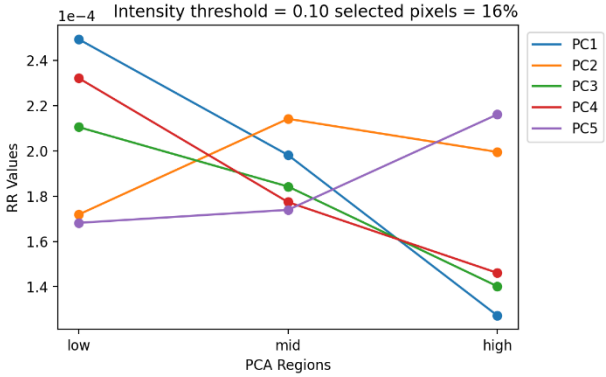

Intensity threshold = 0.10 selected pixels = 11%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

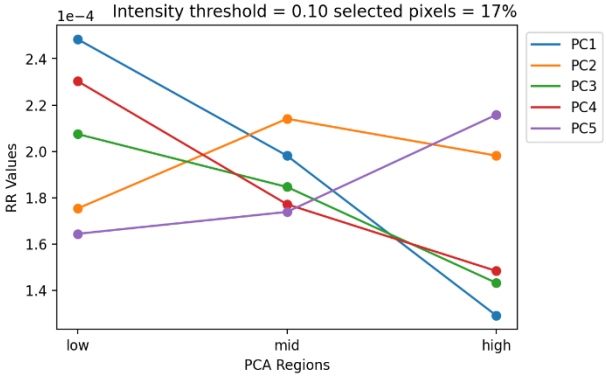

Intensity threshold = 0.10 selected pixels = 12%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

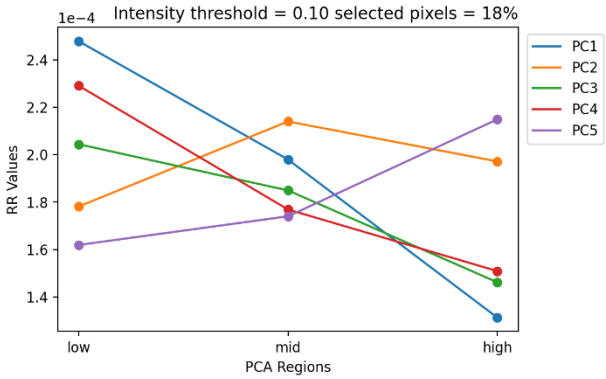

Intensity threshold = 0.10 selected pixels = 13%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

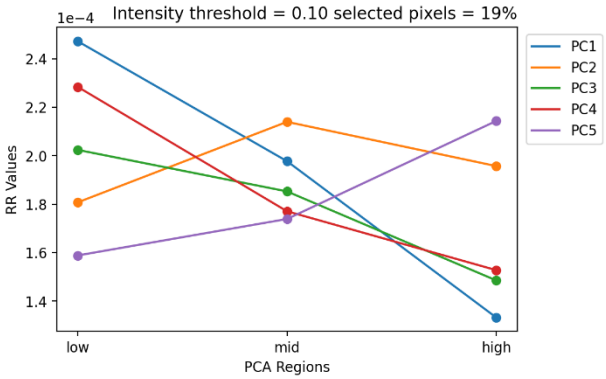

Intensity threshold = 0.10 selected pixels = 14%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

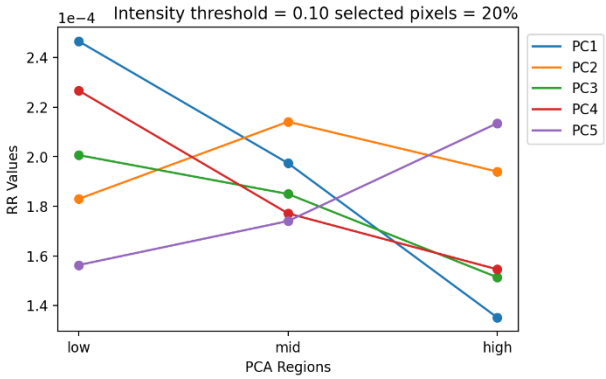

Intensity threshold = 0.10 selected pixels = 15%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

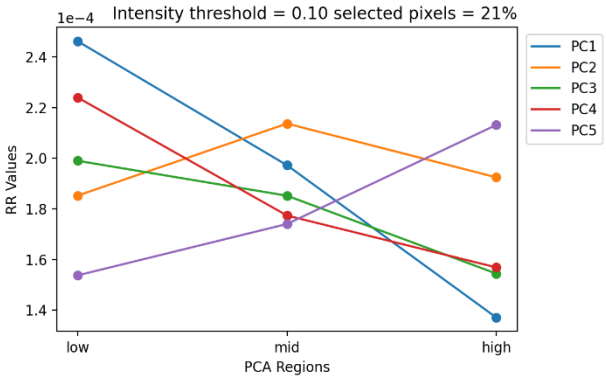

Intensity threshold = 0.10 selected pixels = 16%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

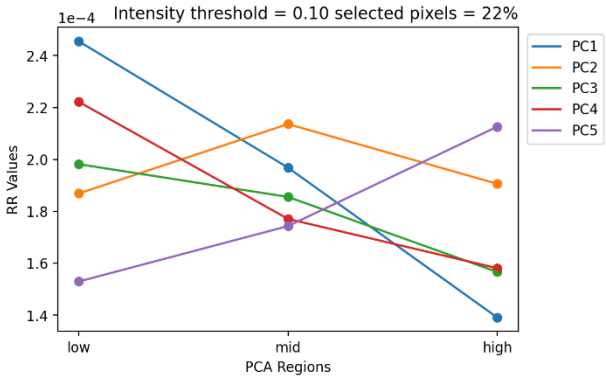

Intensity threshold = 0.10 selected pixels = 17%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

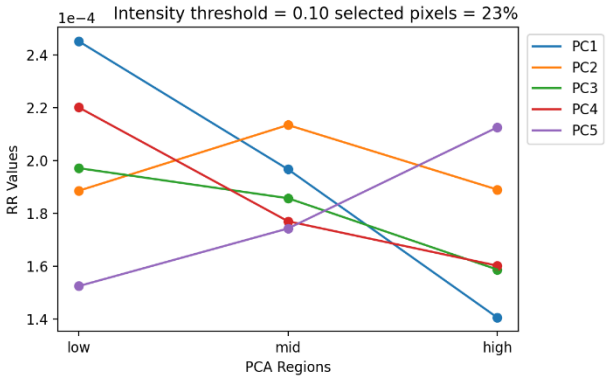

Intensity threshold = 0.10 selected pixels = 18%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

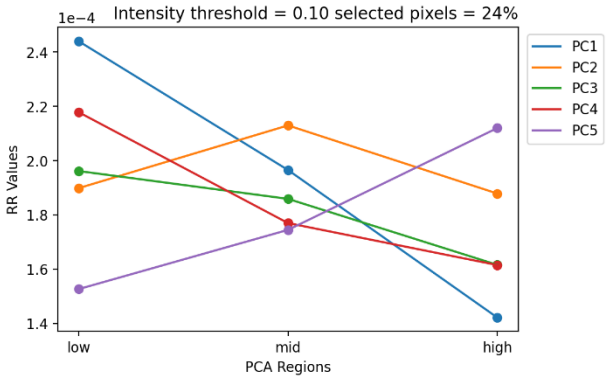

Intensity threshold = 0.10 selected pixels = 19%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

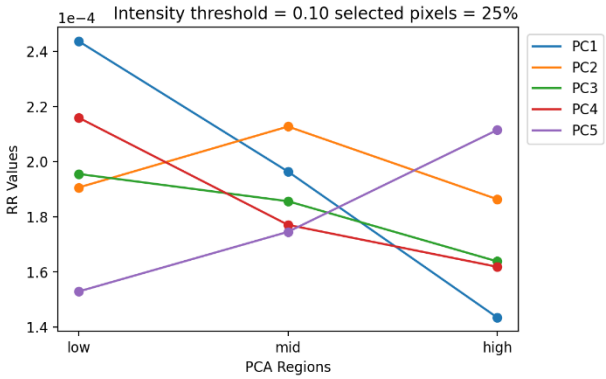

Intensity threshold = 0.10 selected pixels = 20%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

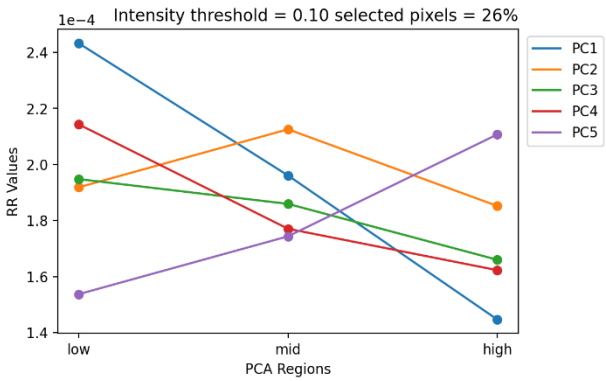

Intensity threshold = 0.10 selected pixels = 21%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

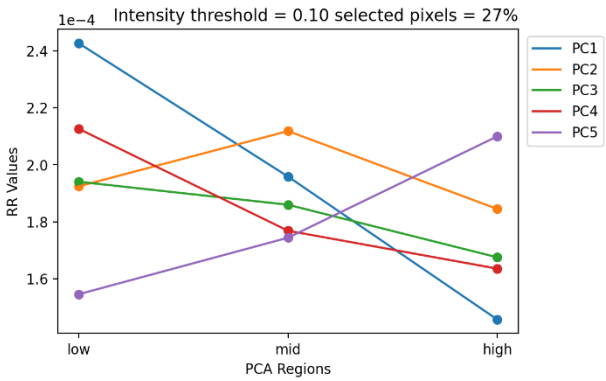

Intensity threshold = 0.10 selected pixels = 22%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

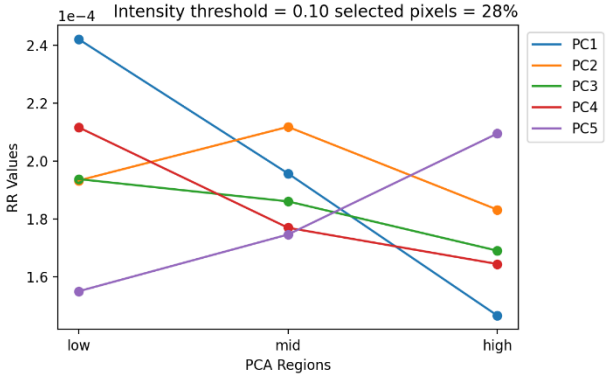

Intensity threshold = 0.10 selected pixels = 23%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

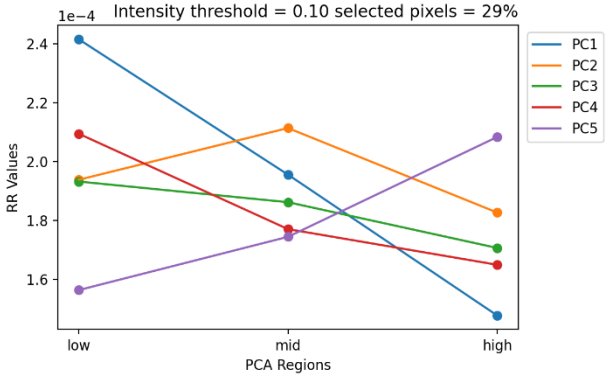

Intensity threshold = 0.10 selected pixels = 24%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 25%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 26%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 27%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 28%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.10 selected pixels = 29%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

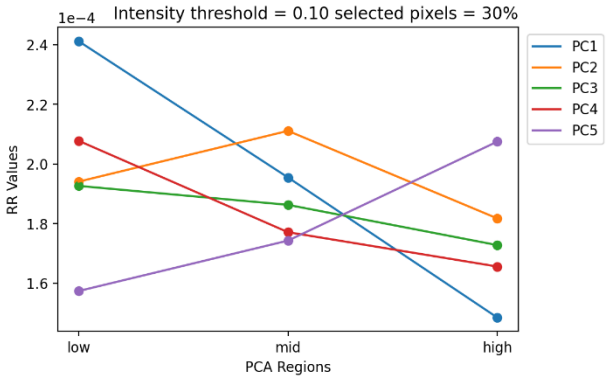
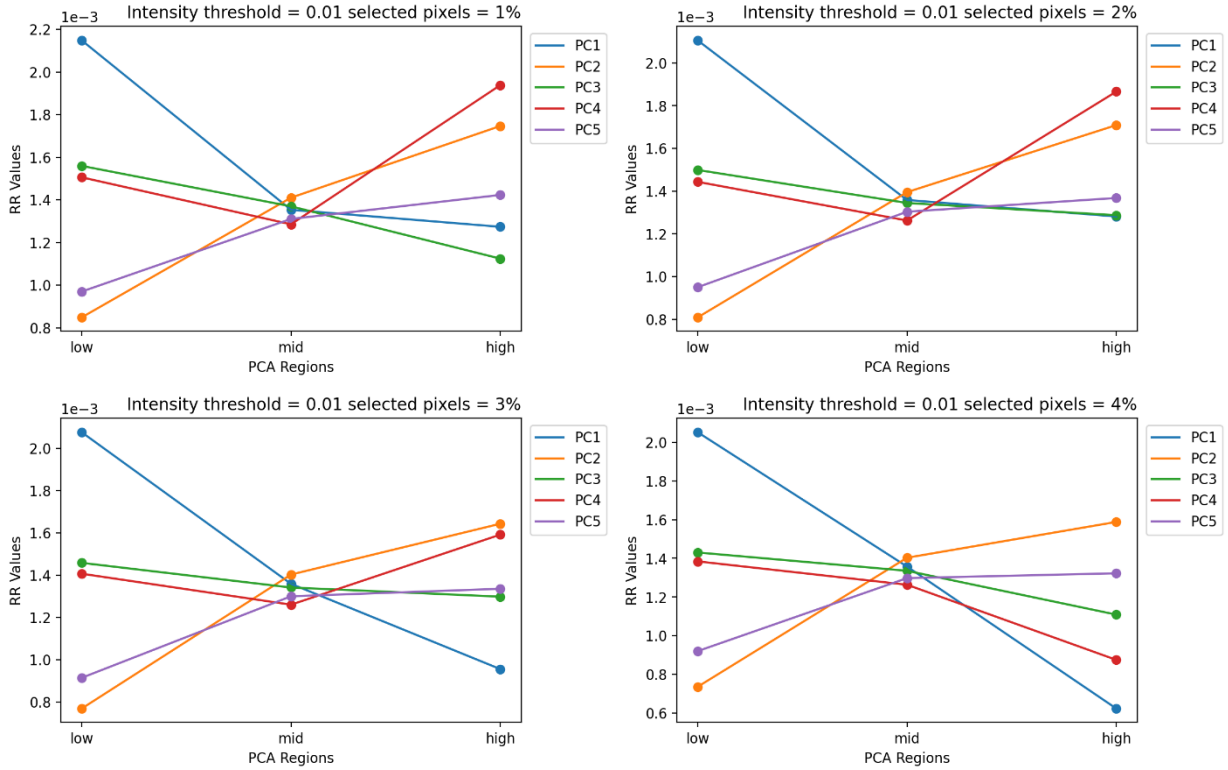
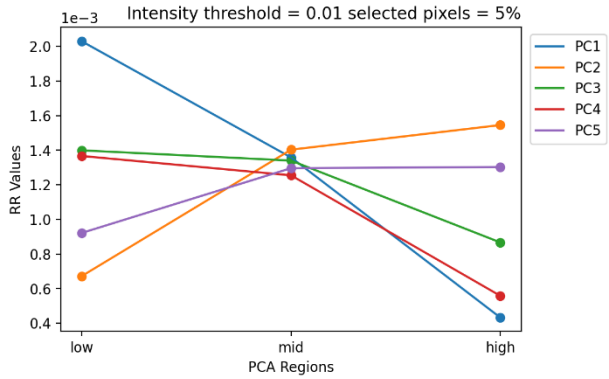


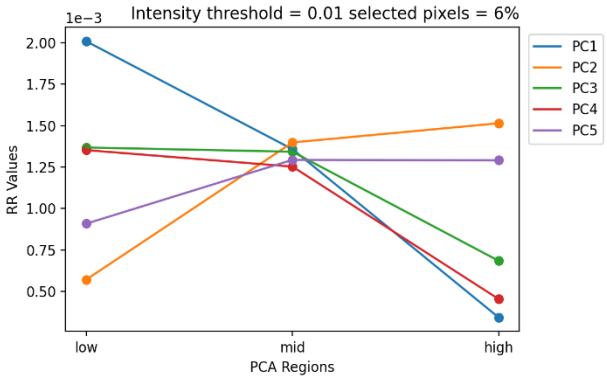
Figure S15: 2-D V plots for local normalization with non-normalized PCA results for intensity threshold 0.10.

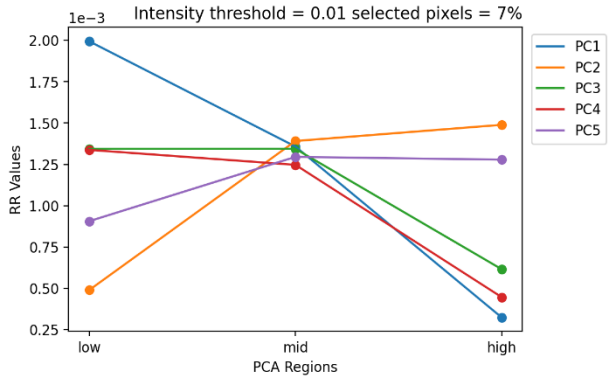
2-D V plots for global normalization with non-normalized PCA results

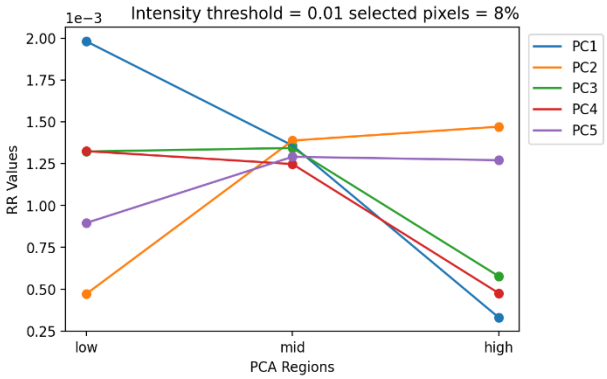
Non-normalized PCA results were used for pixel selection and no RMS pre-processing for global normalization. The method only showed one V plot for PC 4 that disappears after 3% selected pixels.

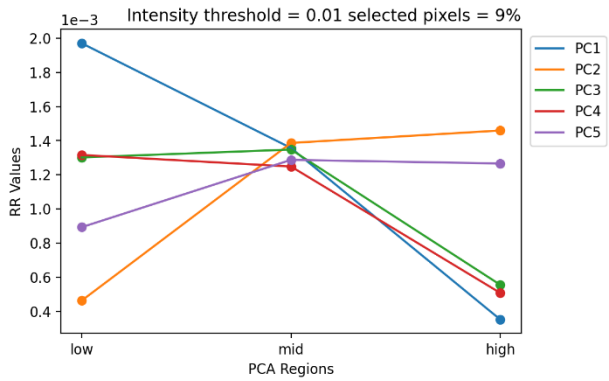


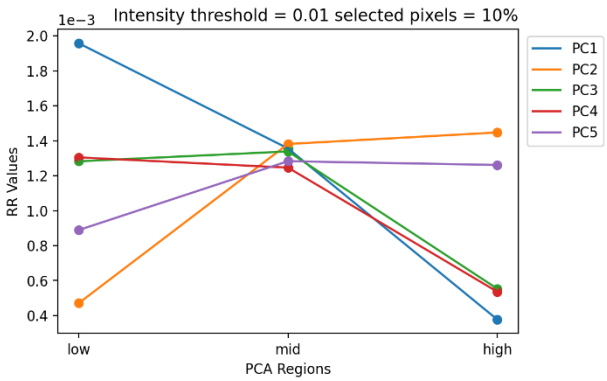

Intensity threshold = 0.01 selected pixels = 5%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

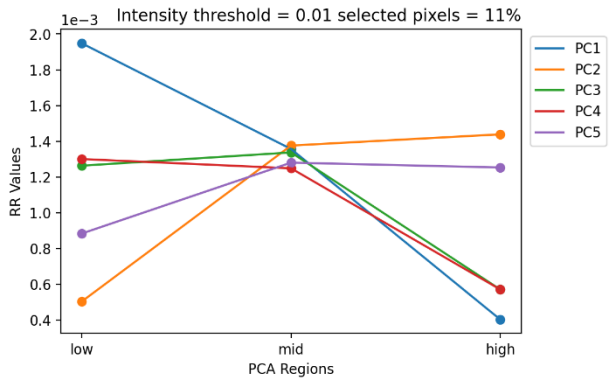

Intensity threshold = 0.01 selected pixels = 6%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

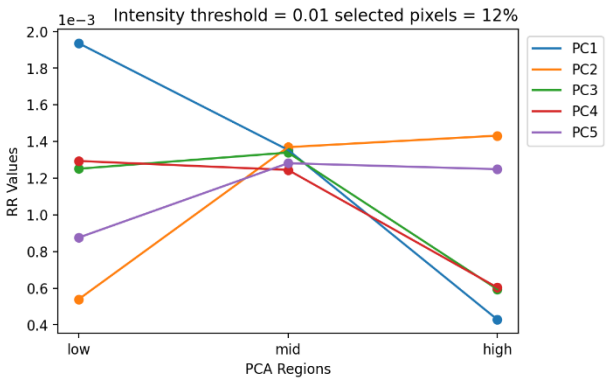

Intensity threshold = 0.01 selected pixels = 7%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

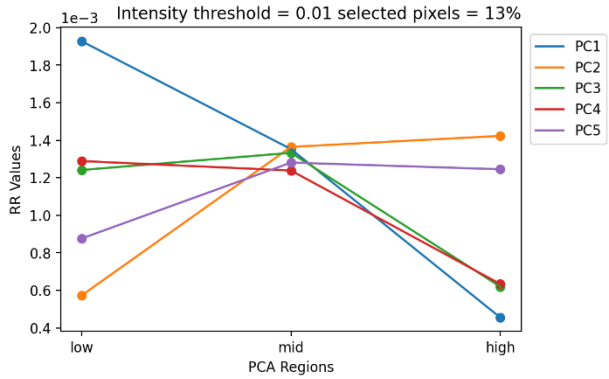

Intensity threshold = 0.01 selected pixels = 8%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

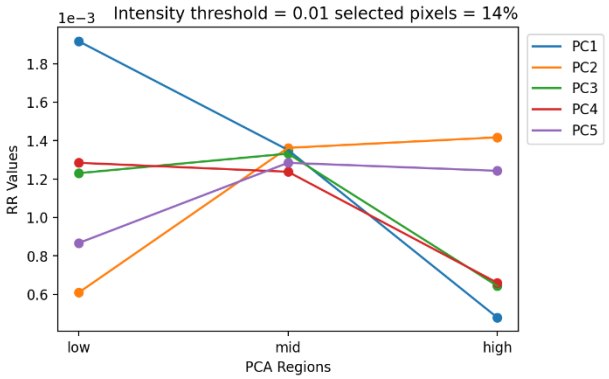

Intensity threshold = 0.01 selected pixels = 9%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

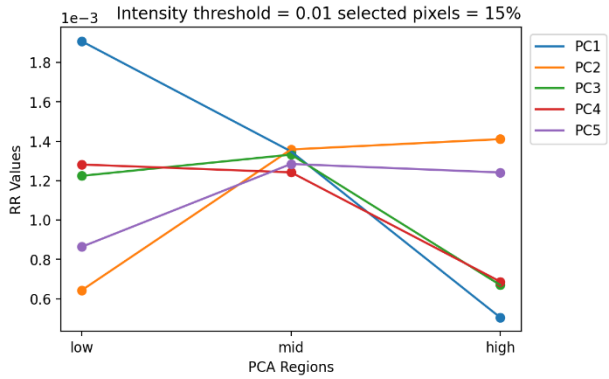

Intensity threshold = 0.01 selected pixels = 10%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

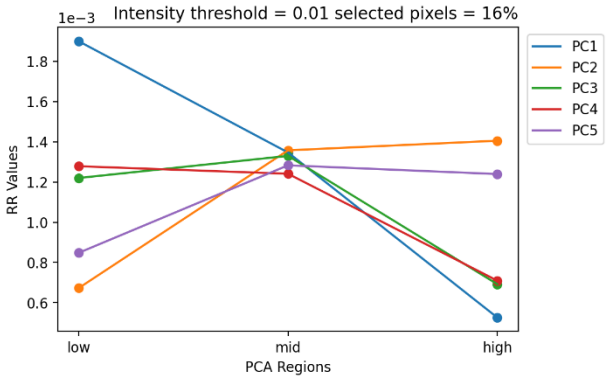

Intensity threshold = 0.01 selected pixels = 11%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

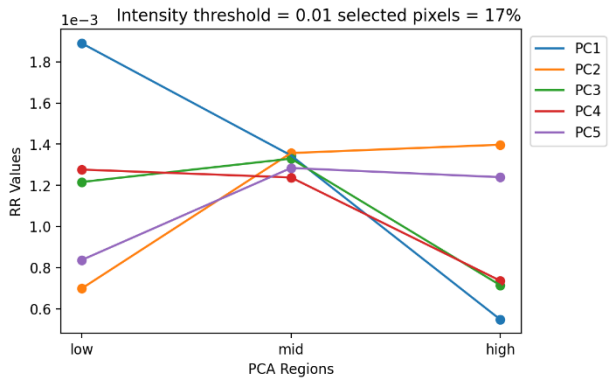

Intensity threshold = 0.01 selected pixels = 12%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

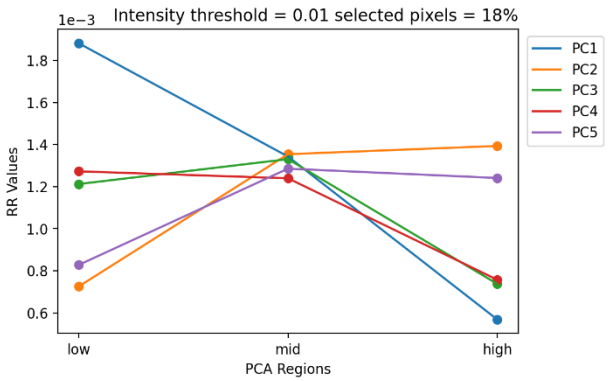

Intensity threshold = 0.01 selected pixels = 13%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

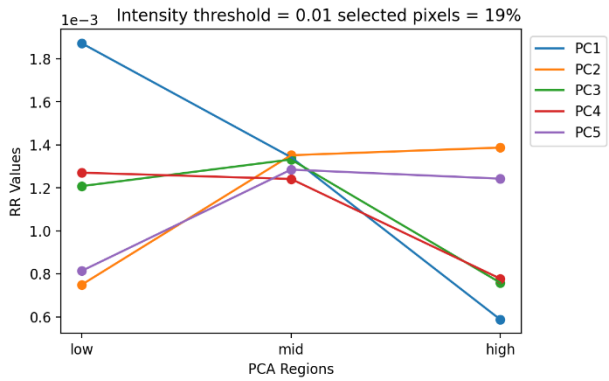

Intensity threshold = 0.01 selected pixels = 14%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

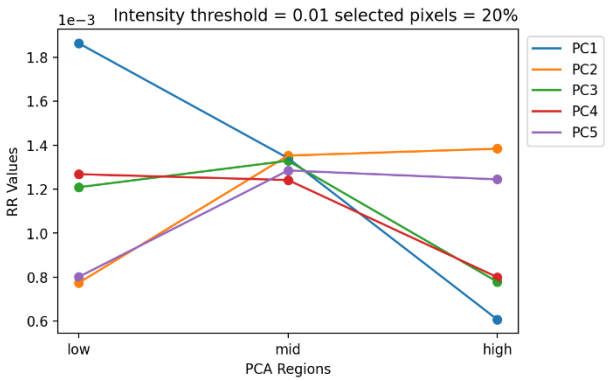

Intensity threshold = 0.01 selected pixels = 15%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

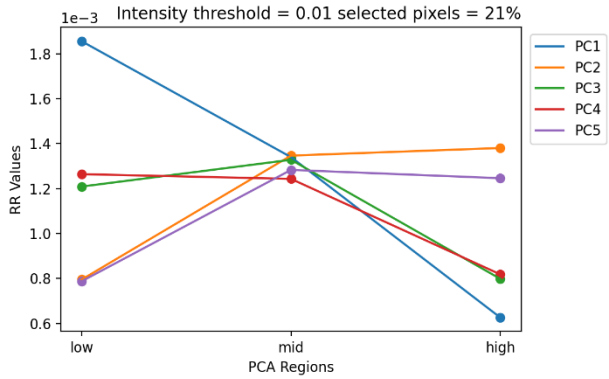

Intensity threshold = 0.01 selected pixels = 16%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

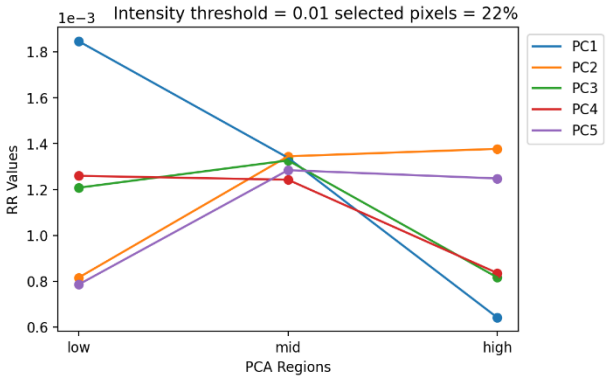

Intensity threshold = 0.01 selected pixels = 17%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

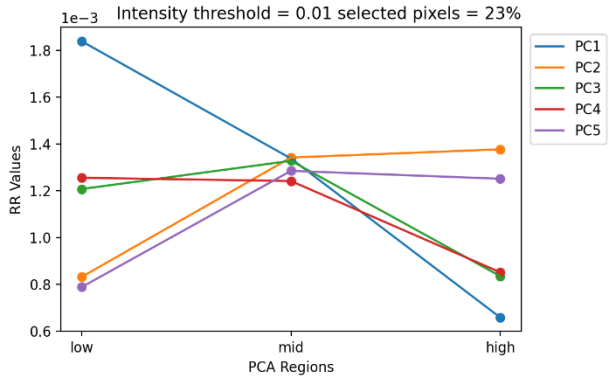

Intensity threshold = 0.01 selected pixels = 18%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

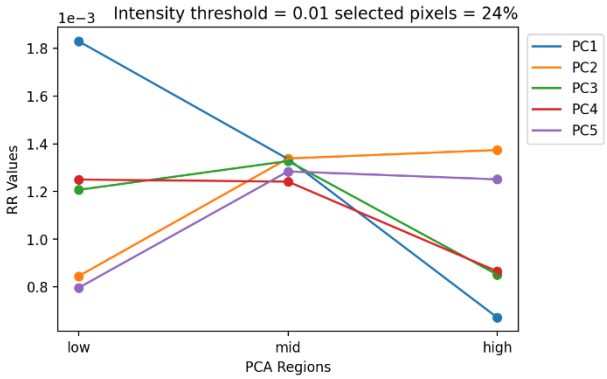

Intensity threshold = 0.01 selected pixels = 19%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

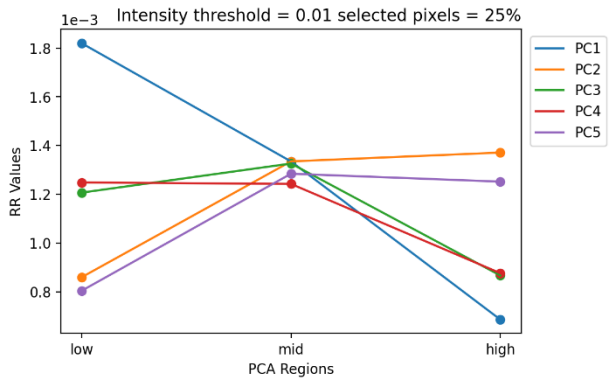

Intensity threshold = 0.01 selected pixels = 20%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

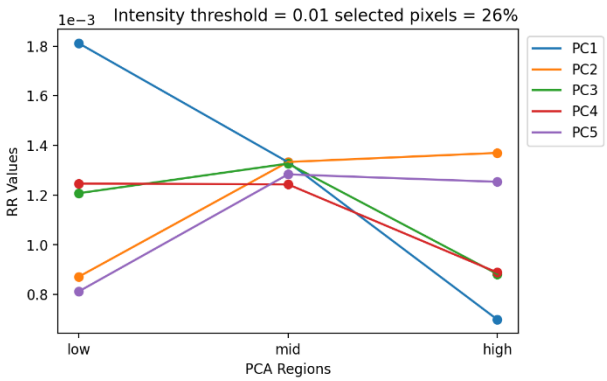

Intensity threshold = 0.01 selected pixels = 21%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 22%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 23%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 24%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 25%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 26%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

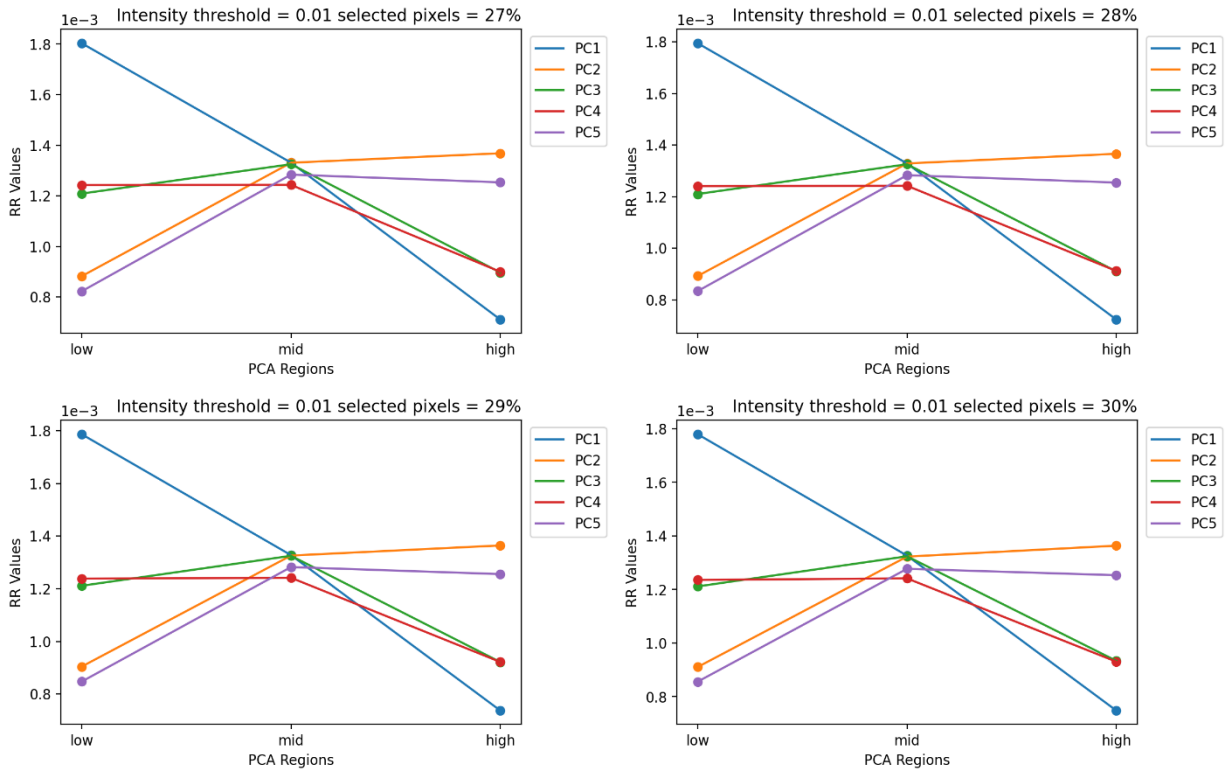
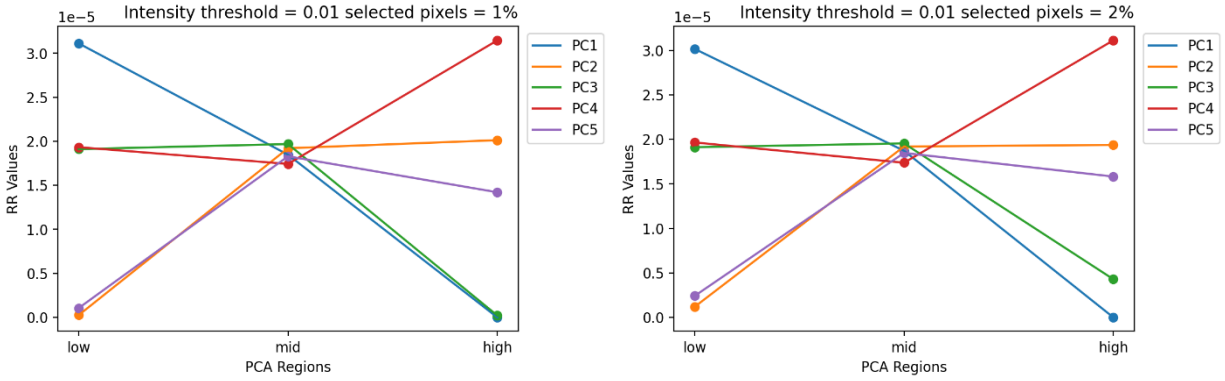
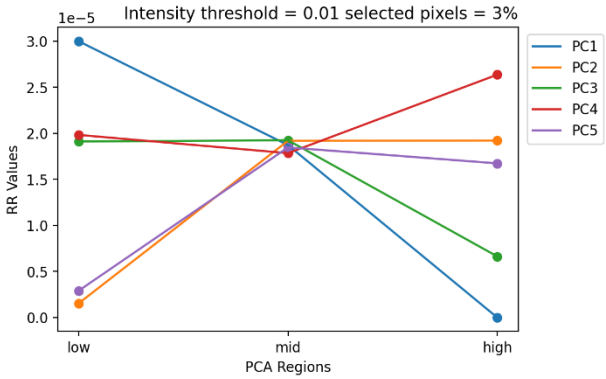


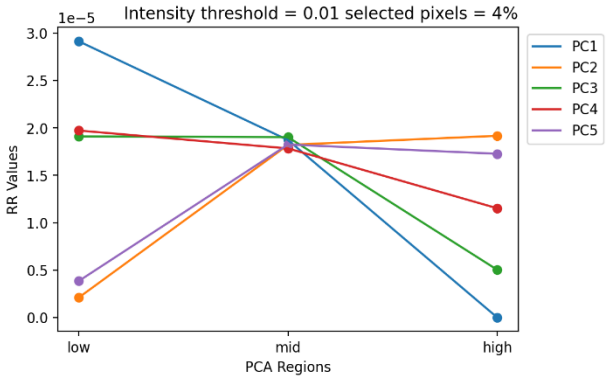
Figure S16: 2-D V plots for global normalization with non-normalized PCA results for intensity threshold 0.01.

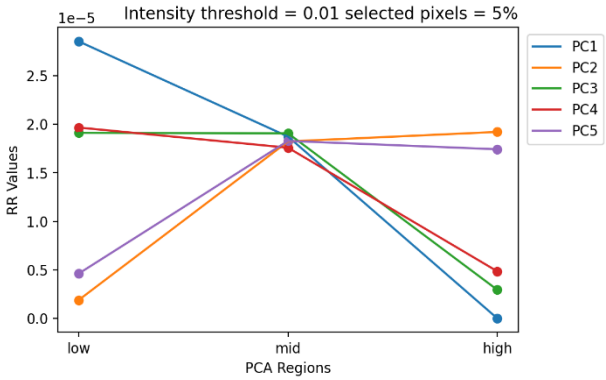
2-D V plots for localTIC normalization with non-normalized PCA results

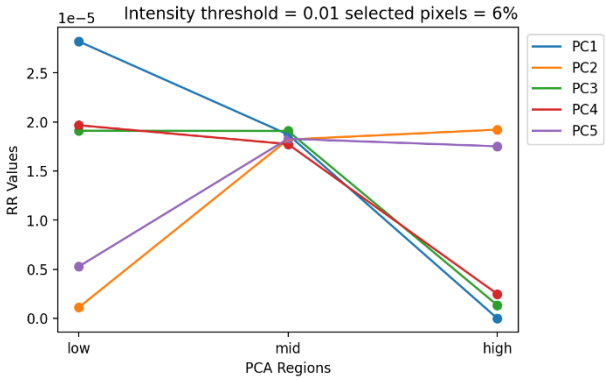
Non-normalized PCA results were used for pixel selection and no RMS pre-processing for global normalization. The method only showed one V plot for PC 4 that disappears after 3% selected pixels. This shows how even though PCA could find biological regions, the workflow can appear to fail simply due to a bad normalization method.

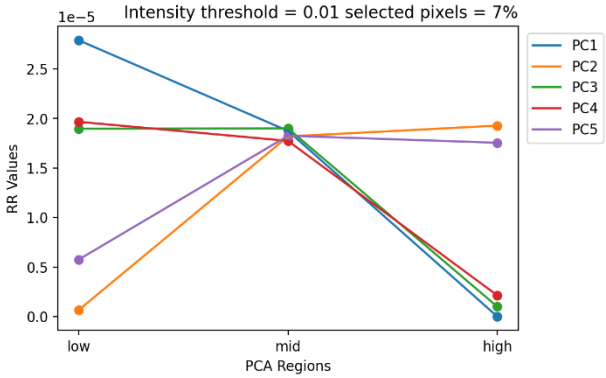


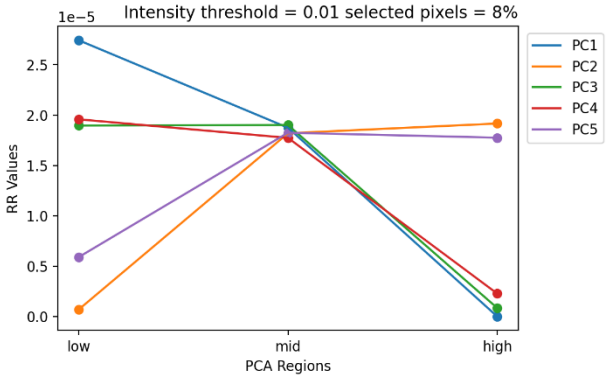

Intensity threshold = 0.01 selected pixels = 3%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

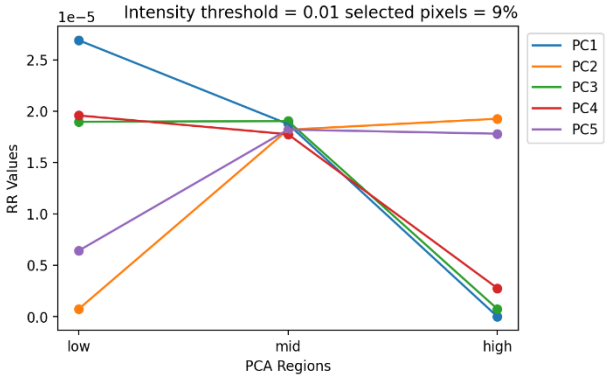

Intensity threshold = 0.01 selected pixels = 4%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

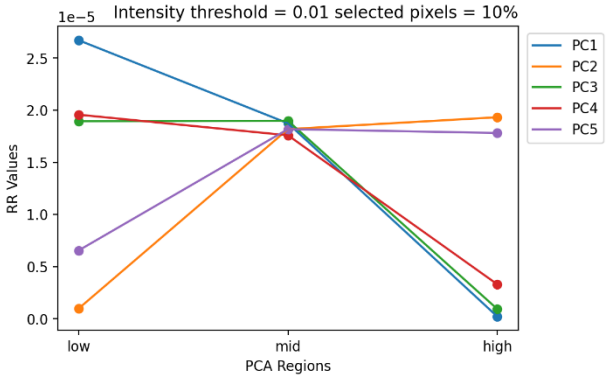

Intensity threshold = 0.01 selected pixels = 5%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

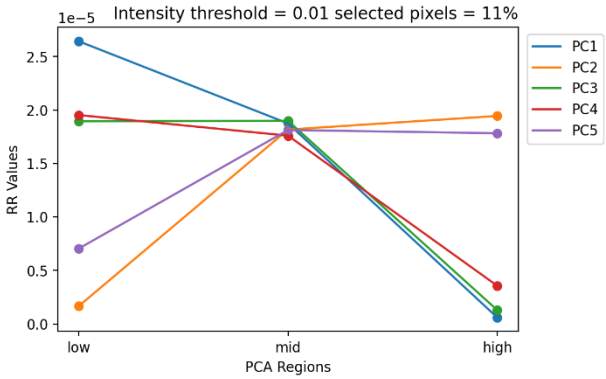

Intensity threshold = 0.01 selected pixels = 6%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

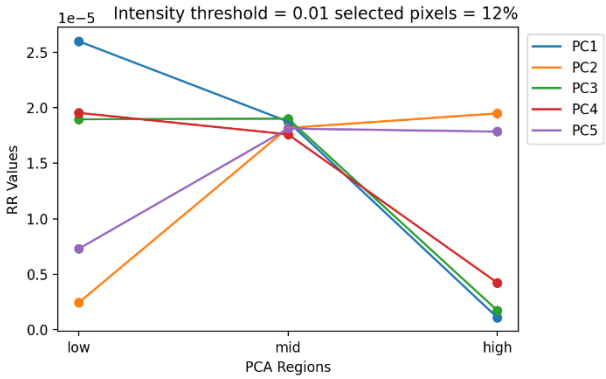

Intensity threshold = 0.01 selected pixels = 7%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

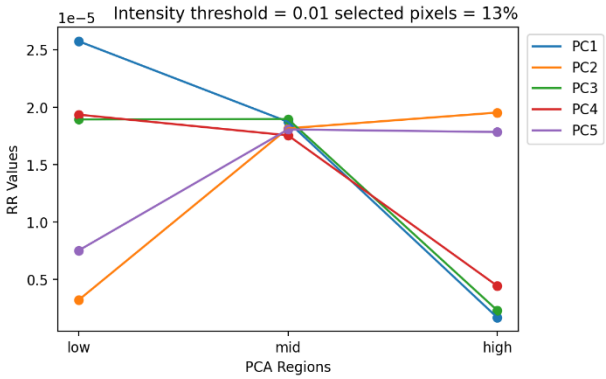

Intensity threshold = 0.01 selected pixels = 8%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

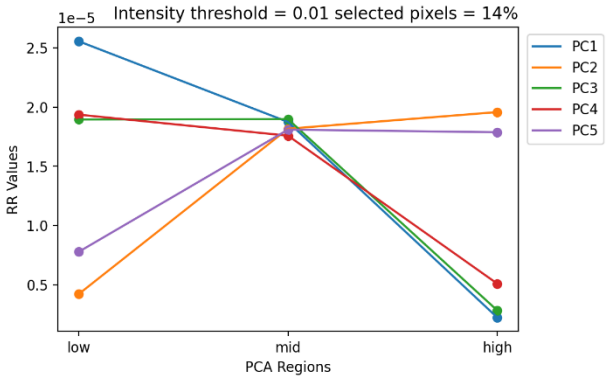

Intensity threshold = 0.01 selected pixels = 9%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

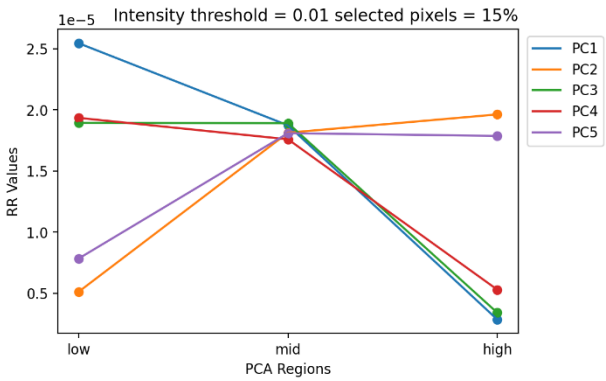

Intensity threshold = 0.01 selected pixels = 10%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

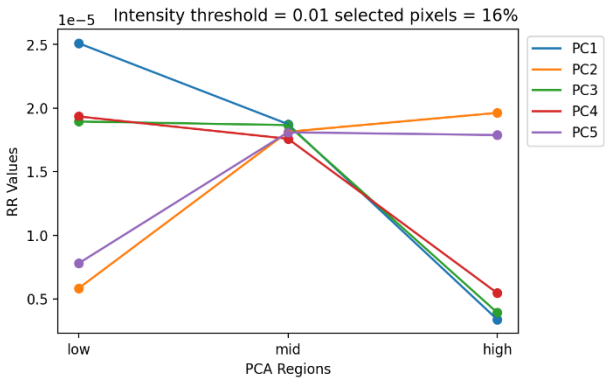

Intensity threshold = 0.01 selected pixels = 11%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

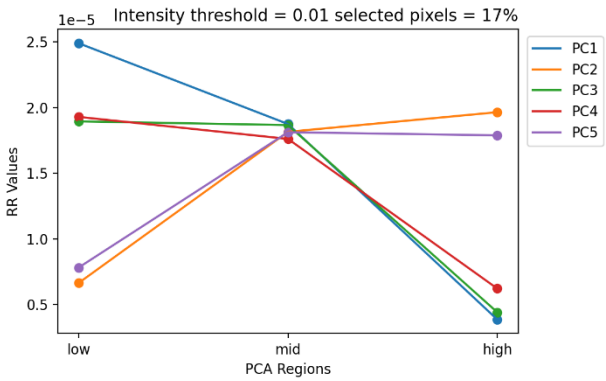

Intensity threshold = 0.01 selected pixels = 12%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

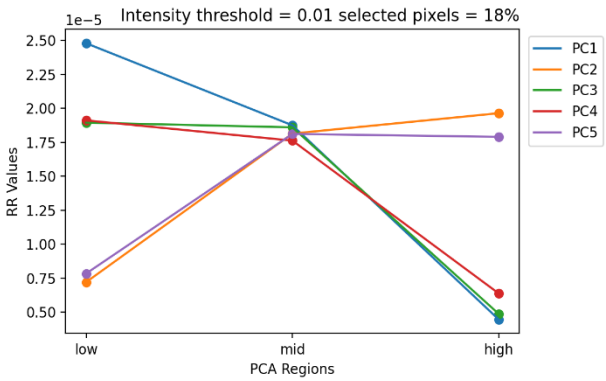

Intensity threshold = 0.01 selected pixels = 13%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

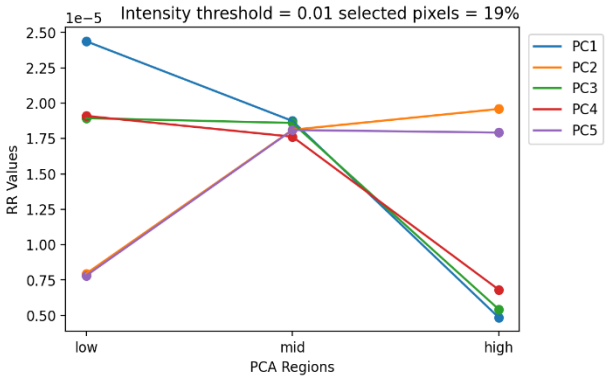

Intensity threshold = 0.01 selected pixels = 14%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

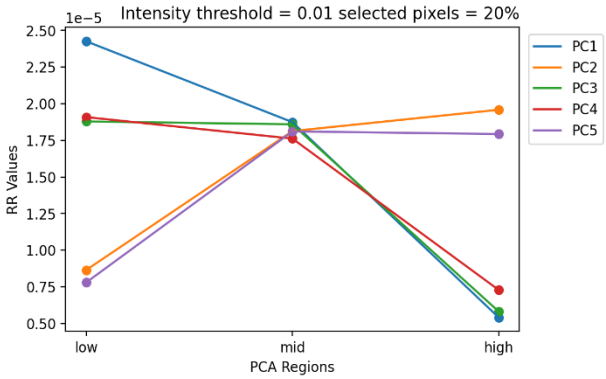

Intensity threshold = 0.01 selected pixels = 15%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

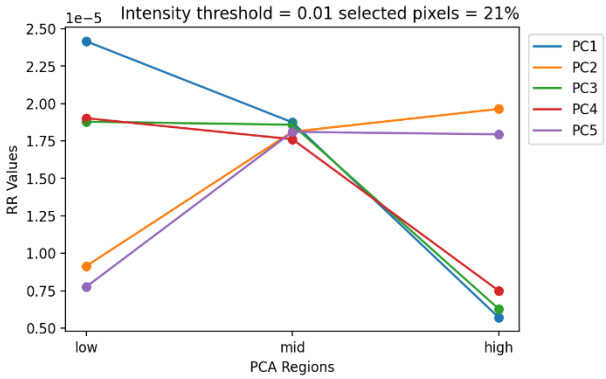

Intensity threshold = 0.01 selected pixels = 16%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

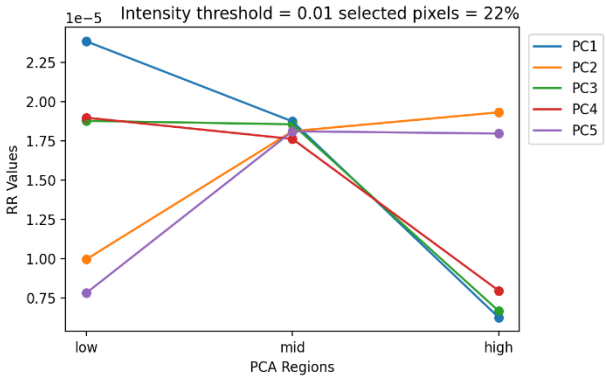

Intensity threshold = 0.01 selected pixels = 17%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

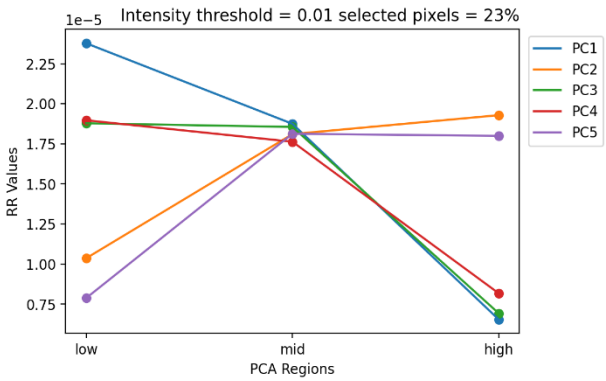

Intensity threshold = 0.01 selected pixels = 18%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

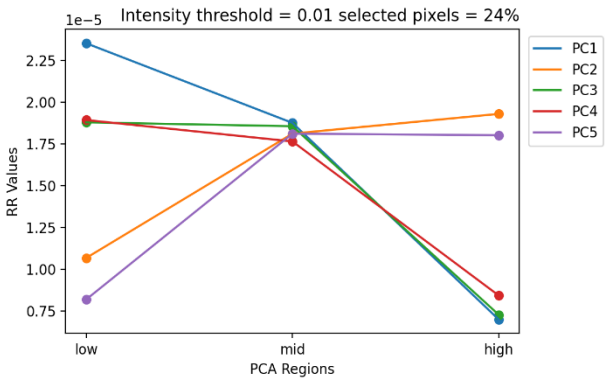

Intensity threshold = 0.01 selected pixels = 19%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

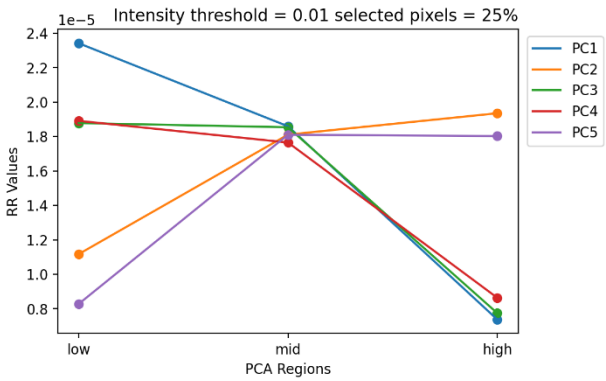

Intensity threshold = 0.01 selected pixels = 20%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

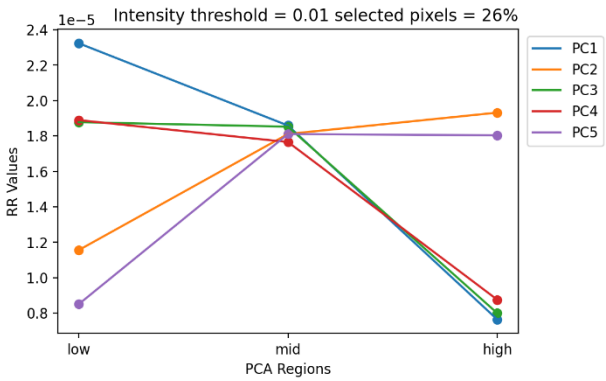

Intensity threshold = 0.01 selected pixels = 21%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 22%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 23%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 24%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 25%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 26%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

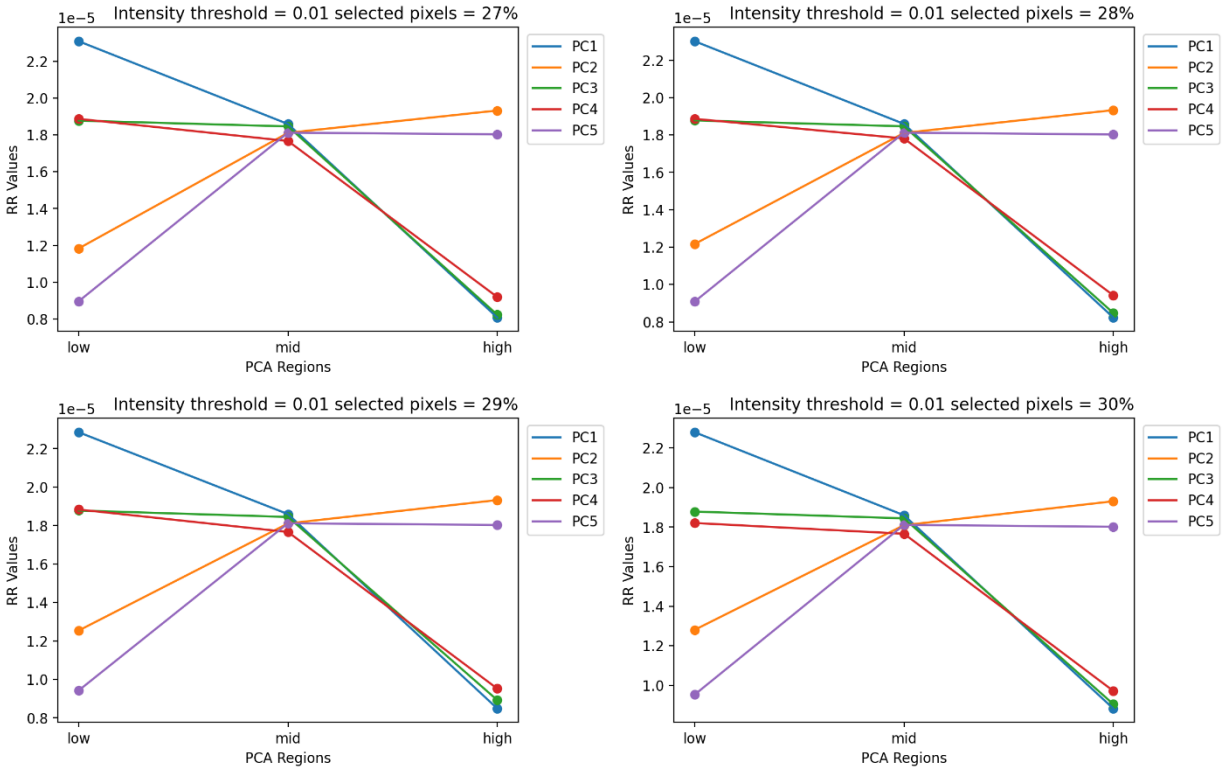


Figure S17: 2-D V plots for localTIC normalization with non-normalized PCA results for intensity threshold 0.01.

Russel-Rao 3-D V Plots

Optimal set of parameters based on the E-index calculations. A-E correspond to PC 1-5, respectively. All PCs follow the expected trend of decreasing similarity as the coincidence threshold increases. Intensity threshold was set to 0.1 and the percent selected pixels was 1%.

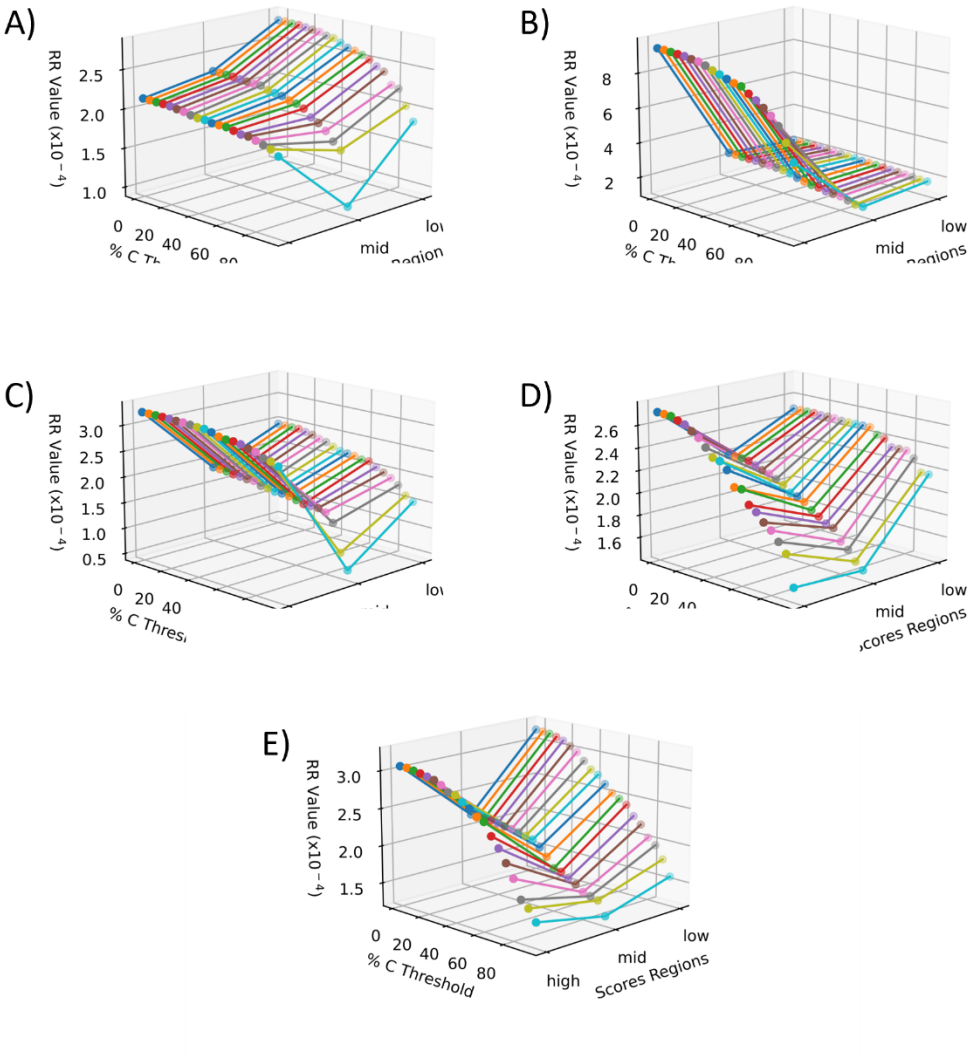
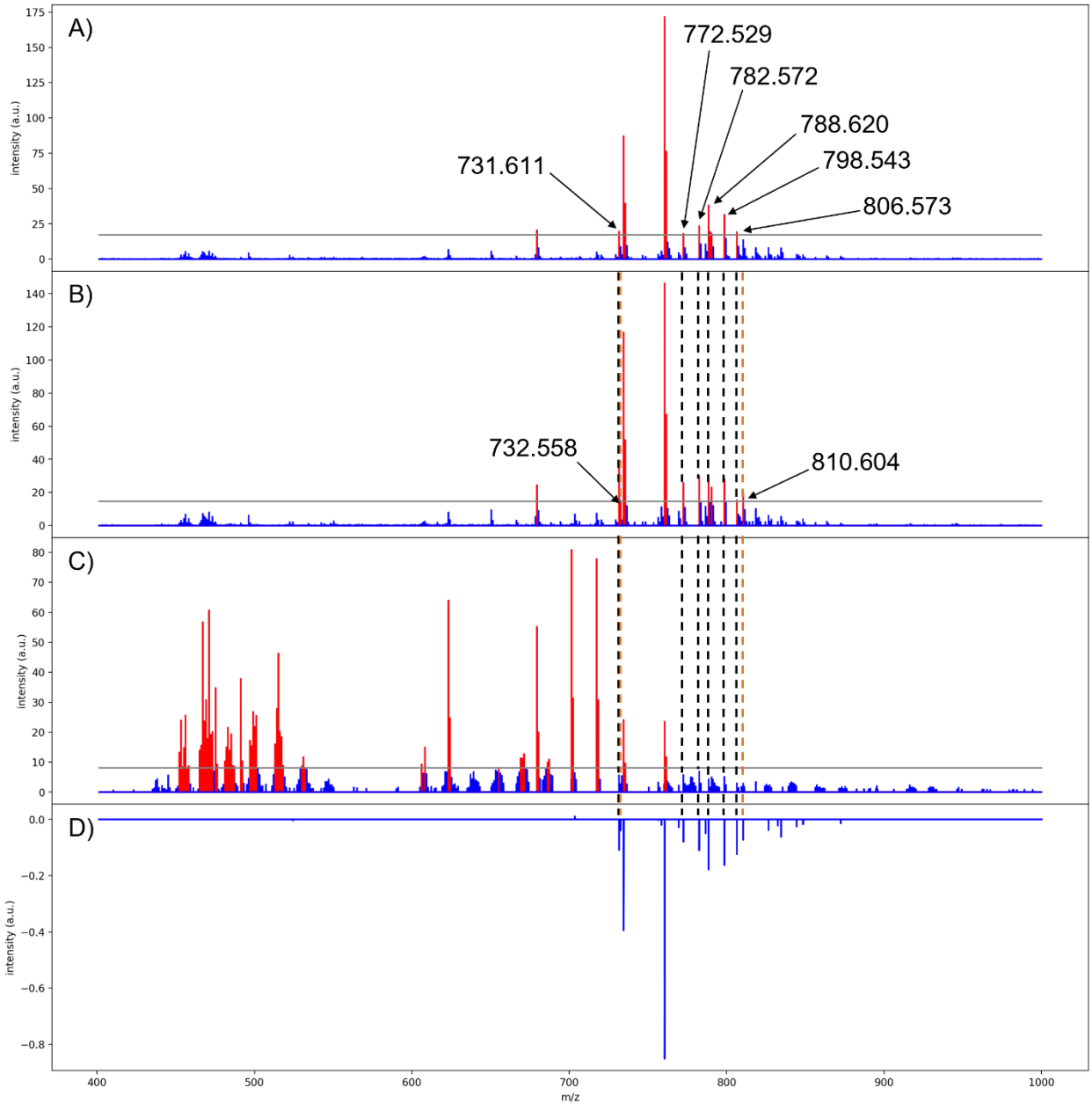


Figure S18: 3-D V plots for local normalization with RMS normalized PCA results for 0.10 of intensity threshold and 1% of selected pixels.

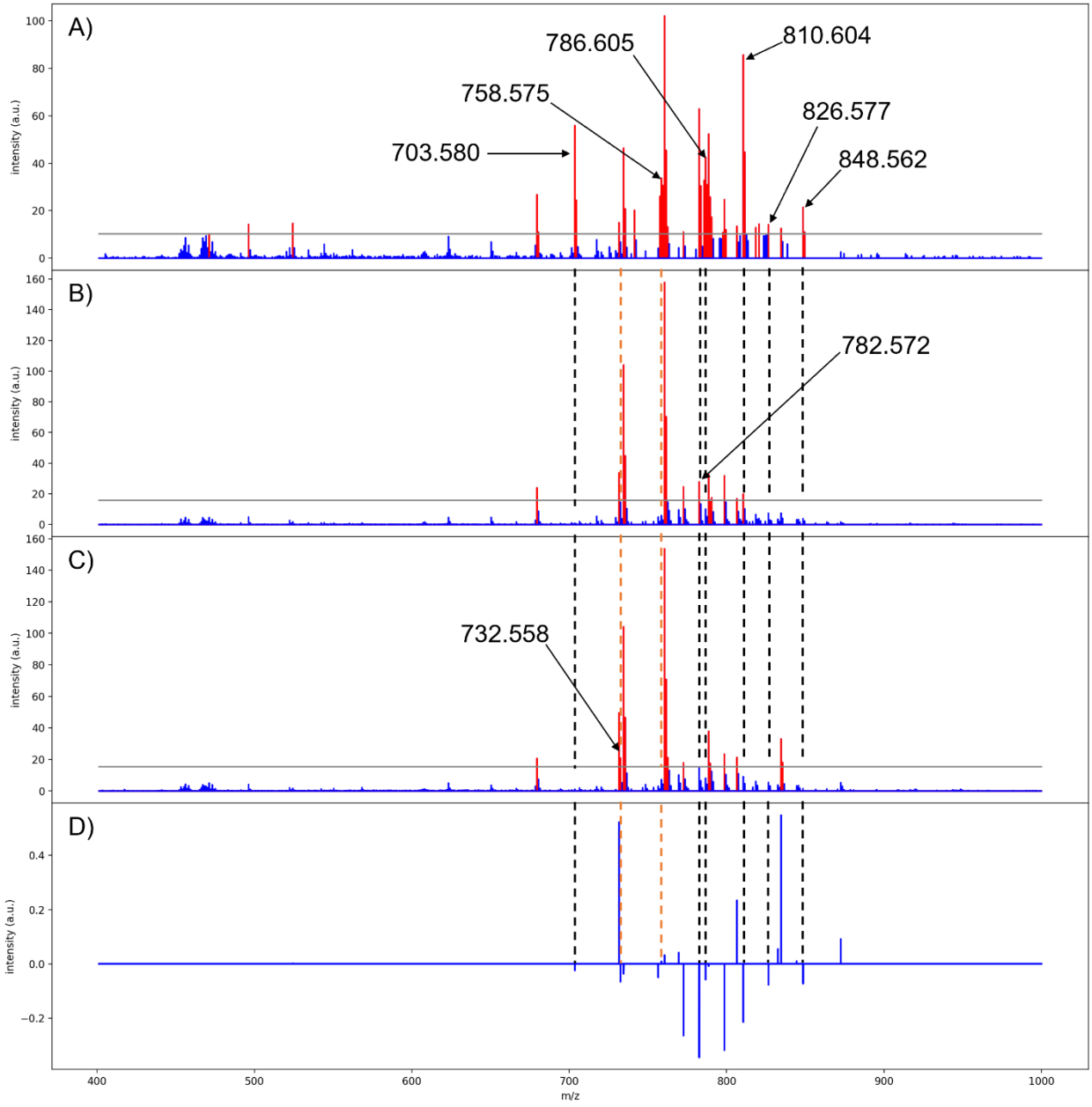
Medoid Spectra

Local normalization with 1% selected pixels, Intensity threshold 0.10, and RMS normalizes PCA results

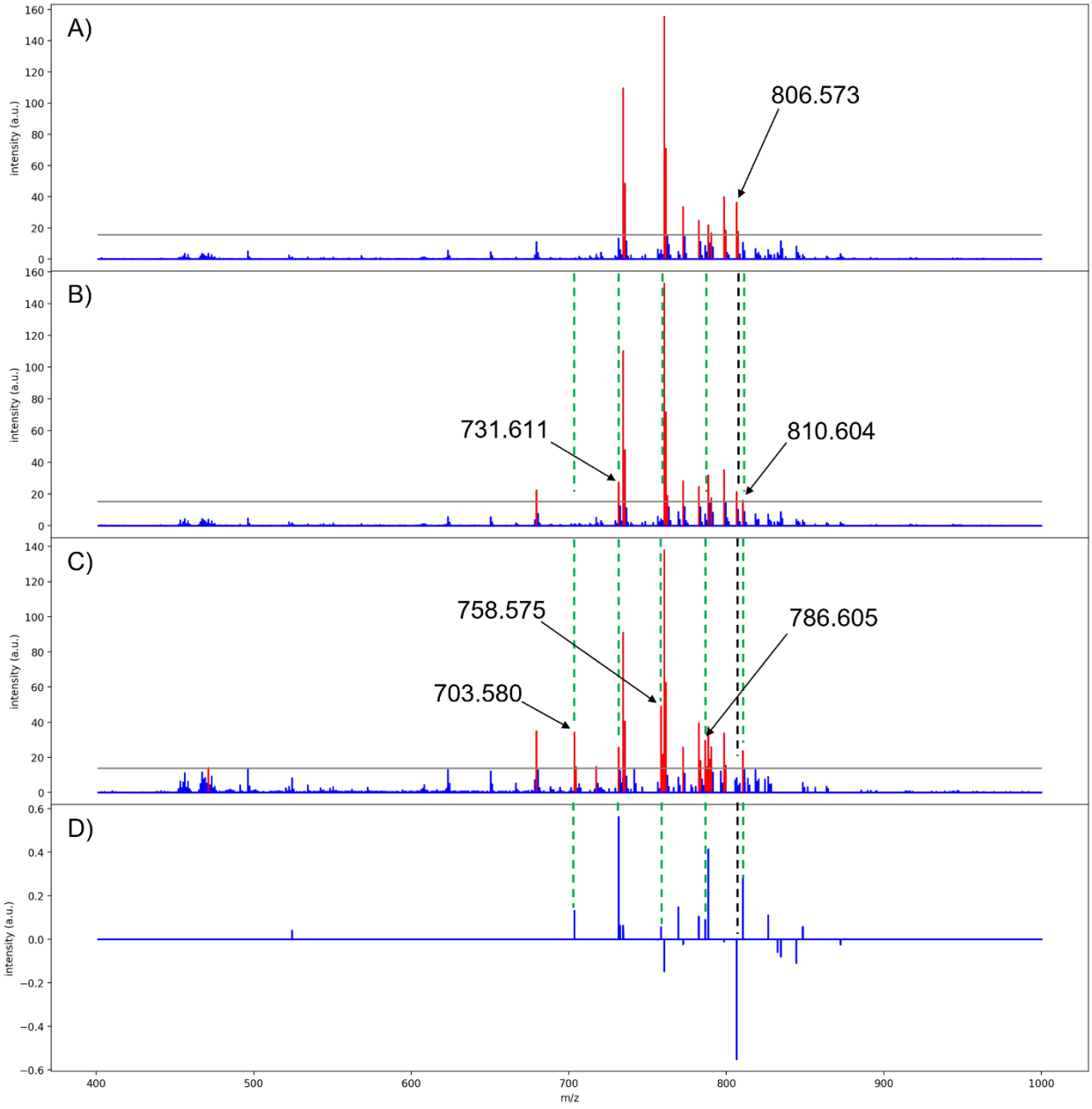
PC 2



PC 3


A)
786.605
810.604
758.575
826.577
703.580
848.562
B)
782.572
C)
732.558
D)
intensity (a.u.)
m/z
400
500
600
700
800
900
1000

PC 4


A)
806.573
B)
731.611
810.604
C)
758.575
786.605
703.580
D)
intensity (a.u.)
m/z

PC 5

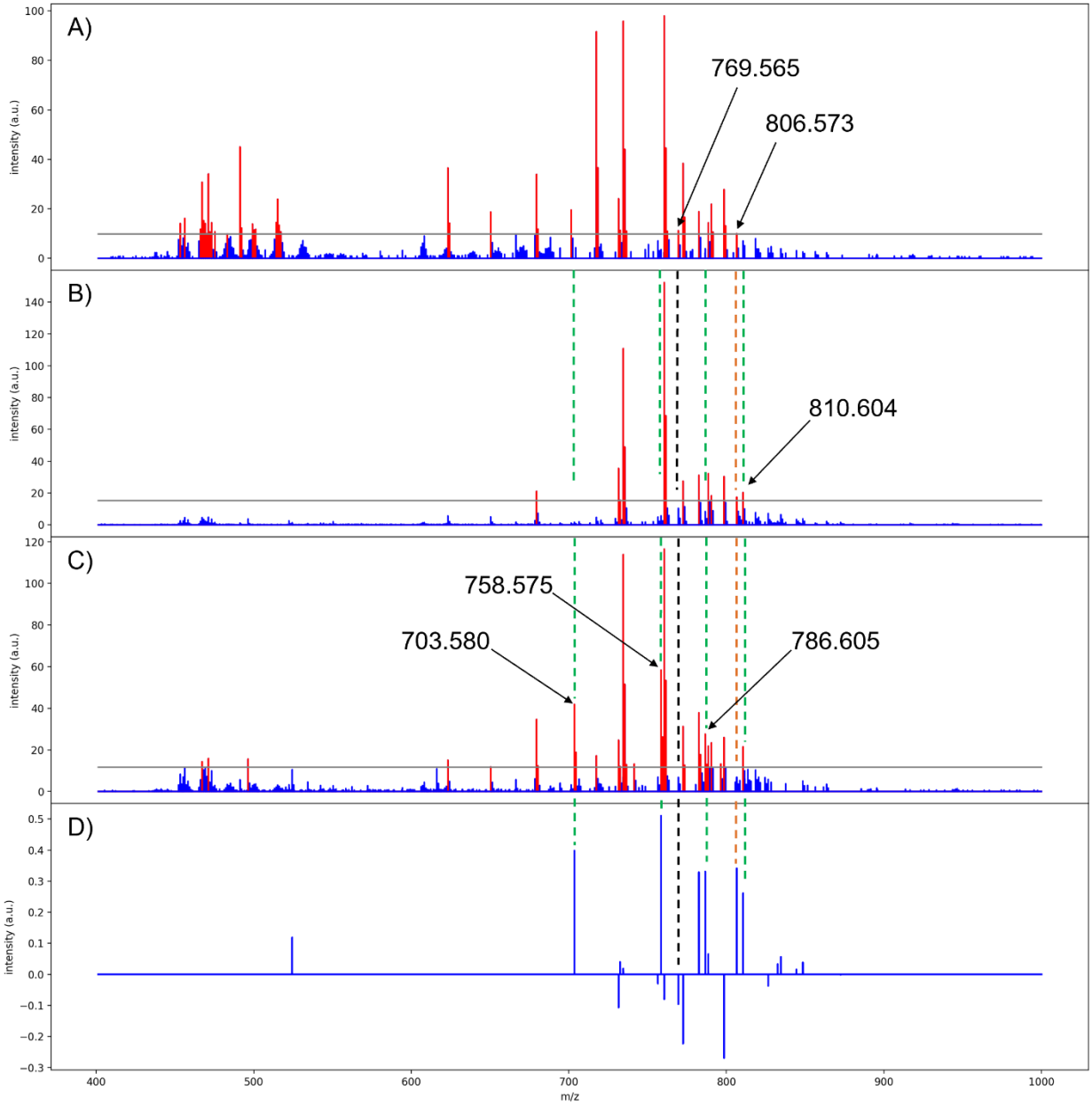
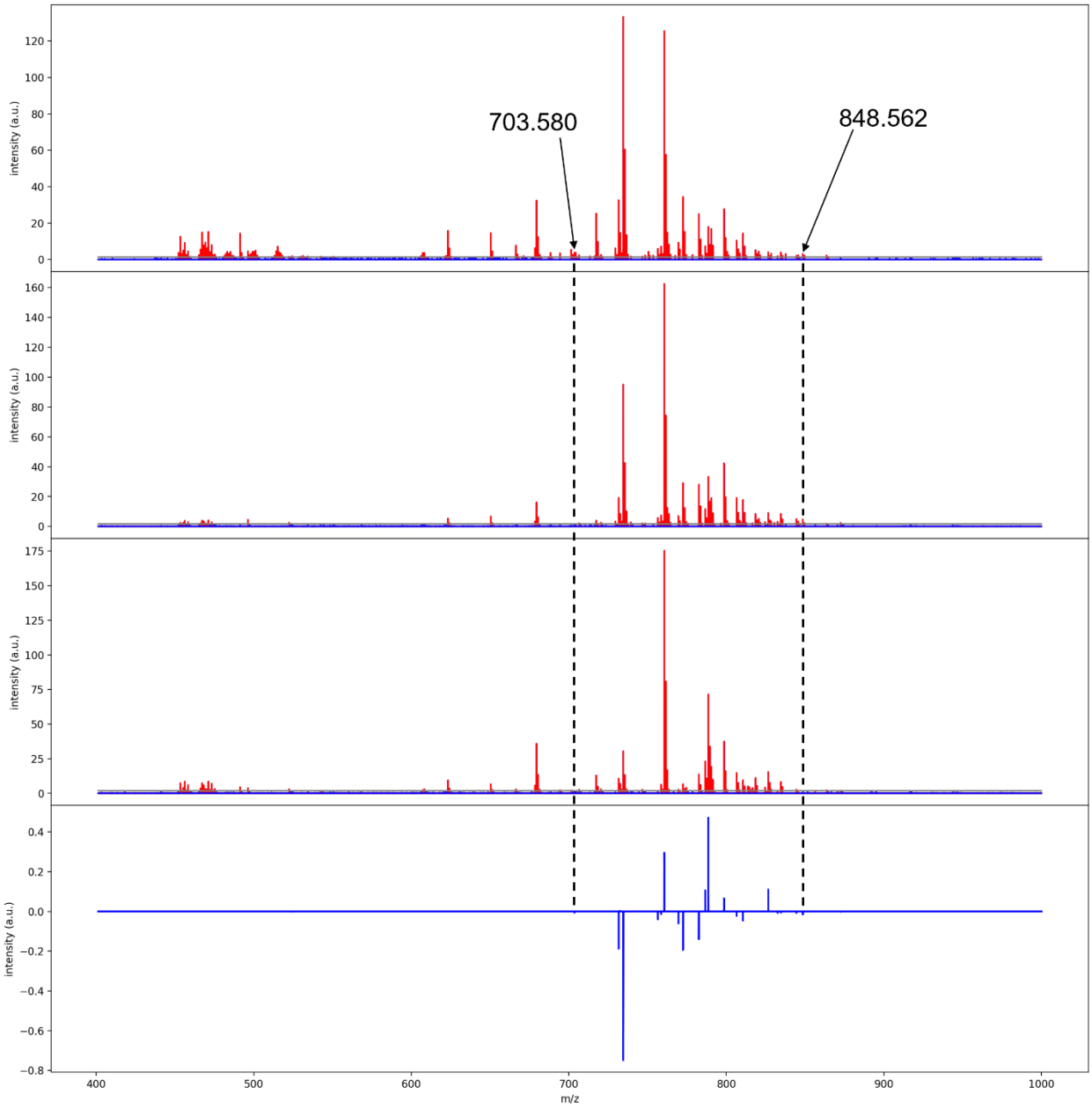


Figure S19: Medoid spectra of the three score groups for PC 2-5 for local normalization with RMS normalized PCA results for 0.10 of intensity threshold and 1% of selected pixels.

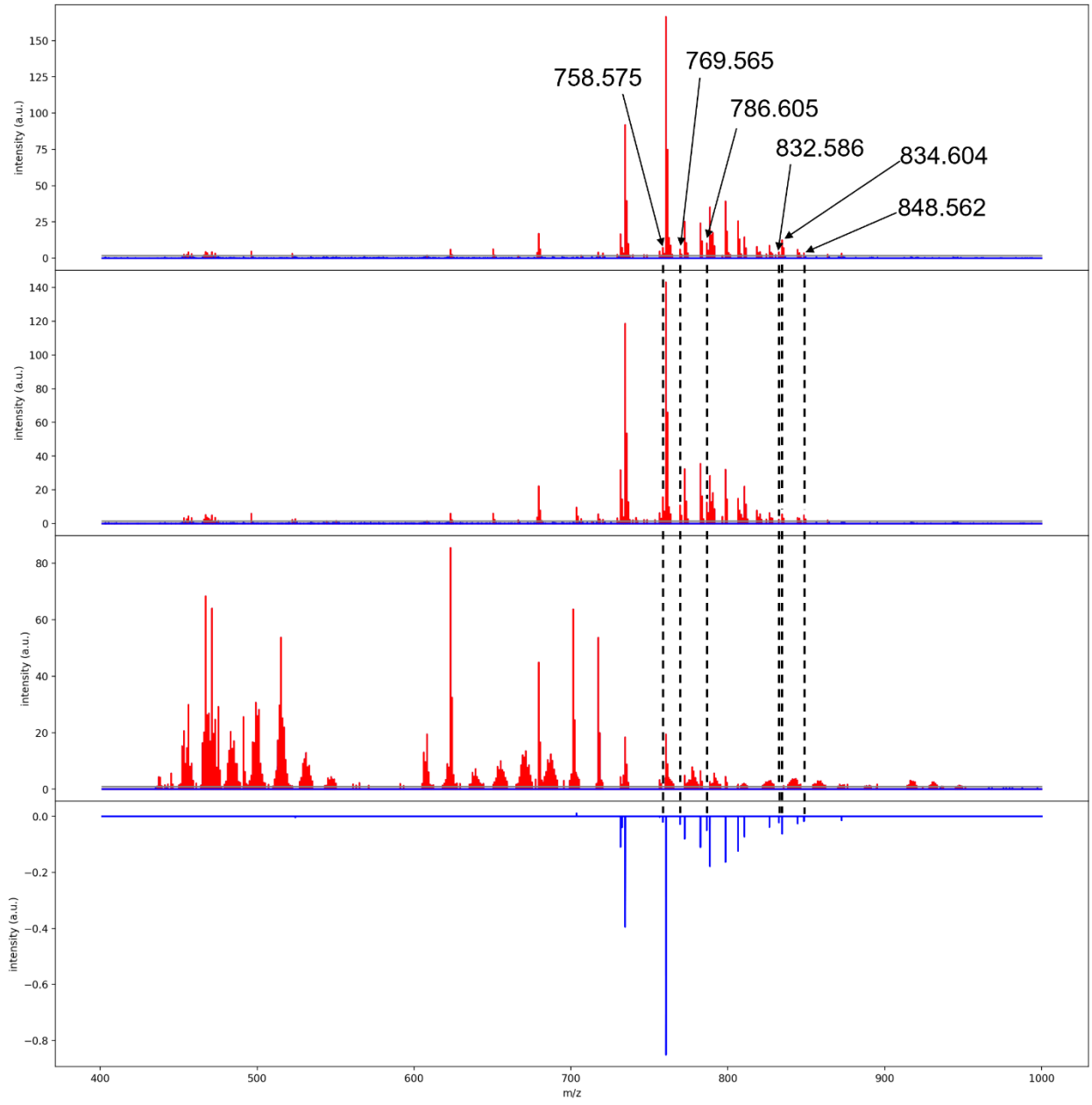
Local normalization with 1% selected pixels, Intensity threshold 0.01, and RMS normalizes PCA results

Significantly fewer peaks are correlated to the PCA loadings due to the intensity threshold being lower than the range of variations for most of the selected PCA peaks.

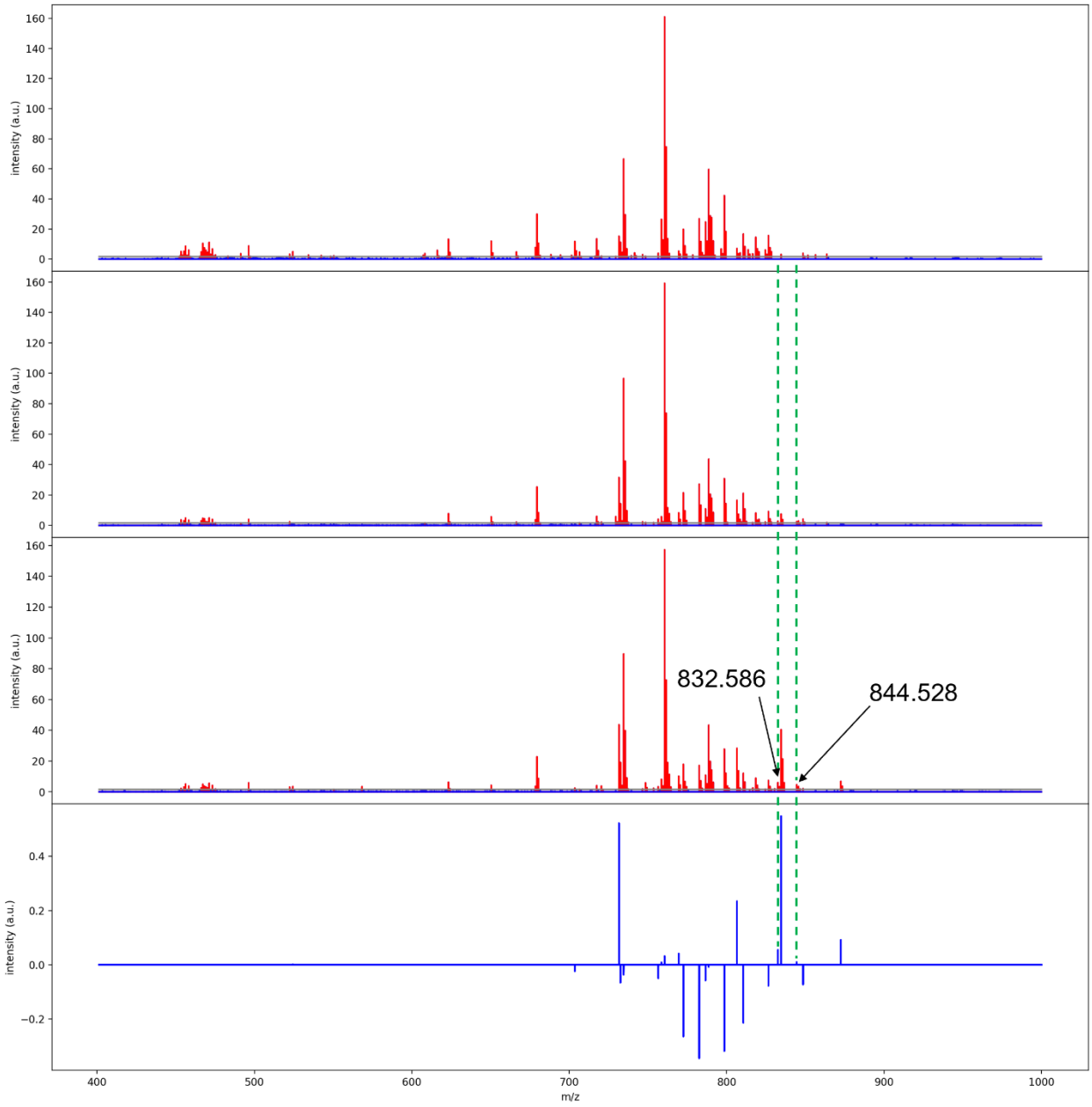
PC 1



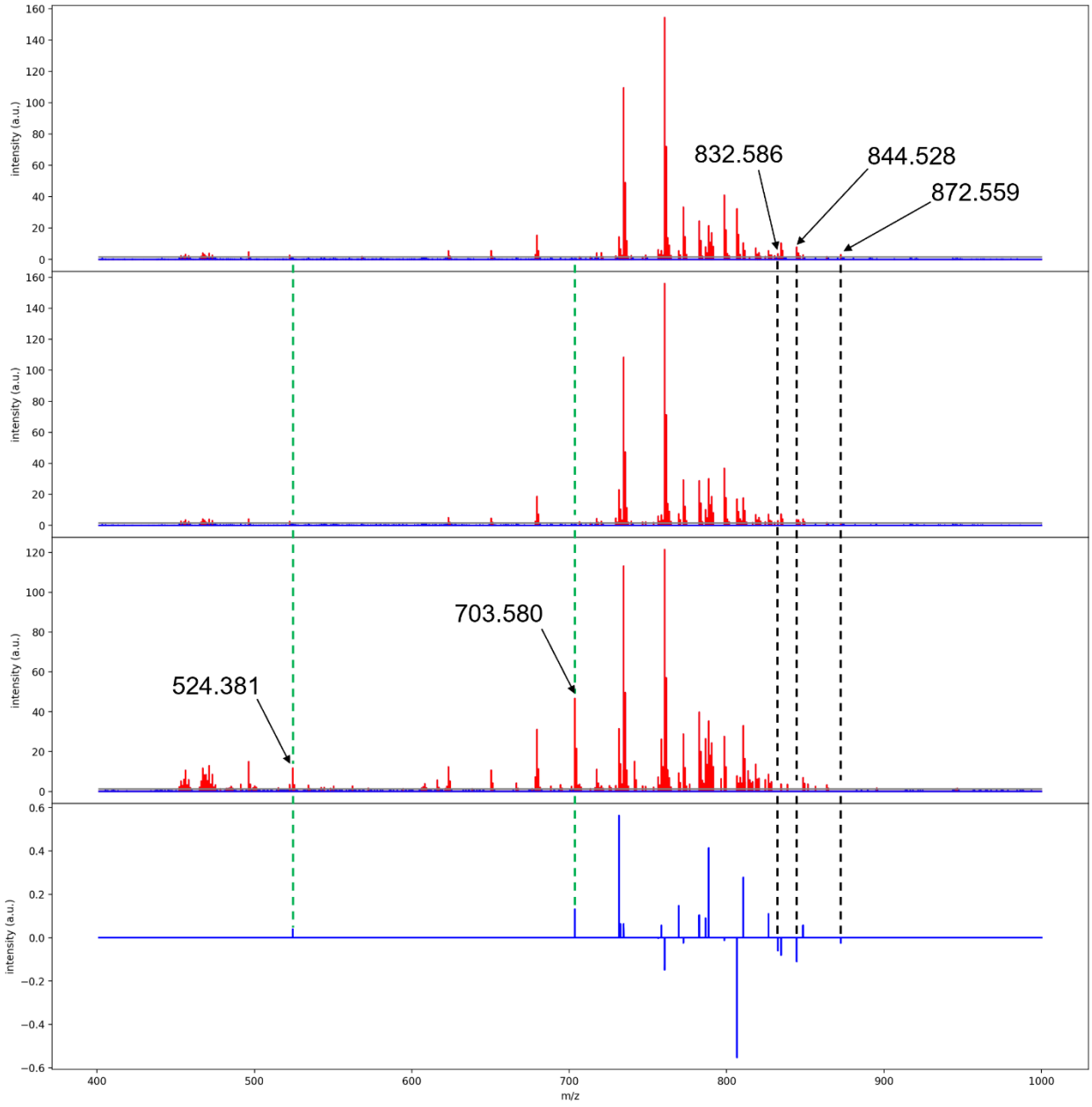
PC 2



PC 3



PC 4



PC 5

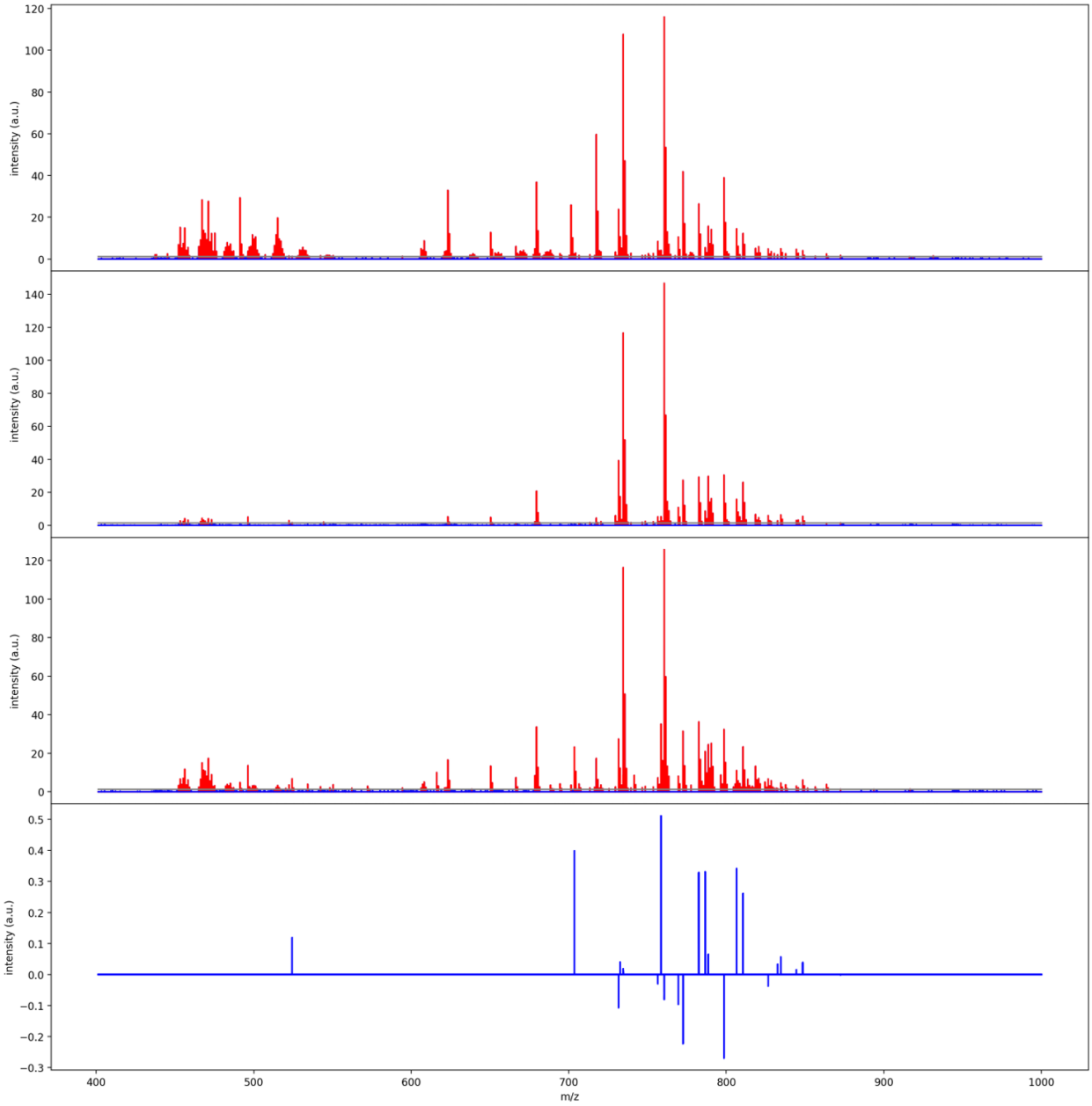


Figure S20: Medoid spectra of the three score groups for PC 1-5 for local normalization with RMS normalized PCA results for 0.01 of intensity threshold and 1% of selected pixels.

Mouse brain image A

Imaging conditions

Ten micrometer thick transverse mouse brain sections were prepared using CM 3050S cryostat (Leica Biosystems, Vista, CA) and stored in a -80°C freezer. The tissue sections were placed in the desiccator for 30 min prior to MALDI matrix application. A 2,5-Dihydroxybenzoic acid (DHB) MALDI matrix layer was applied using a home-built sublimation apparatus. The mouse brain sections were then stored in the desiccator for another 30 min before matrix-assisted laser desorption/ionization (MALDI) imaging with a 7T solariX Fourier transform ion cyclotron resonance mass spectrometer (FTICR) (Bruker Daltonics, Billerica, MA). Imaging parameters were optimized for known lipid profiles[24] in positive ion mode between *m/z* 400-1000. A laser power of 37% with 750 shots per pixel and a free induced decay (FID) of 0.4893 s were chosen. The resolving power for *m/z* 772.5255 was 34090 based on the full width half maximum (FWHM) mass resolution. Spectra file sizes were set to 256 kB and 98% data reduction was done during acquisition. Spatial resolution and SmartWalk sampling pattern were set to 150 µm and the final image contained 4,211 pixels with a file size of 4.77 GB.

Principal component analysis

PCA was calculated using SCiLS Lab pro by Bruker Daltonics with no normalization, scaling, nor denoising.

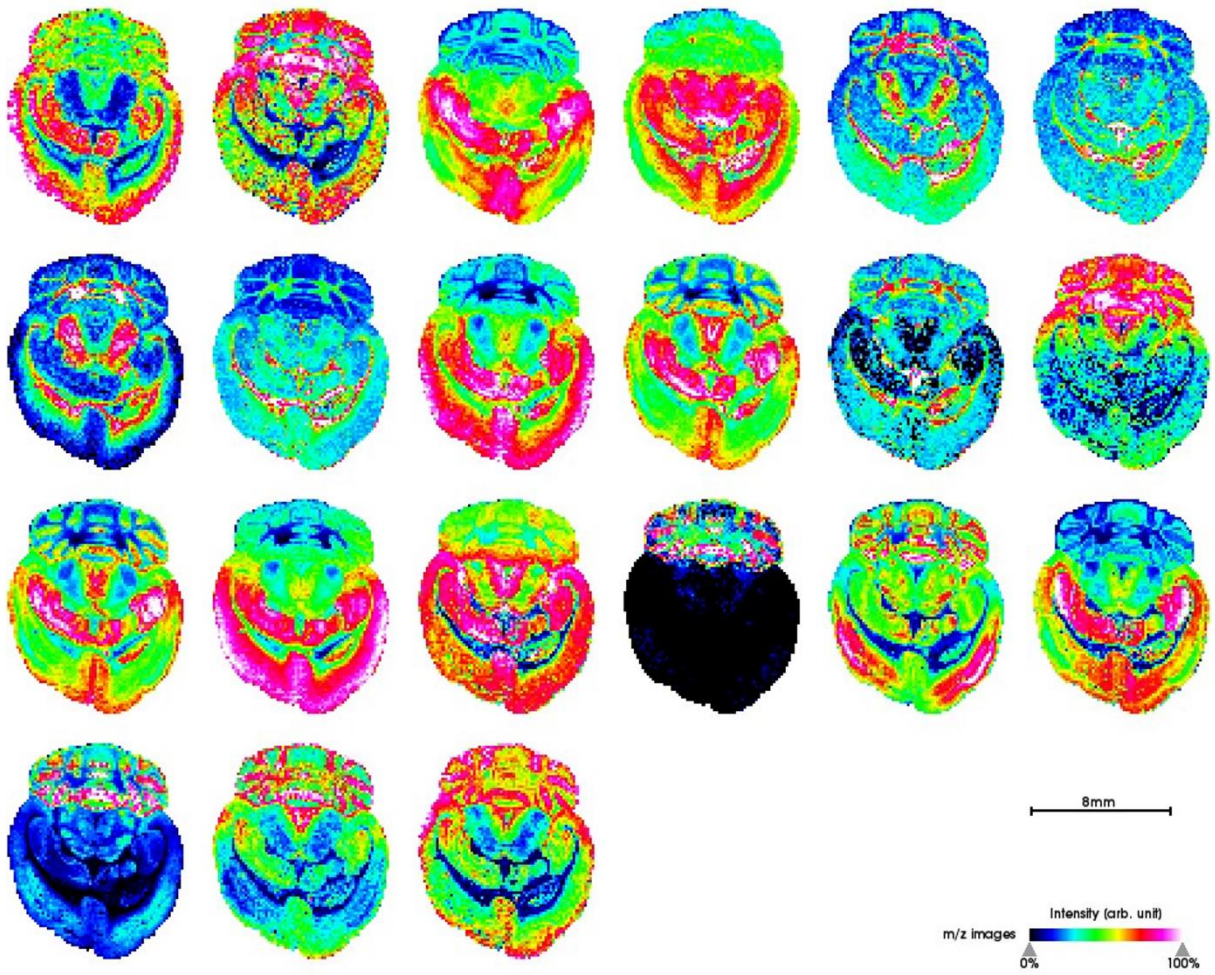


Figure S21: Ion images for PCA. From top left to bottom right, *m/z* are as follows: 459.890, 496.351, 554.295, 561.534, 732.560, 732.600, 734.560, 753.594, 756.557, 758.579, 760.570, 769.567, 772.531, 782.557, 798.510, 806.501, 810.578, 834.583, 844.507, 848.548, and 872.542.

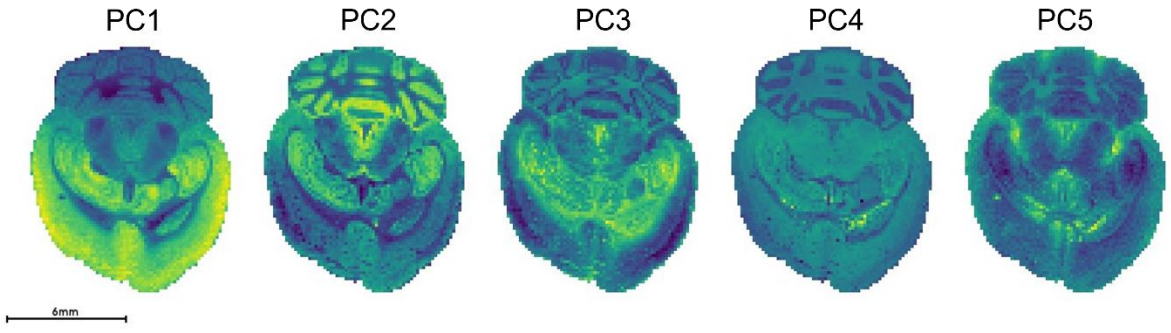


Figure S22: Spatial-expression images for PC 1-5.

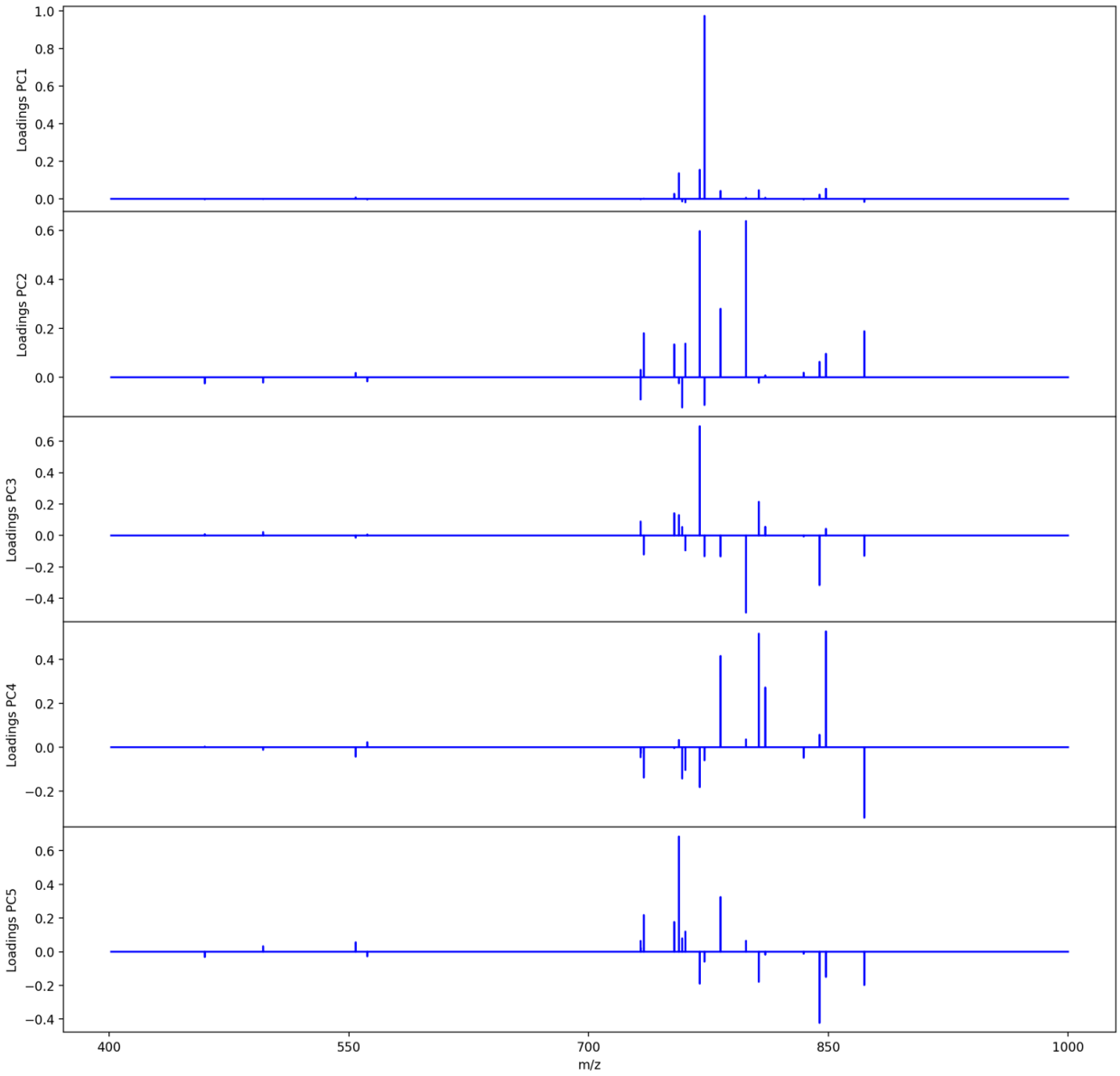


Figure S23: Pseudo-spectra for PC 1-5 from top to bottom.

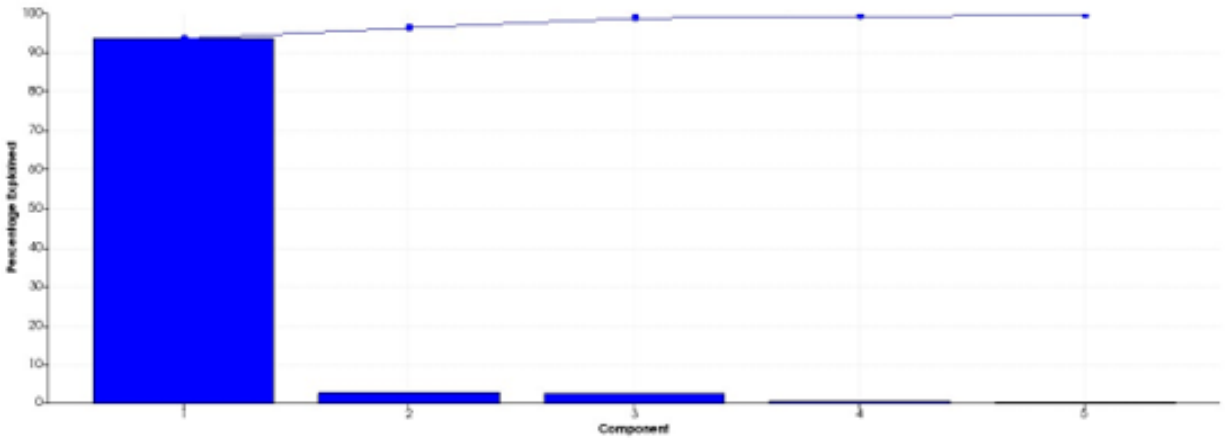


Figure S24: Pareto plot for PC 1-5. Explained variance for each PC is as follows: 93.55%, 2.74%, 2.49%, 0.50%, and 0.24%, respectively. The cumulative sum is 93.55%, 96.29%, 98.77%, 99.27%, and 99.51%

E-index Plots

All E-index calculations failed to find optimal set of parameters and did not exhibit any unique trends. The intensity threshold that resulted in the largest E value from local normalizationwas selected as the optimal intensity threshold and the selected pixel percentage was hand-picked.

local normalization from non-normalized PCA results

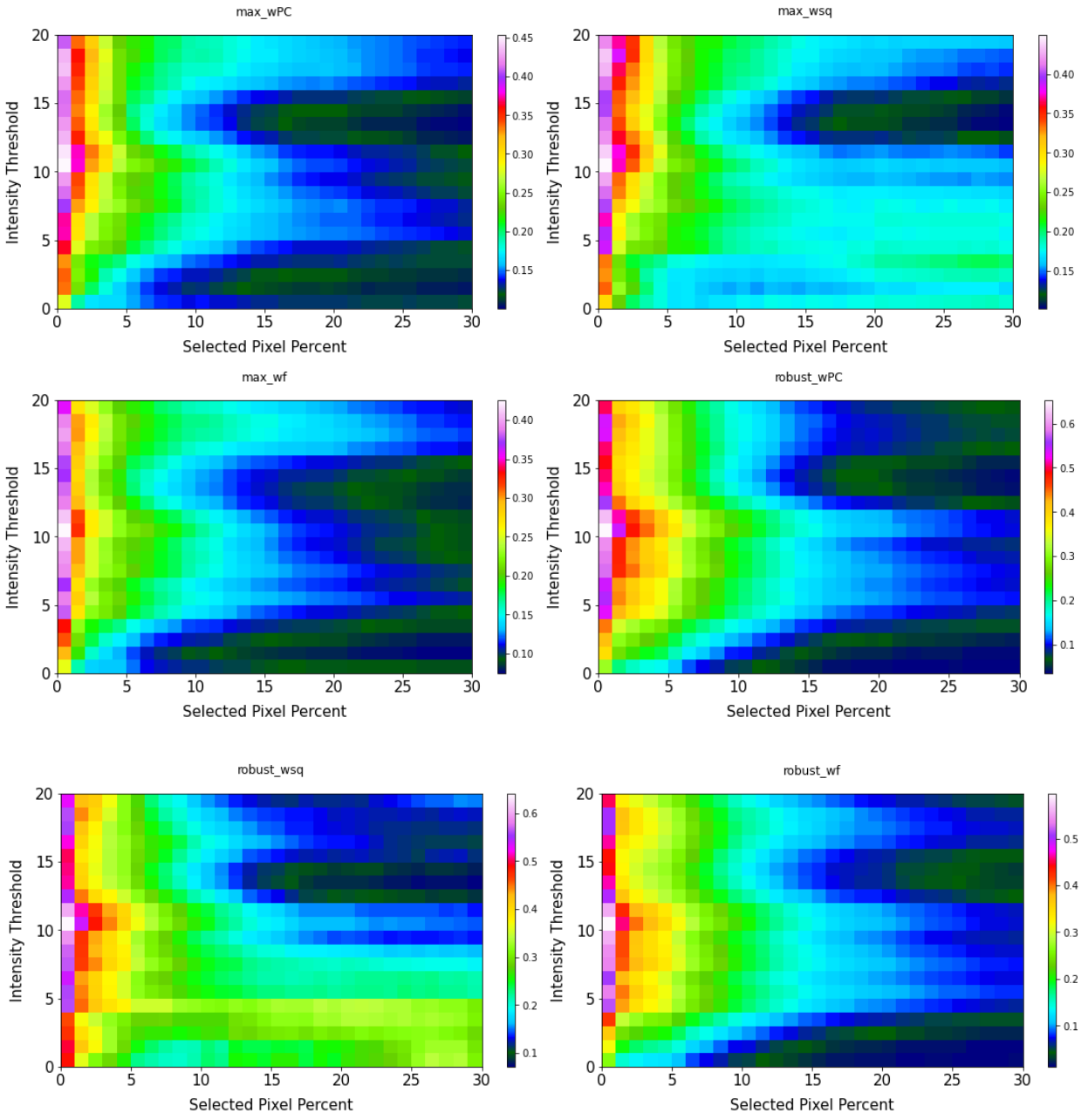


Figure S25: E-index plots for local normalization from non-normalized PCA results.

global normalization from non-normalized PCA results

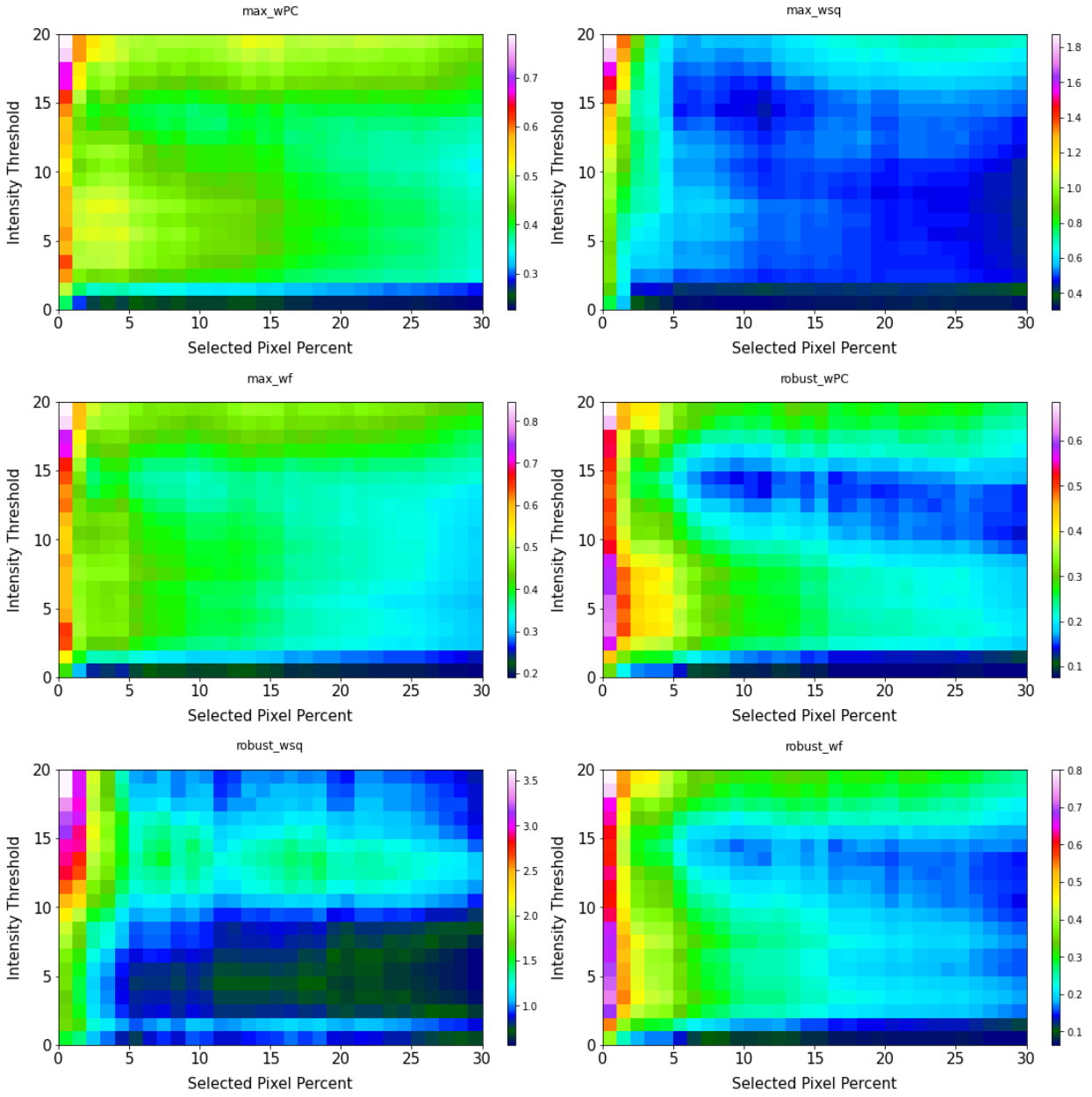


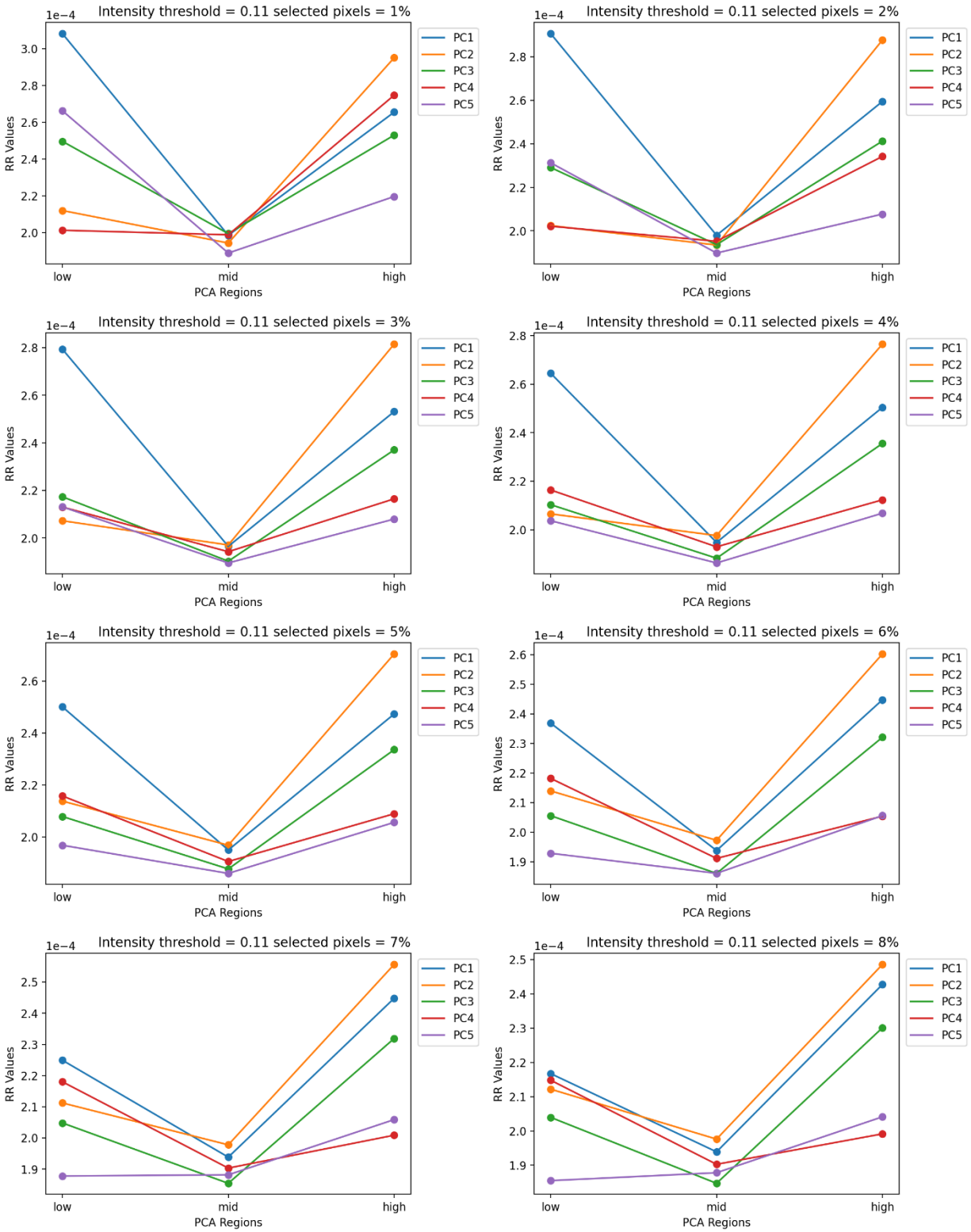
Figure S26: E-index plots for global normalization from non-normalized PCA results.

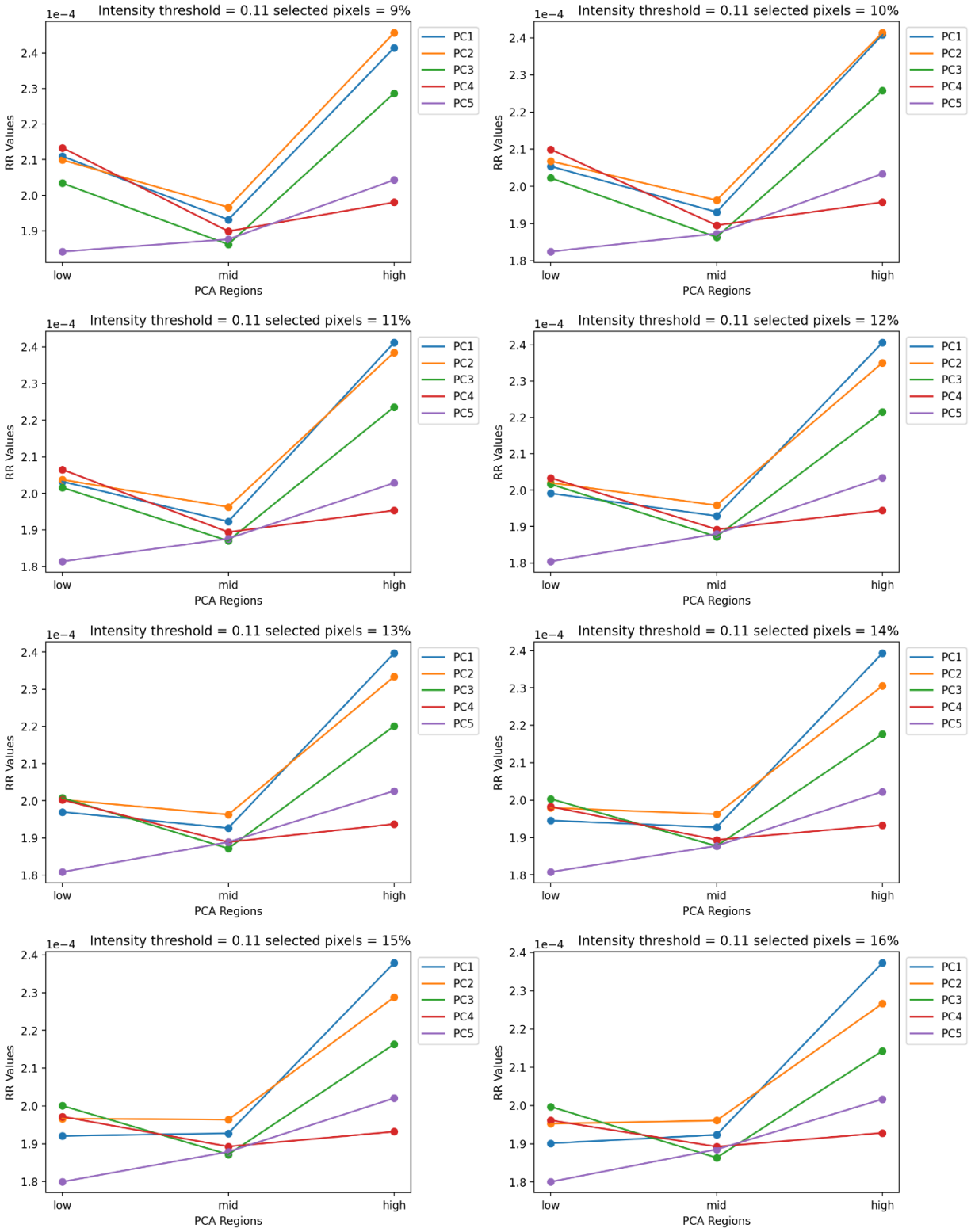
Russel-Rao 2-D V Plots

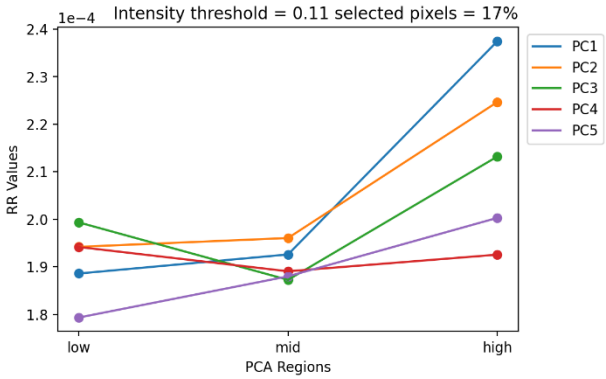
Optimal parameters were found to be intensity threshold of 0.11 with 8% selected pixels and local normalization.

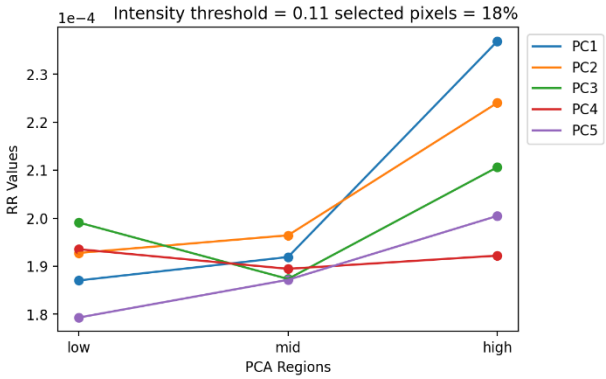
No RMS pre-processing was done.

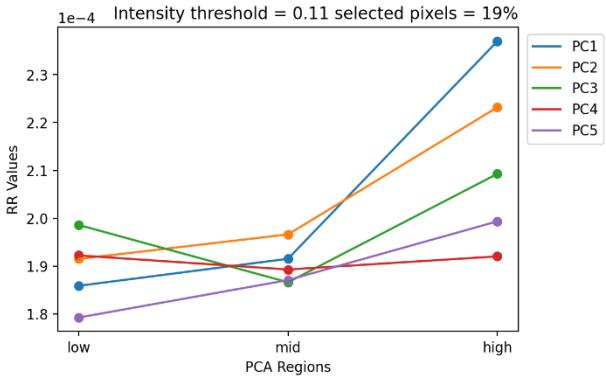
2-D V plots for local normalization with non-normalized PCA results

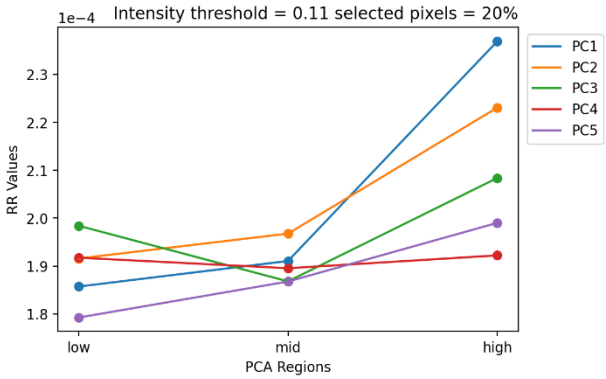


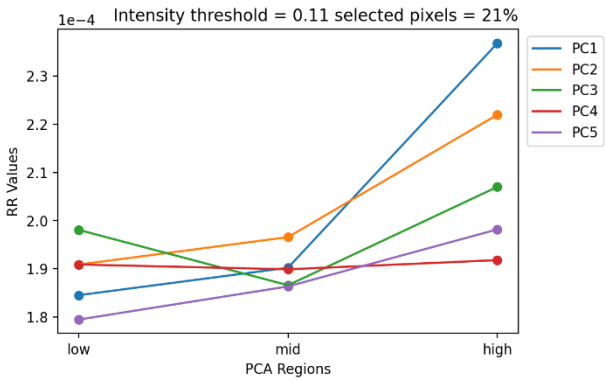

Intensity threshold = 0.11 selected pixels = 9%
Intensity threshold = 0.11 selected pixels = 10%
Intensity threshold = 0.11 selected pixels = 11%
Intensity threshold = 0.11 selected pixels = 12%
Intensity threshold = 0.11 selected pixels = 13%
Intensity threshold = 0.11 selected pixels = 14%
Intensity threshold = 0.11 selected pixels = 15%
Intensity threshold = 0.11 selected pixels = 16%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

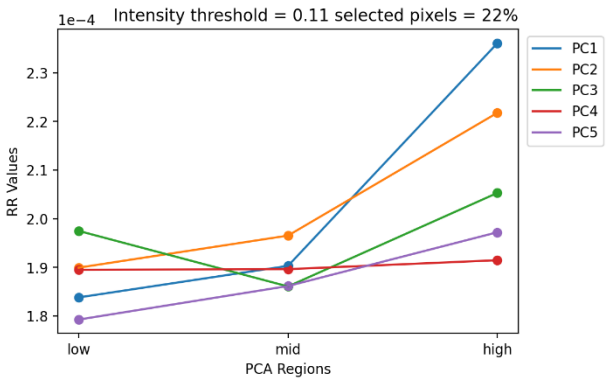

Intensity threshold = 0.11 selected pixels = 17%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

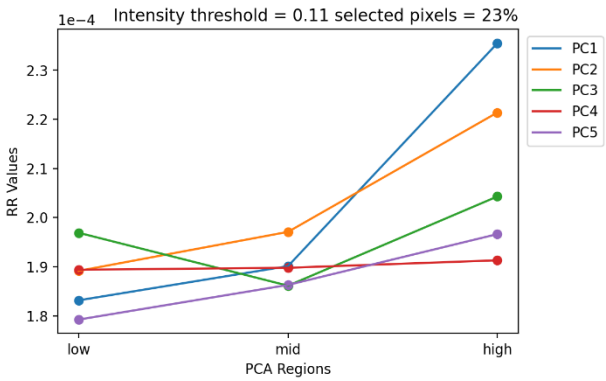

Intensity threshold = 0.11 selected pixels = 18%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

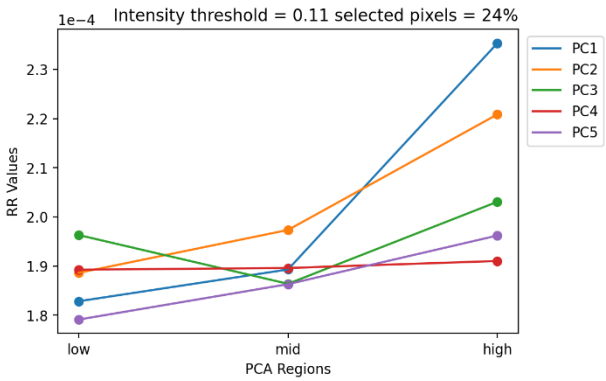

Intensity threshold = 0.11 selected pixels = 19%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.11 selected pixels = 20%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.11 selected pixels = 21%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.11 selected pixels = 22%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.11 selected pixels = 23%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.11 selected pixels = 24%
1e−4
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

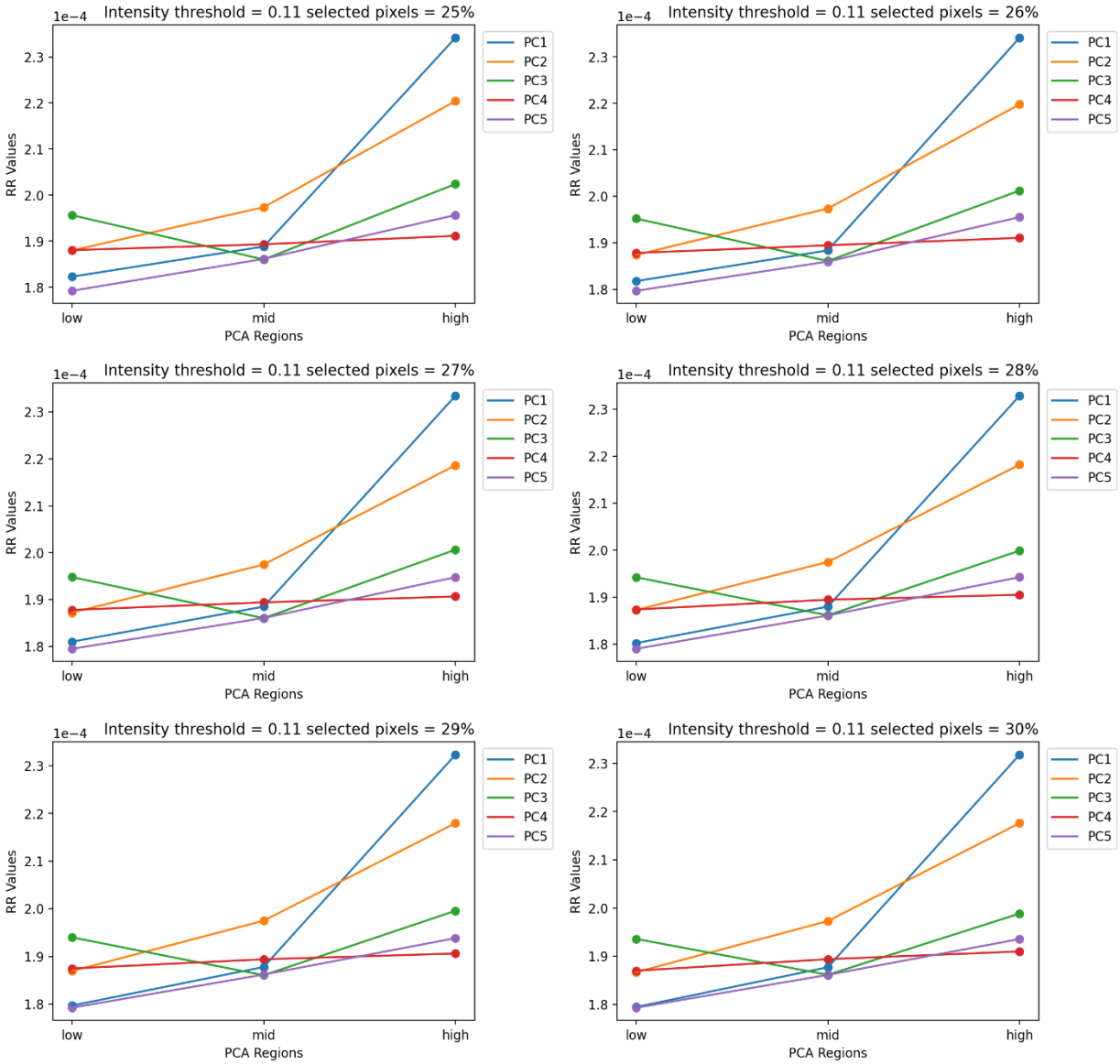
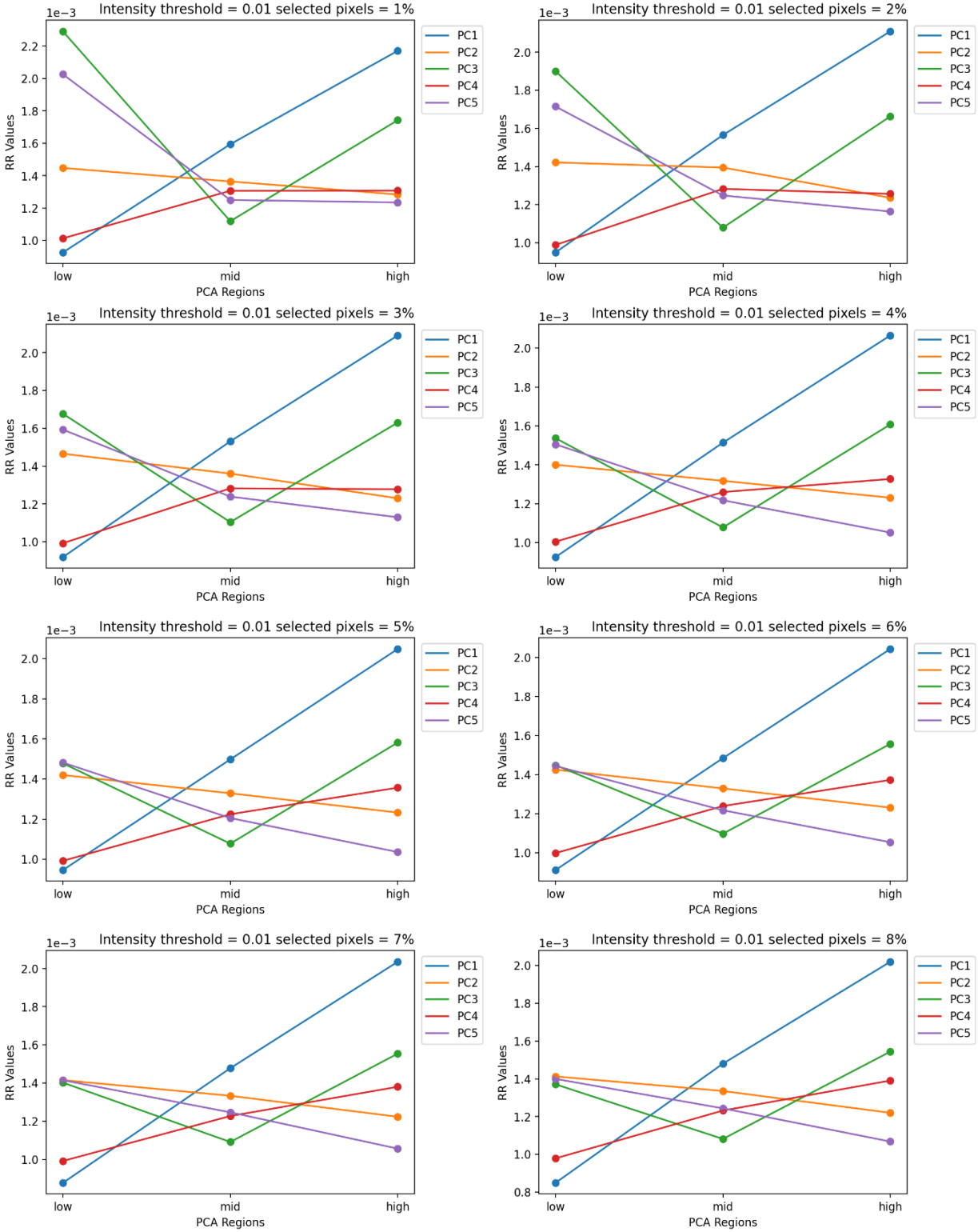
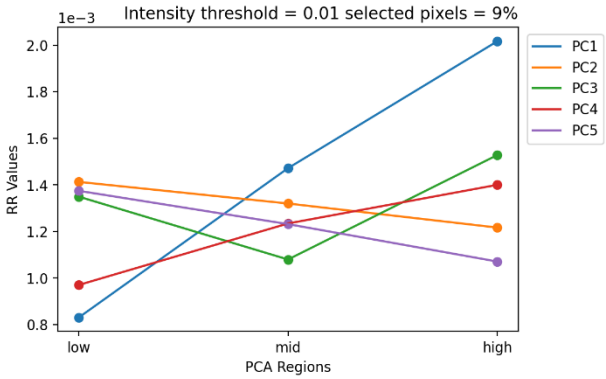


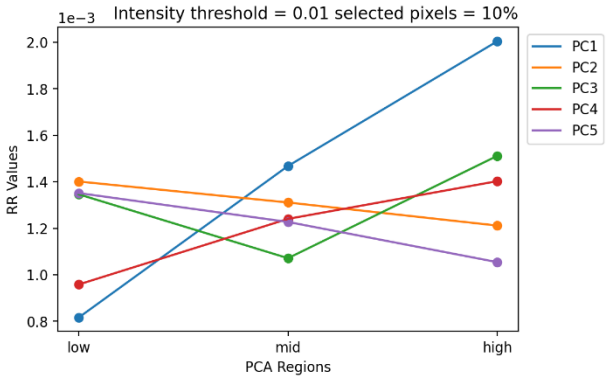
Figure S27: 2-D V plots for local normalization from non-normalized PCA results for 0.11 of intensity threshold.

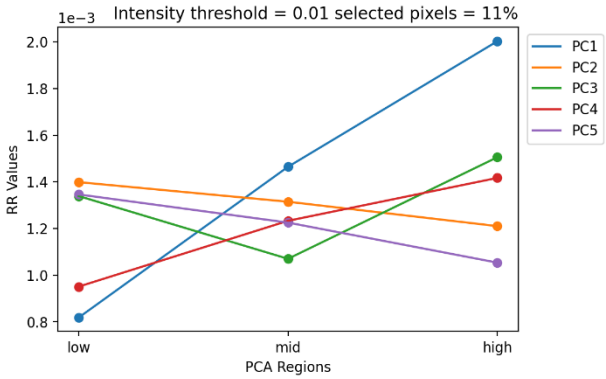
2-D V plots for global normalization with non-normalized PCA results

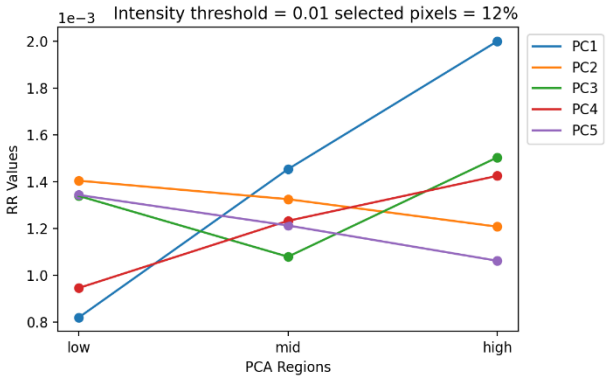
Global normalization, in general, fails to distinguish region similarity with the exception of PC 3.

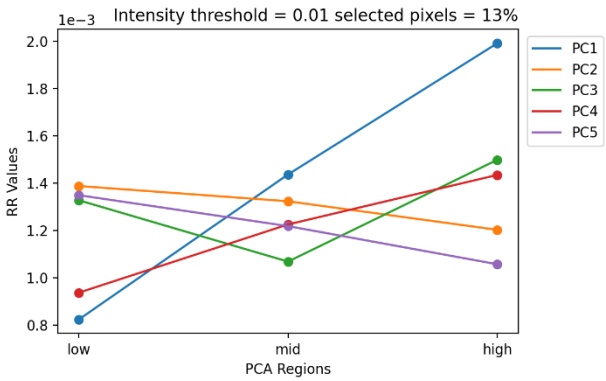

Intensity threshold = 0.01 selected pixels = 1%
Intensity threshold = 0.01 selected pixels = 2%
Intensity threshold = 0.01 selected pixels = 3%
Intensity threshold = 0.01 selected pixels = 4%
Intensity threshold = 0.01 selected pixels = 5%
Intensity threshold = 0.01 selected pixels = 6%
Intensity threshold = 0.01 selected pixels = 7%
Intensity threshold = 0.01 selected pixels = 8%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

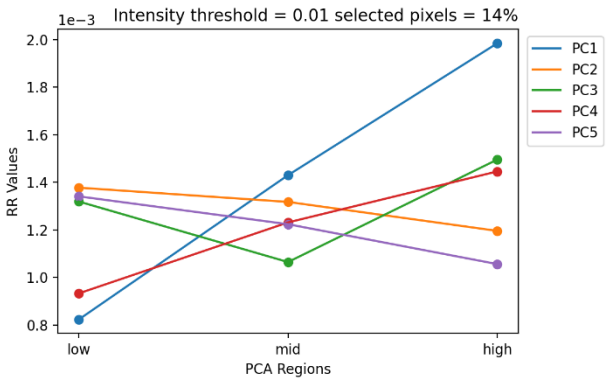

Intensity threshold = 0.01 selected pixels = 9%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

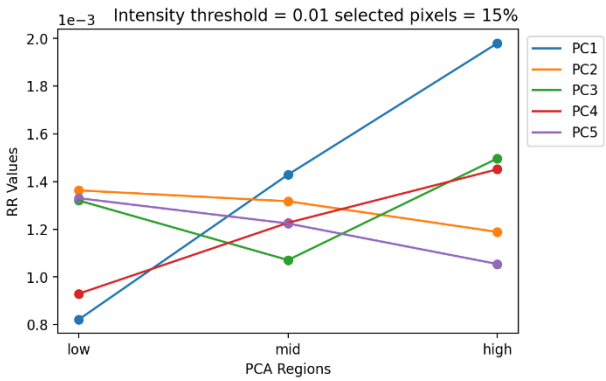

Intensity threshold = 0.01 selected pixels = 10%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

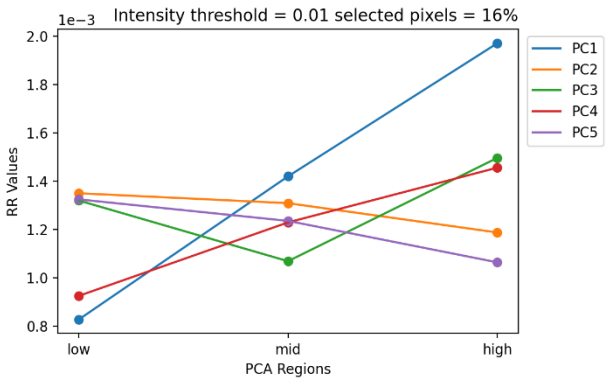

Intensity threshold = 0.01 selected pixels = 11%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

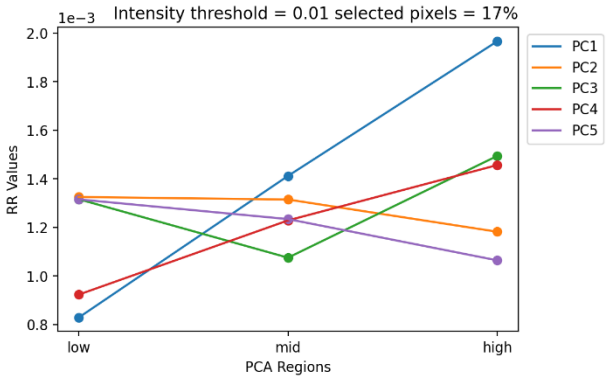

Intensity threshold = 0.01 selected pixels = 12%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

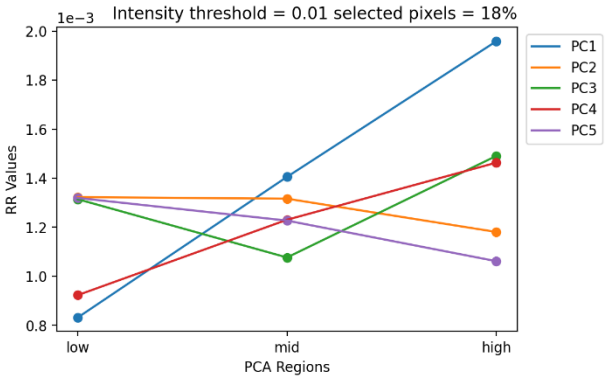

Intensity threshold = 0.01 selected pixels = 13%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

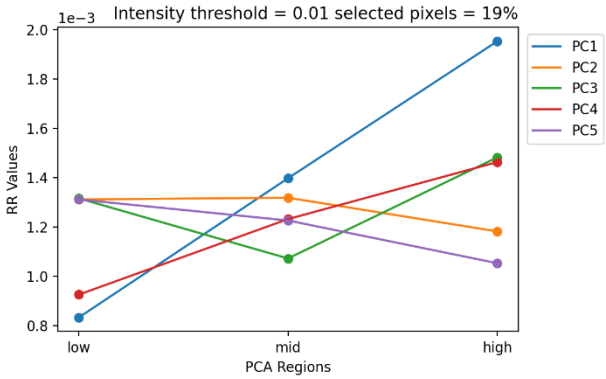

Intensity threshold = 0.01 selected pixels = 14%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

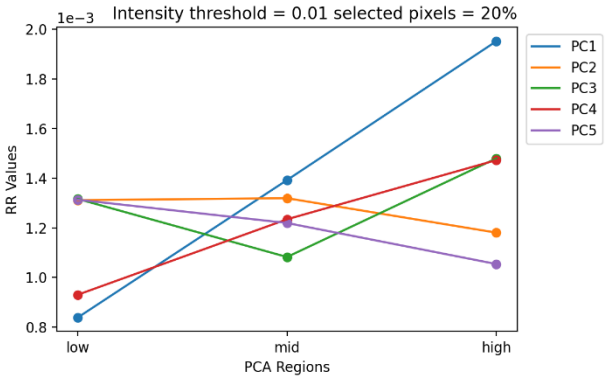

Intensity threshold = 0.01 selected pixels = 15%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

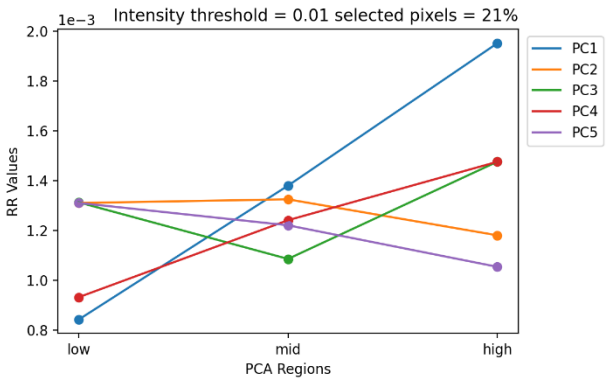

Intensity threshold = 0.01 selected pixels = 16%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

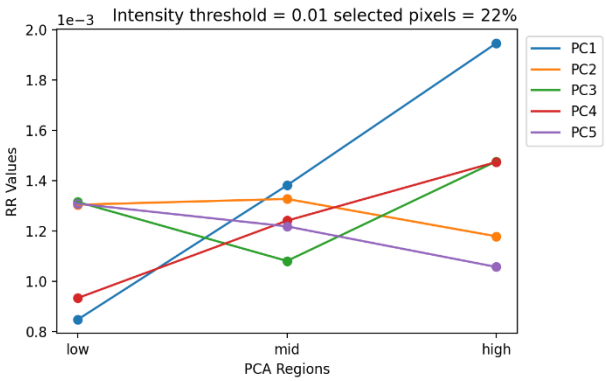

Intensity threshold = 0.01 selected pixels = 17%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

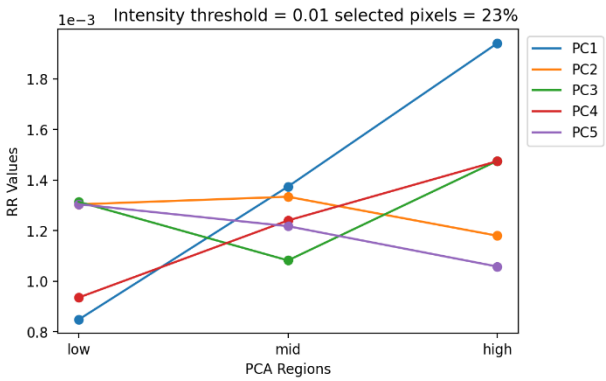

Intensity threshold = 0.01 selected pixels = 18%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

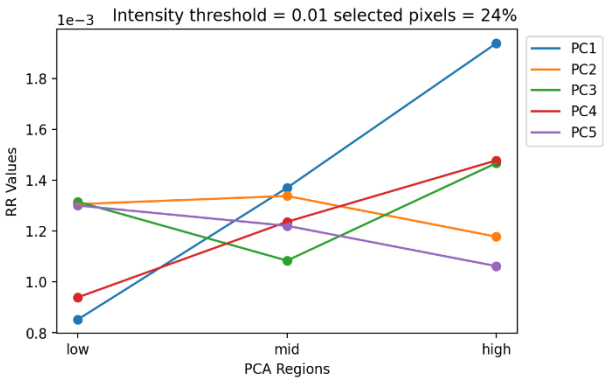

Intensity threshold = 0.01 selected pixels = 19%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 20%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 21%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 22%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 23%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 24%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

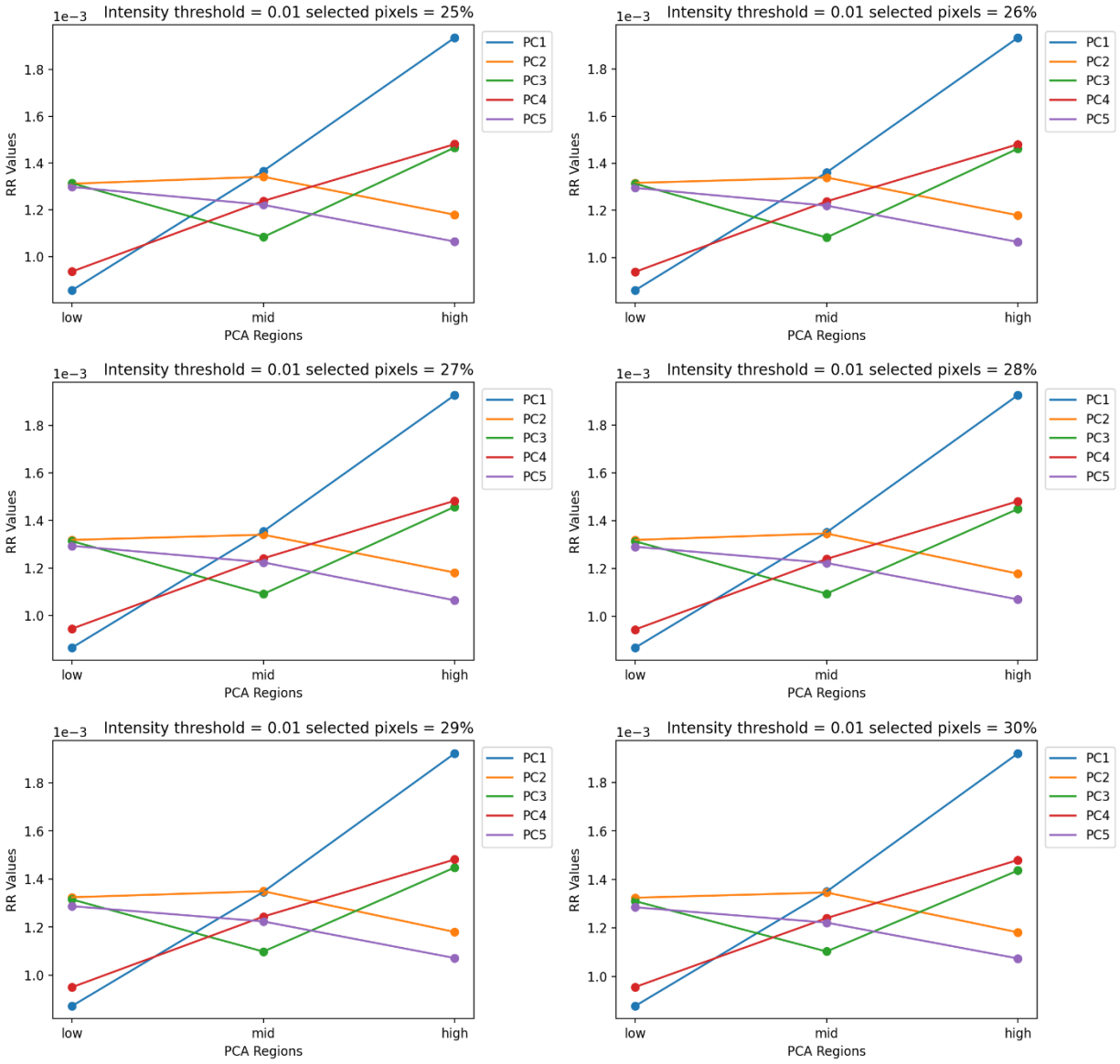
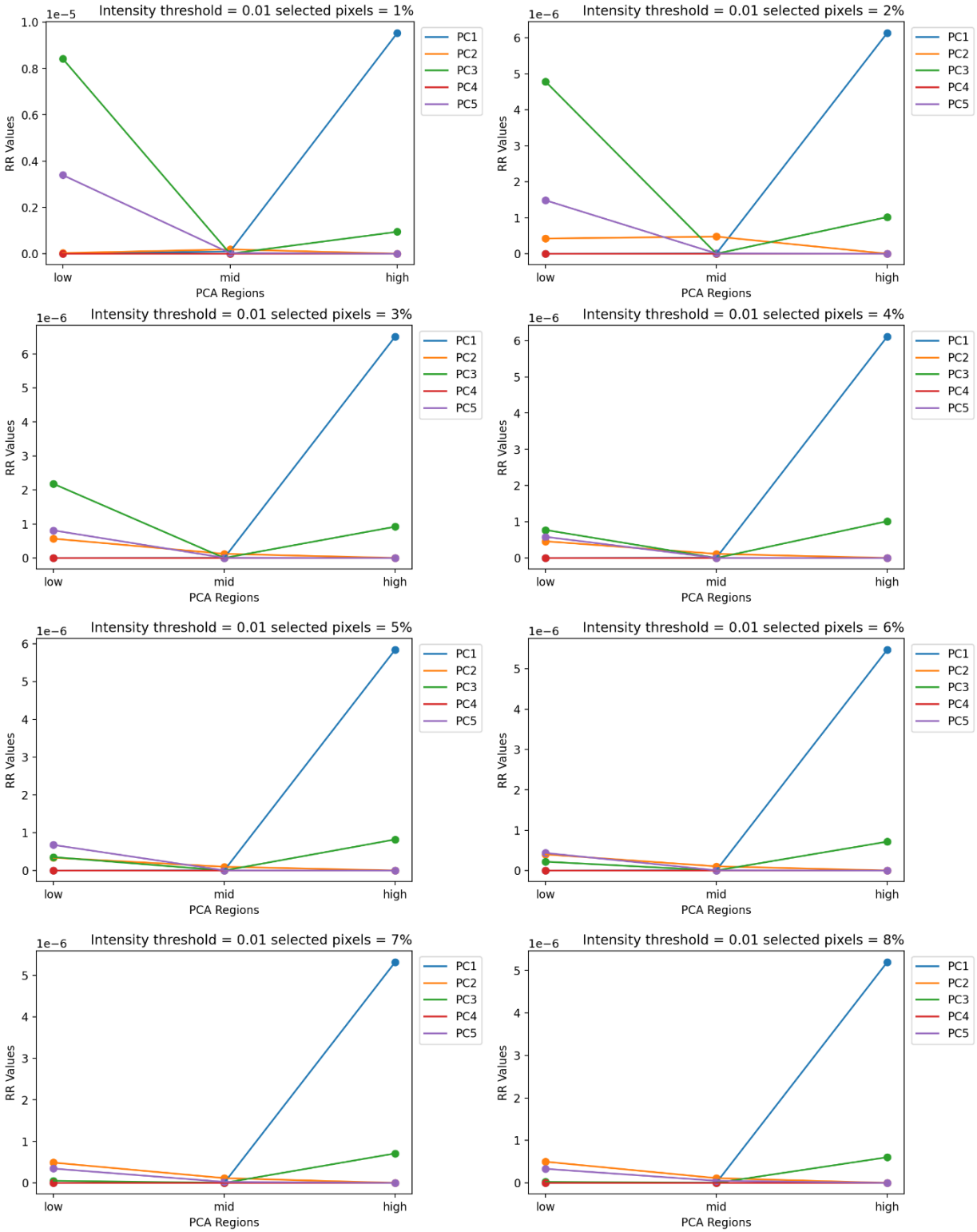
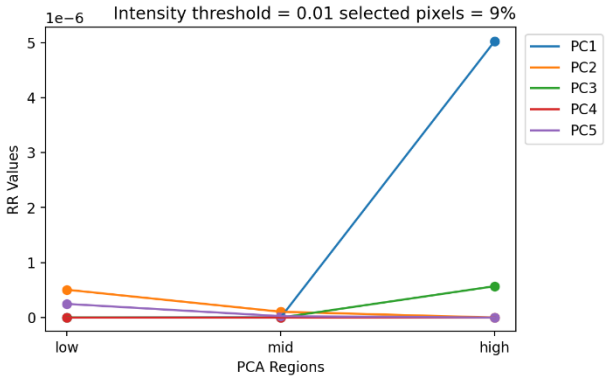


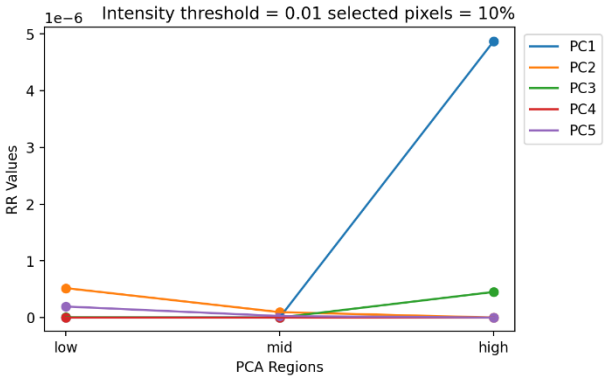
Figure S28: 2-D V plots for global normalization from non-normalized PCA results for 0.01 of intensity threshold.

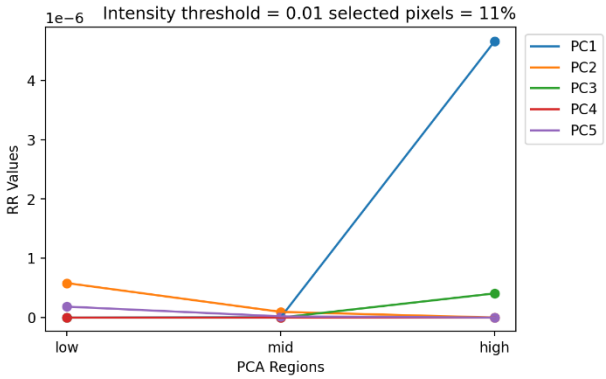
2-D V plots for globalTIC normalization with non-normalized PCA results

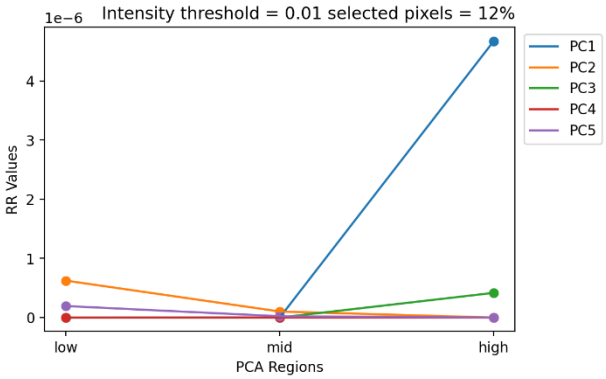
GlobalTIC completely fails to show a distinction of region similarity. This is due to a majority of the data not being above the intensity threshold when normalized to the largest total ion count.

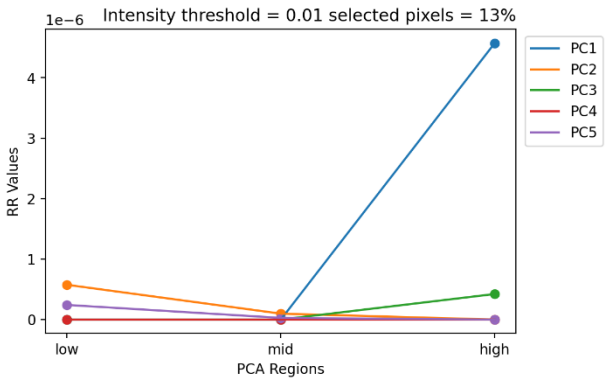

Intensity threshold = 0.01 selected pixels = 1%
1e−5
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5
Intensity threshold = 0.01 selected pixels = 2%
1e−6
Intensity threshold = 0.01 selected pixels = 3%
Intensity threshold = 0.01 selected pixels = 4%
Intensity threshold = 0.01 selected pixels = 5%
Intensity threshold = 0.01 selected pixels = 6%
Intensity threshold = 0.01 selected pixels = 7%
Intensity threshold = 0.01 selected pixels = 8%

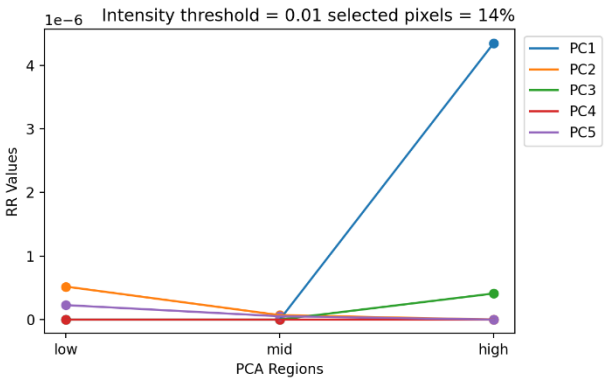

Intensity threshold = 0.01 selected pixels = 9%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

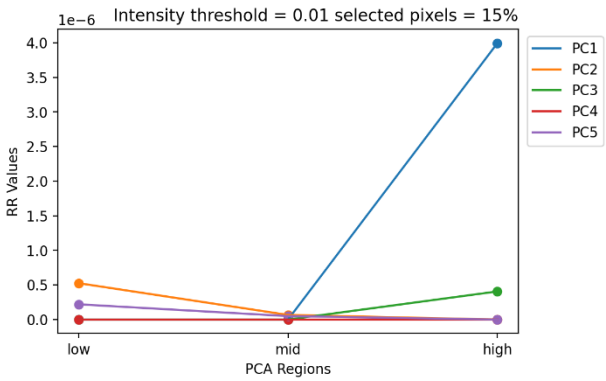

Intensity threshold = 0.01 selected pixels = 10%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

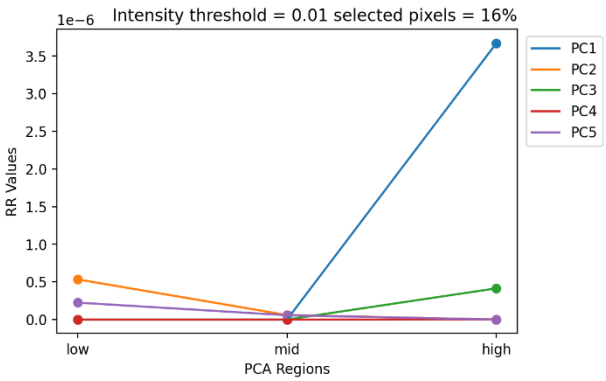

Intensity threshold = 0.01 selected pixels = 11%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

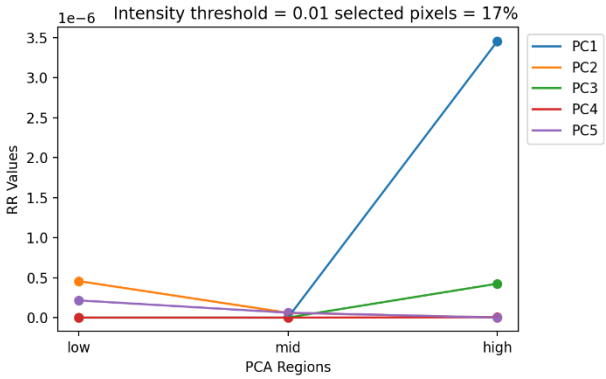

Intensity threshold = 0.01 selected pixels = 12%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

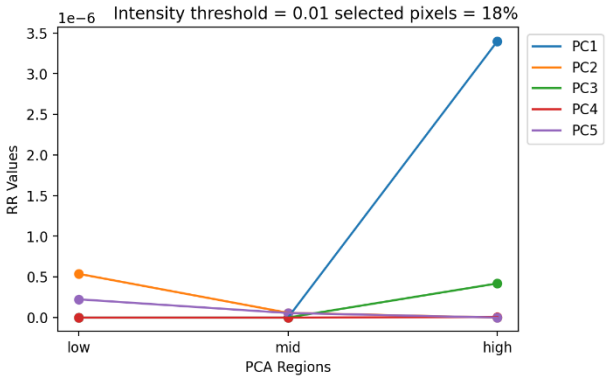

Intensity threshold = 0.01 selected pixels = 13%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

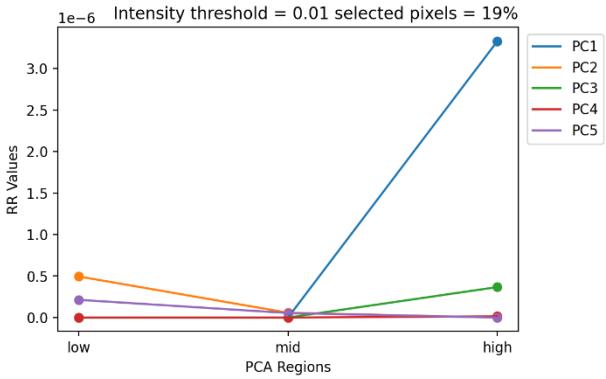

Intensity threshold = 0.01 selected pixels = 14%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

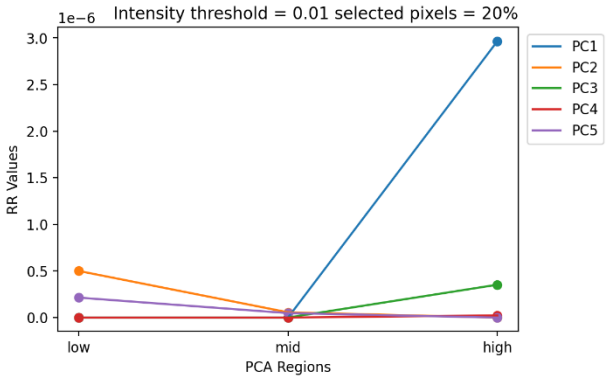

Intensity threshold = 0.01 selected pixels = 15%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

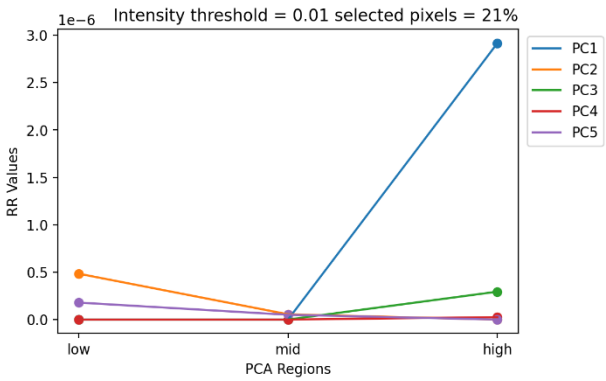

Intensity threshold = 0.01 selected pixels = 16%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

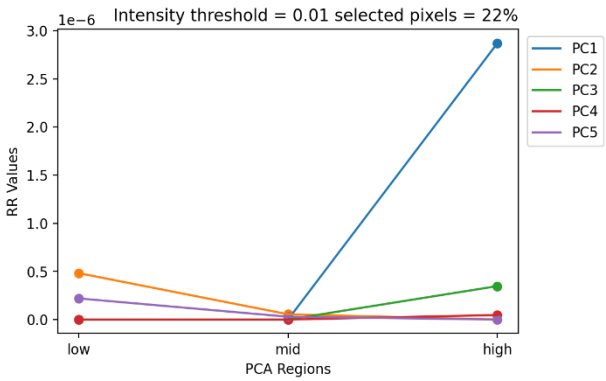

Intensity threshold = 0.01 selected pixels = 17%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

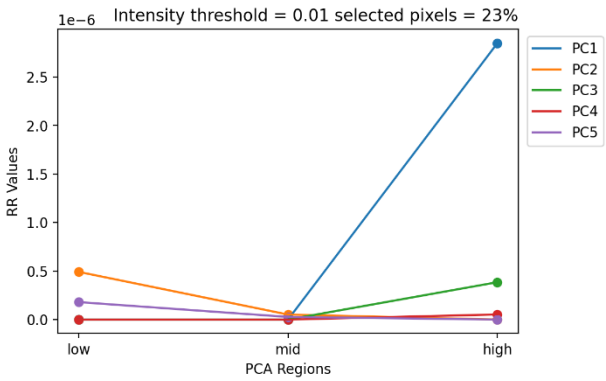

Intensity threshold = 0.01 selected pixels = 18%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

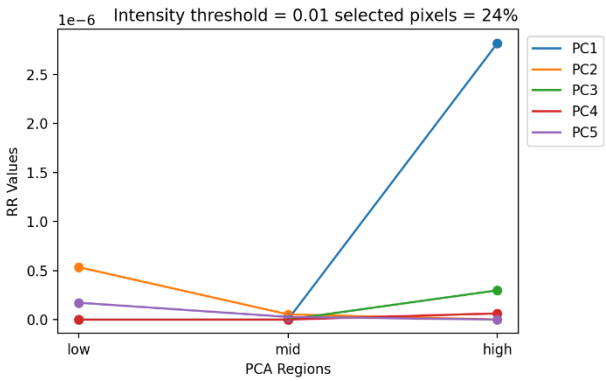

Intensity threshold = 0.01 selected pixels = 19%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 20%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 21%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 22%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 23%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 24%
1e−6
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

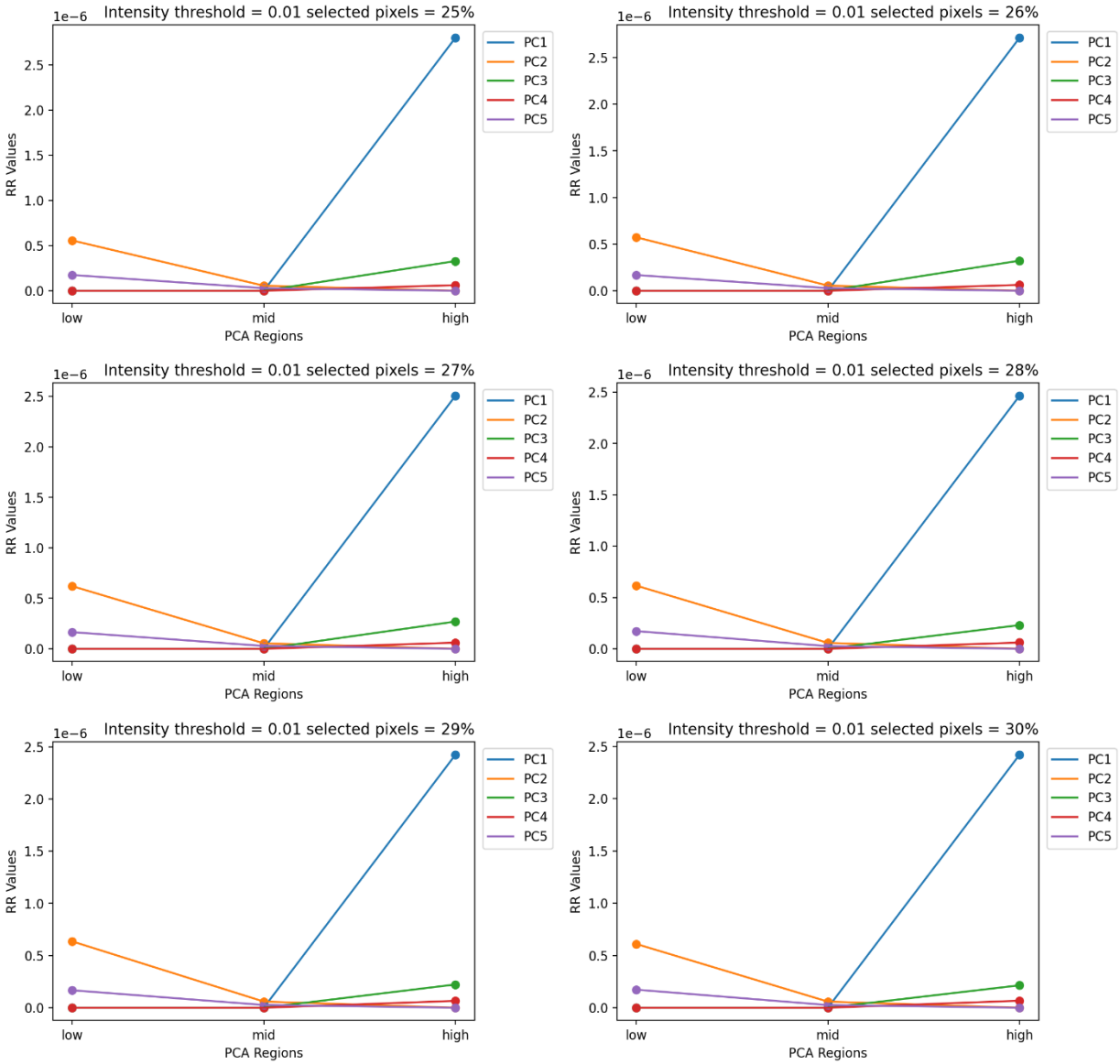


Figure S29: 2-D V plots for globalTIC normalization from non-normalized PCA results for 0.01 of intensity threshold.

Russel-Rao 3-D V plots for optimal parameters

Optimal parameters: intensity threshold = 0.11, selected pixels percent = 8%, normalization = local.

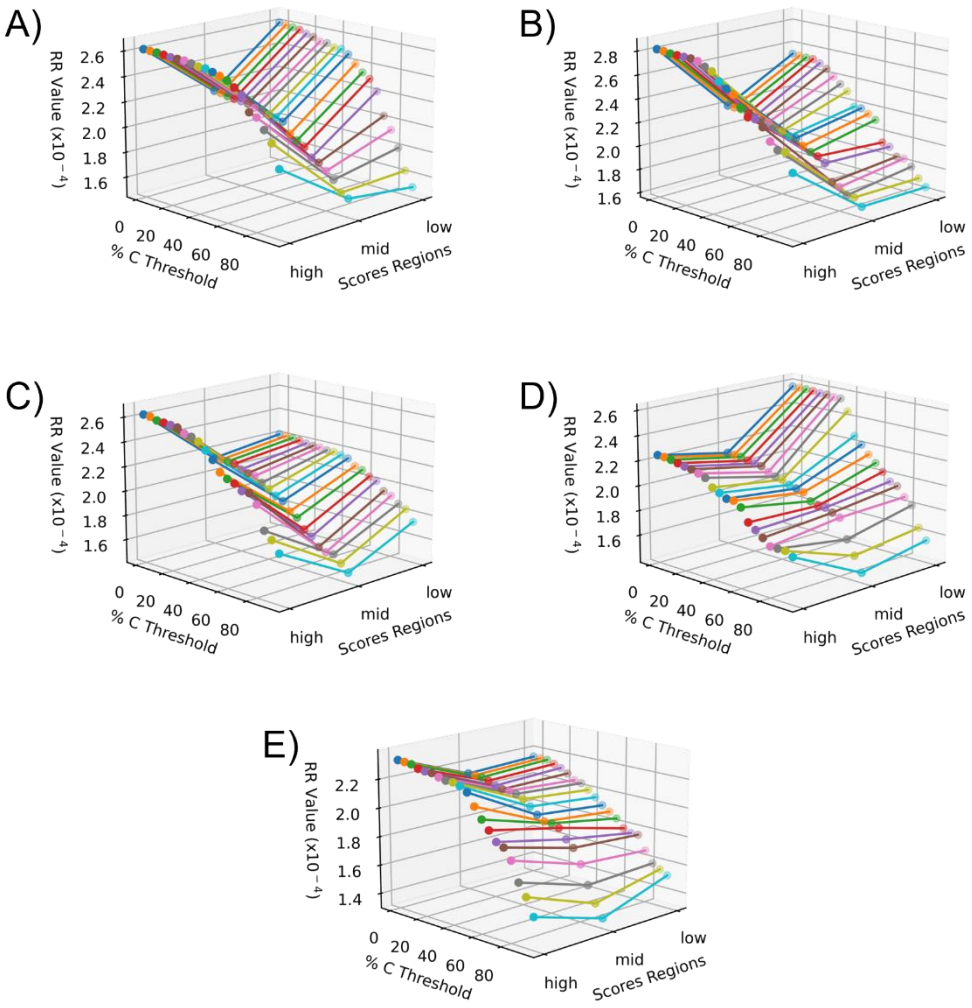


Figure S30: 3-D V plots for local normalization from non-normalized PCA results for 0.11 of intensity threshold and 8% of selected pixels.

Mouse brain image B

Imaging conditions

Ten micrometer thick transverse mouse brain sections were prepared using CM 3050S cryostat (Leica Biosystems, Vista, CA) and stored in a -80°C freezer. The tissue sections were placed in the desiccator for 30 min prior to MALDI matrix application. A

2,5-Dihydroxyacetophenone (DHA) MALDI matrix layer was applied using a home-built sublimation apparatus. The mouse brain sections were then stored in the desiccator for another 30 min before matrix-assisted laser desorption/ionization (MALDI) imaging with a 7T solariX Fourier transform ion cyclotron resonance mass spectrometer (FTICR) (Bruker Daltonics, Billerica, MA). Imaging parameters were optimized for known lipid profiles[24] in positive ion mode between *m/z* 400-2000. A laser power of 26% with 500 shots per pixel was chosen. The resolving power for *m/z* 798.541 was 33000 based on the full width half maximum (FWHM) mass resolution. Spectra file sizes were set to 256 kB and 98% data reduction was done during acquisition. Spatial resolution and SmartWalk sampling pattern were set to 100 µm and the final image contained 10,318 pixels with a file size of 12.0 GB.

Principal component analysis

PCA was calculated using SCiLS Lab pro by Bruker Daltonics with no normalization, scaling, nor denoising.

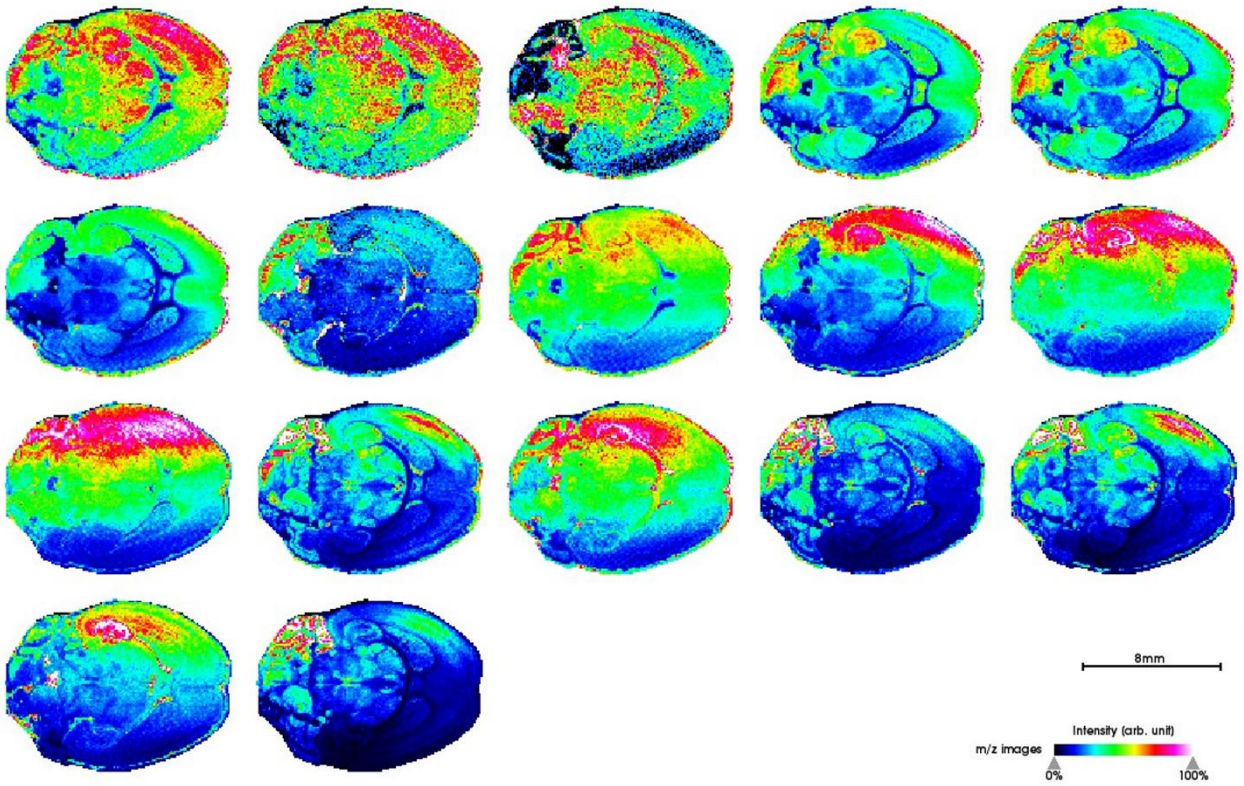


Figure S31: Ion images of *m/z* values used for PCA. From top left to bottom right *m/z* are as follows: 554.290, 555.292, 558.320, 731.278, 732.608, 734.199, 758.568, 760.584, 772.460, 782.564, 798.542, 806.467, 810.454, 834.455, 844.468, 848.515, and 872.556.

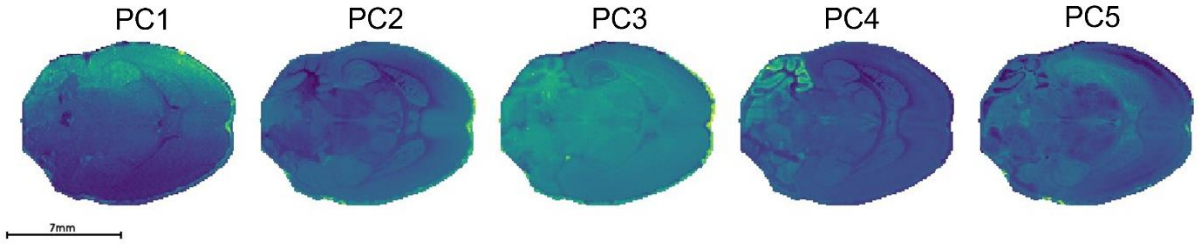


Figure S32: Spatial-expression images for PC 1-5. Although some parts of brain structures can be discerned, they are not strongly expressed by the score values. This shows PCA failed to identify biological regions with strong correlations, largely due to the poor ion images.

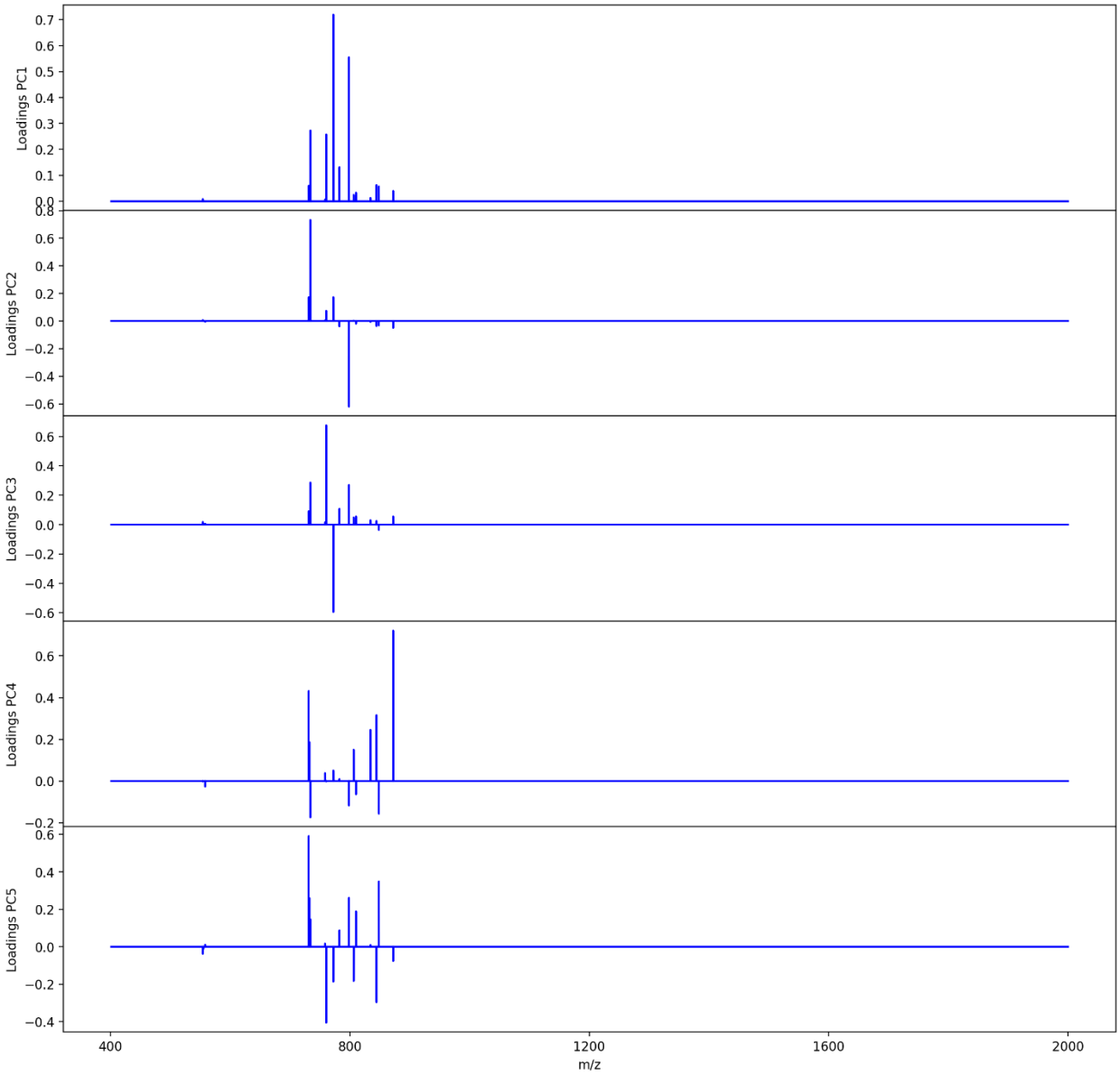


Figure S33: Pseudo-spectra for PC 1-5 from top to bottom, respectively.

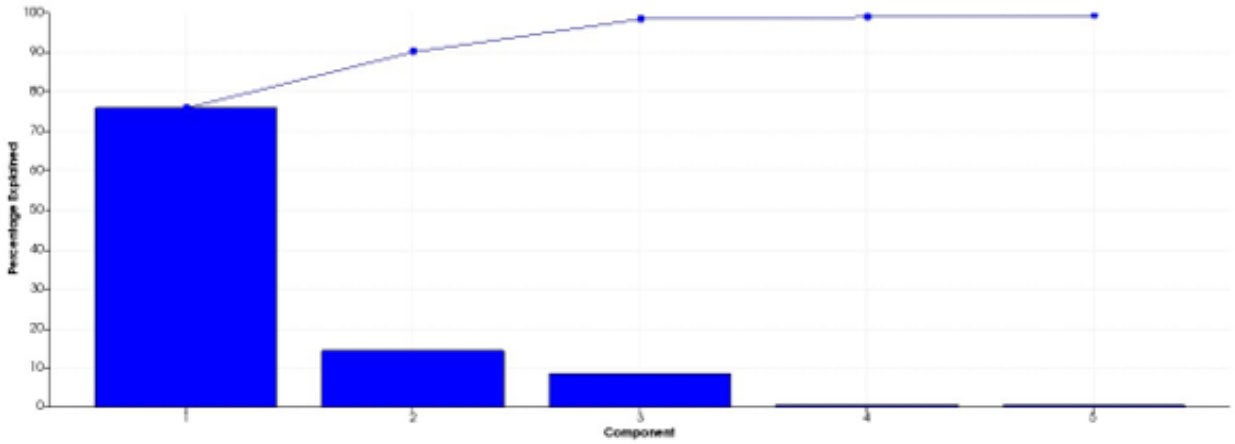
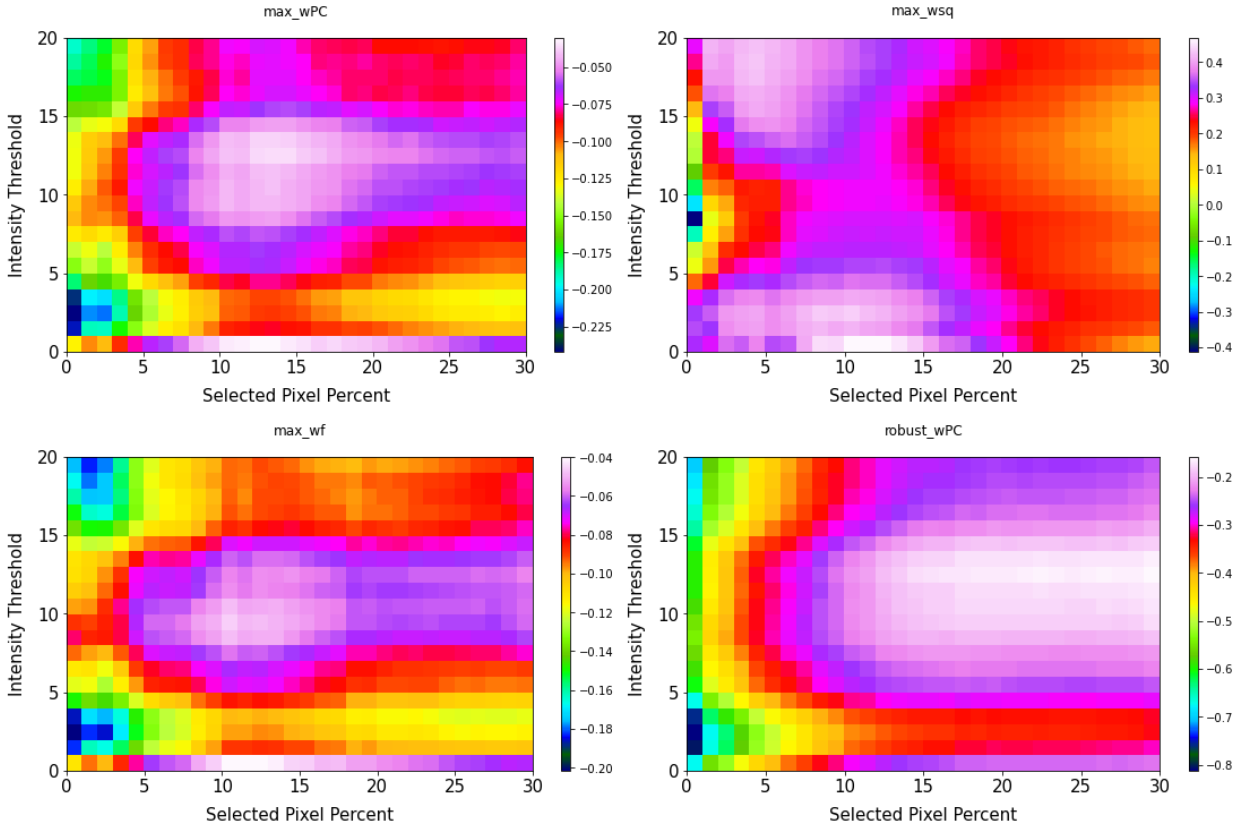


Figure S34: Pareto plot of explained variance for PC 1-5. Explained variance for PC 1-5 is as follows: 75.79%, 14.23%, 8.322%, 0.5471%, and 0.3682%. Cumulative sum of explained variance for PC 1-5 is as follows: 75.79%, 90.02%, 98.34%, 98.89%, and 99.25%.

E-index Plots

local normalization from non-normalized PCA results

All the E-values resulted in negative values except for when the squared weight function was used (a few points of the E_{m_wsq} resulted in negative values but mostly positive). These values were the result of a “Λ” shape meaning the mid region was found to have higher similarity than the low and high regions. This is likely due to the PCA failing to strongly correlate biological regions in the mouse brain image. The positive values seen with the weighted squared functions are a result of the inverse sum coefficient and the absolute value function carrying the negative signs. The absolute value function was used to help weed out the negative differences between the low/high regions and the mid, assuming most of the results were positive differences.



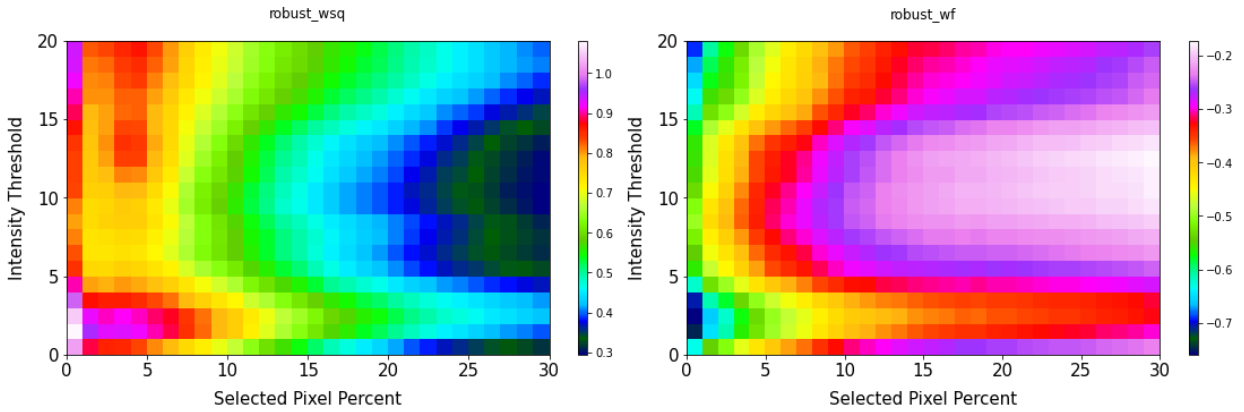
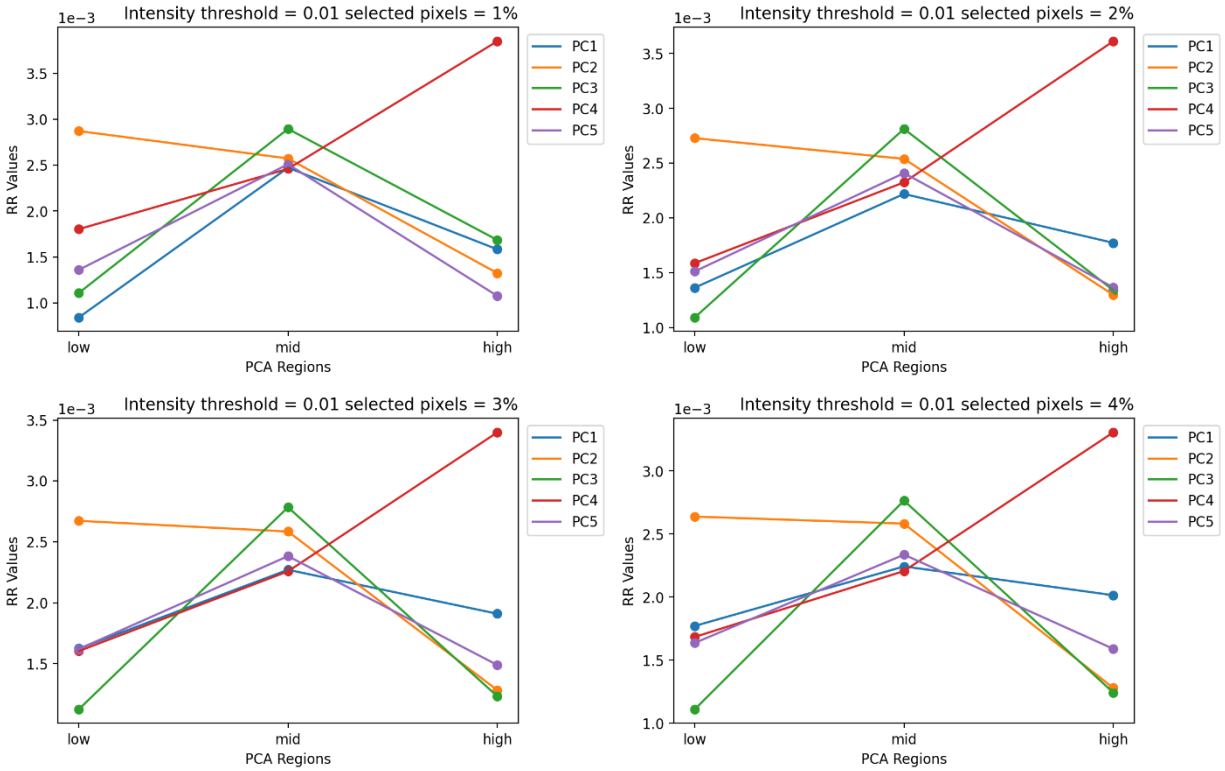


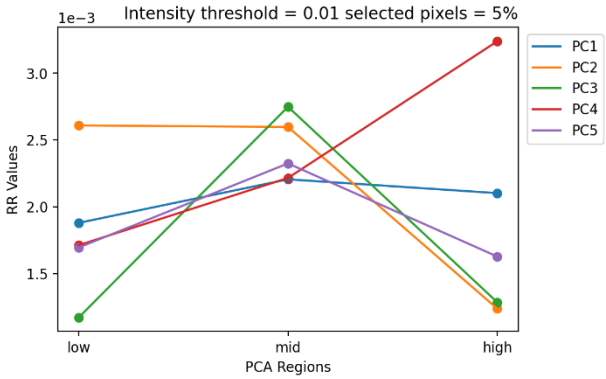
Figure S35: E-index plots for local normalization from non-normalized PCA results.

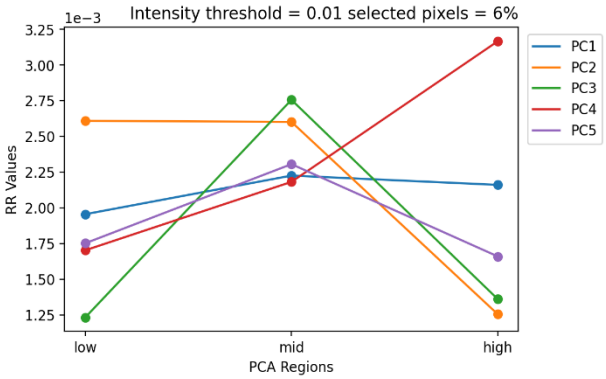
Russel-Rao 2-D V Plots

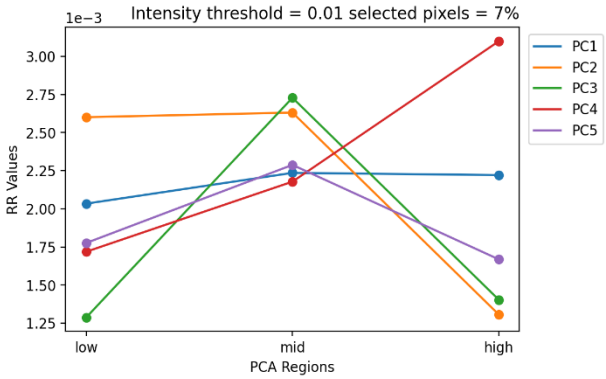
2-D V plots for local normalization with non-normalized PCA results

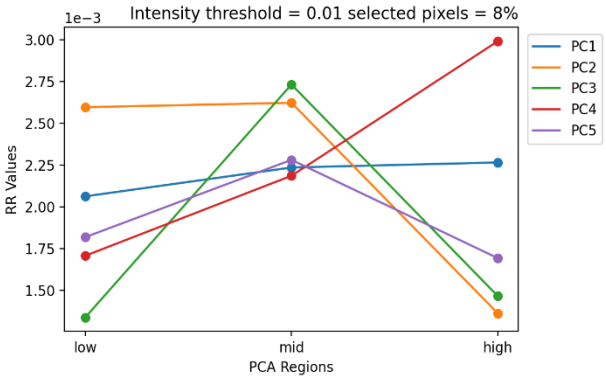
All tested combinations of normalization methods, intensity threshold, and selected pixel percentages results in the “Λ” shape.

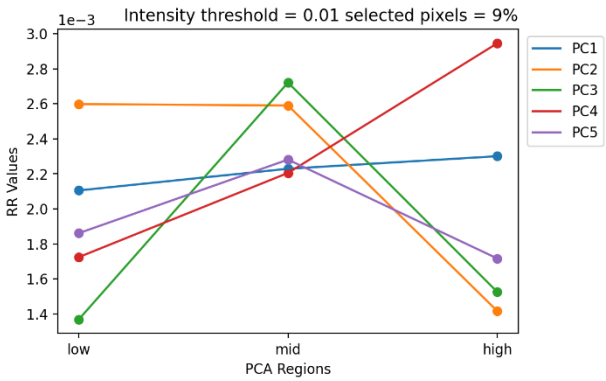


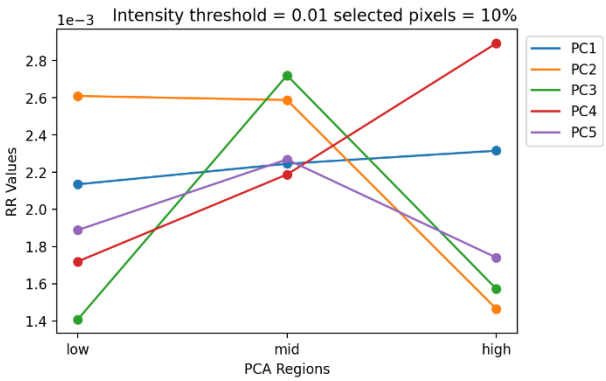

Intensity threshold = 0.01 selected pixels = 5%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

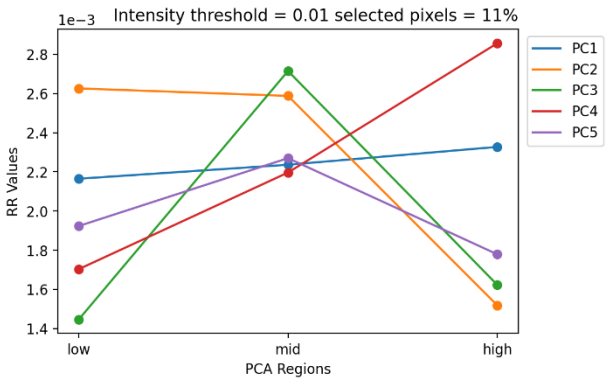

Intensity threshold = 0.01 selected pixels = 6%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

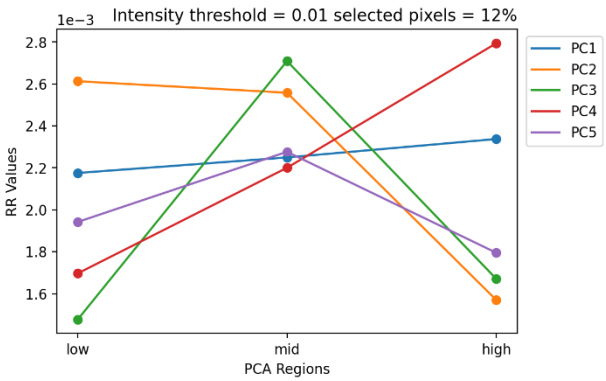

Intensity threshold = 0.01 selected pixels = 7%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

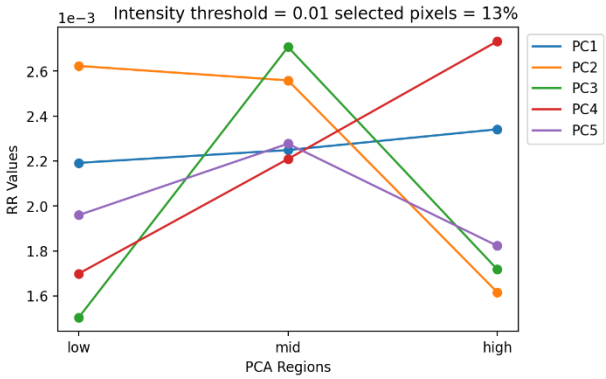

Intensity threshold = 0.01 selected pixels = 8%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

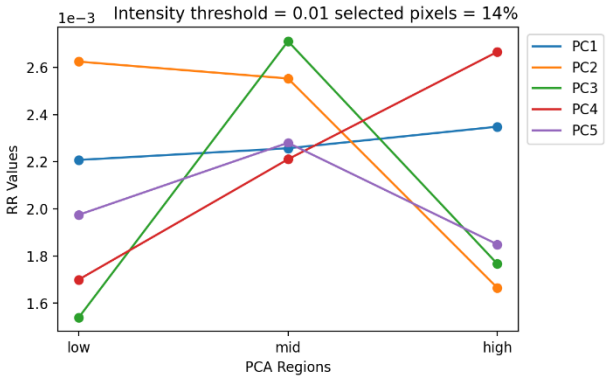

Intensity threshold = 0.01 selected pixels = 9%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

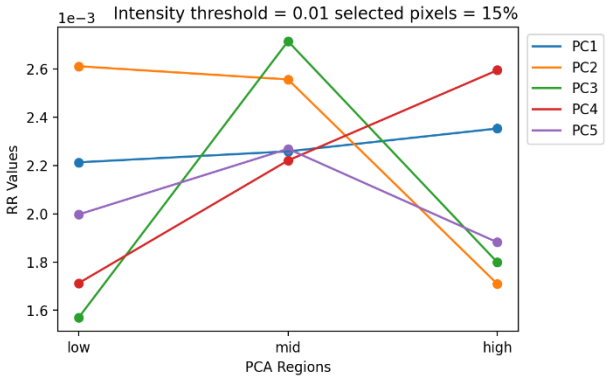

Intensity threshold = 0.01 selected pixels = 10%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

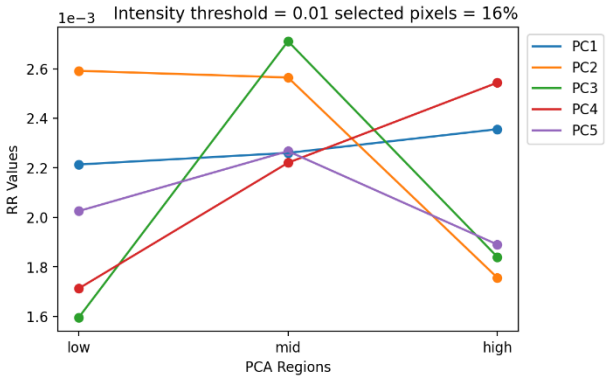

Intensity threshold = 0.01 selected pixels = 11%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

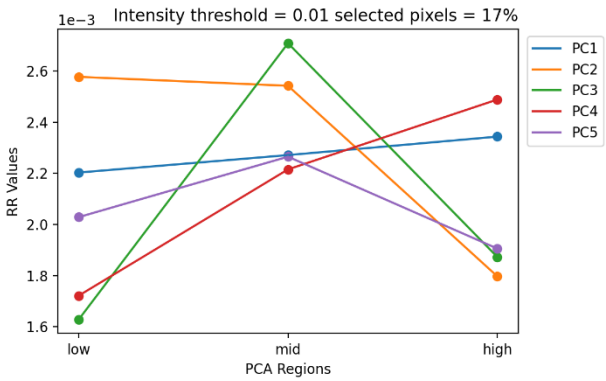

Intensity threshold = 0.01 selected pixels = 12%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

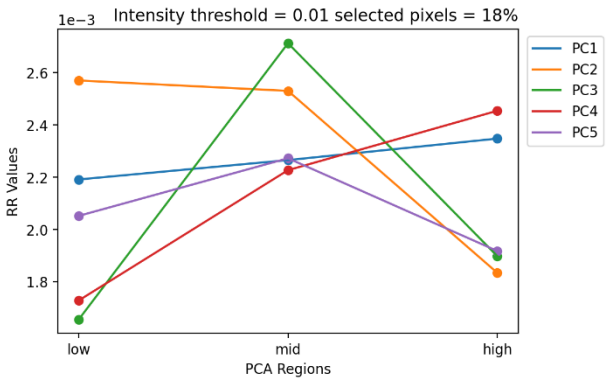

Intensity threshold = 0.01 selected pixels = 13%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

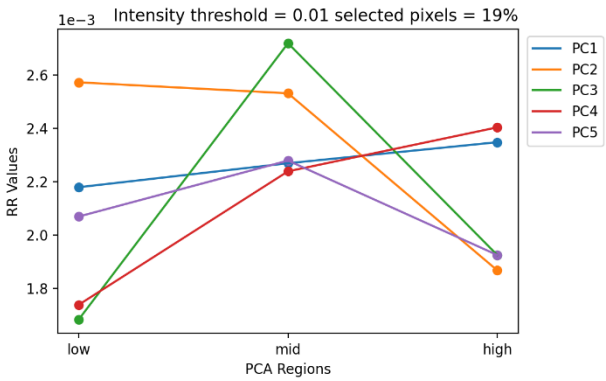

Intensity threshold = 0.01 selected pixels = 14%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

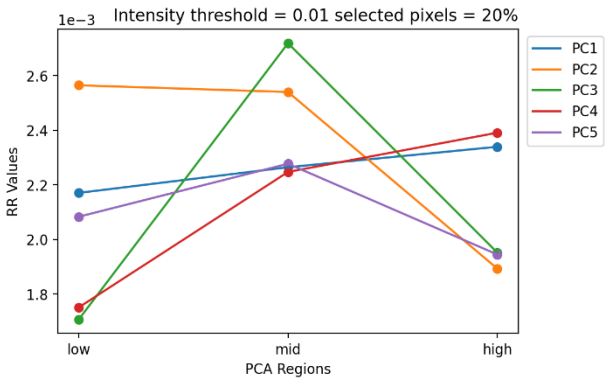

Intensity threshold = 0.01 selected pixels = 15%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

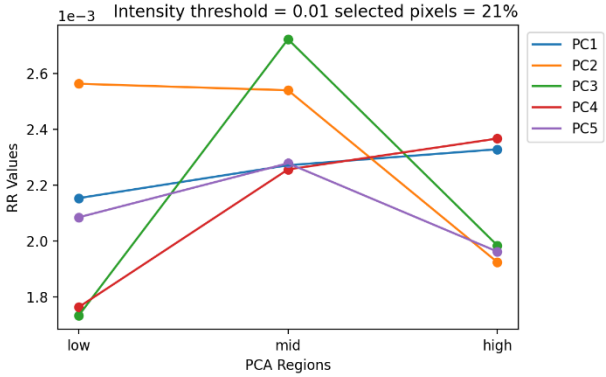

Intensity threshold = 0.01 selected pixels = 16%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

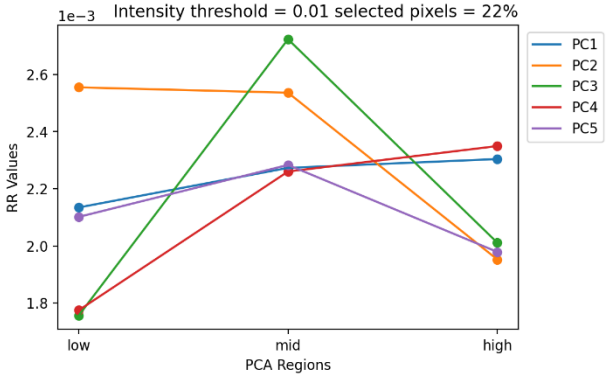

Intensity threshold = 0.01 selected pixels = 17%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

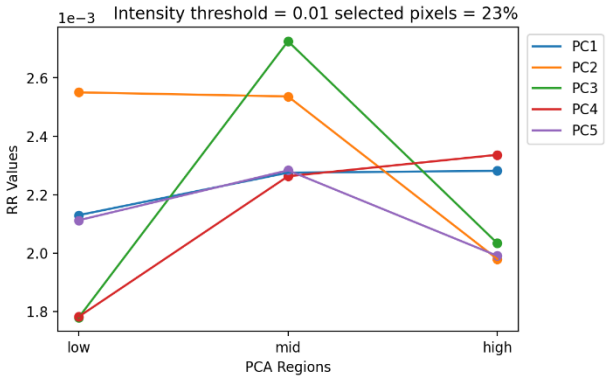

Intensity threshold = 0.01 selected pixels = 18%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

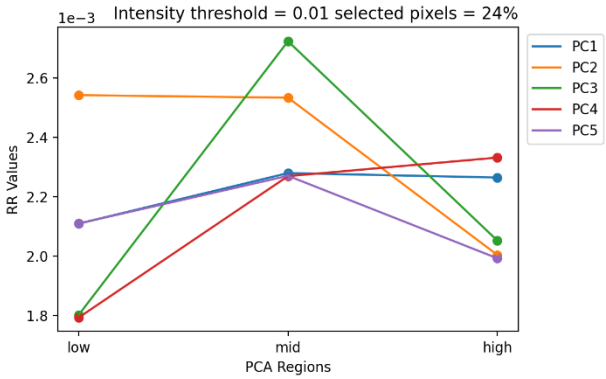

Intensity threshold = 0.01 selected pixels = 19%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

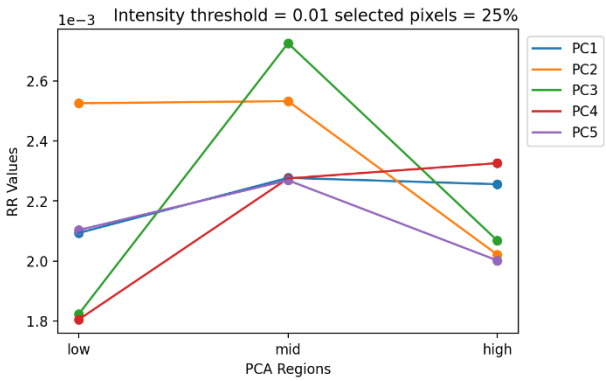

Intensity threshold = 0.01 selected pixels = 20%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

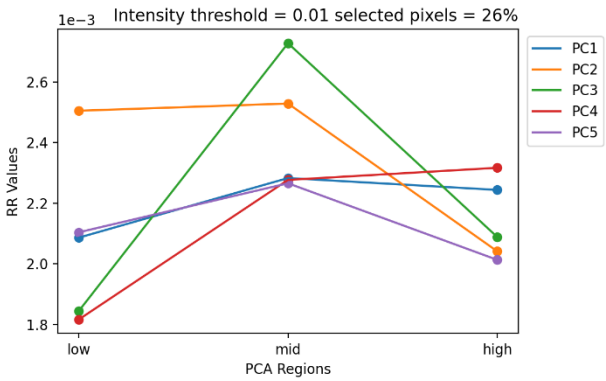

Intensity threshold = 0.01 selected pixels = 21%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

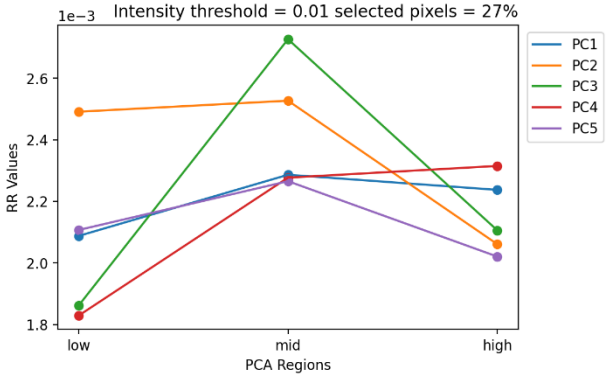

Intensity threshold = 0.01 selected pixels = 22%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

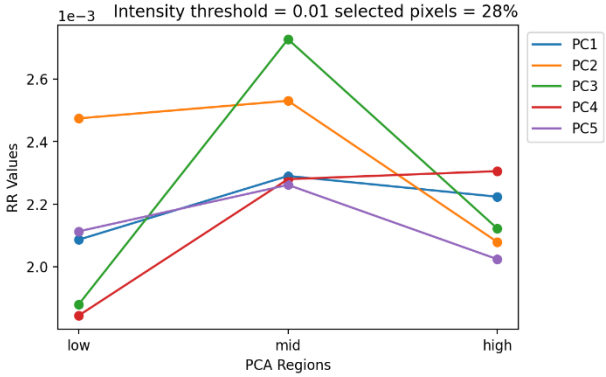

Intensity threshold = 0.01 selected pixels = 23%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 24%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 25%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 26%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 27%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5


Intensity threshold = 0.01 selected pixels = 28%
1e−3
RR Values
PCA Regions
low
mid
high
PC1
PC2
PC3
PC4
PC5

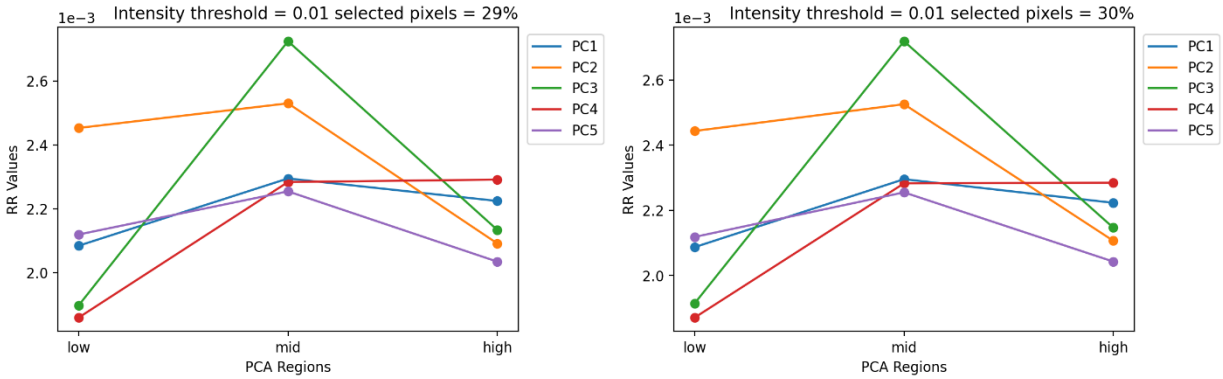


Figure S36: 2-D V plots for local normalization with non-normalized PCA results for intensity threshold 0.01

Mouse brain image C

Imaging conditions

Ten micrometer thick transverse mouse brain sections were prepared using CM 3050S cryostat (Leica Biosystems, Vista, CA) and stored in a -80°C freezer. The tissue sections were placed in the desiccator for 30 min prior to MALDI matrix application. A 2,5-Dihydroxyacetophenone (DHA) MALDI matrix layer was applied using a home-built sublimation apparatus. The mouse brain sections were then stored in the desiccator for another 30 min before matrix-assisted laser desorption/ionization (MALDI) imaging with a 7T solariX Fourier transform ion cyclotron resonance mass spectrometer (FTICR) (Bruker Daltonics, Billerica, MA). Imaging parameters were optimized for known lipid profiles[24] in positive ion mode between *m/z* 400-2000. A laser power of 20% with 50 shots per pixel was chosen. The resolving power for *m/z* 760.584 was 39520 based on the full width half maximum (FWHM) mass resolution. Spectra file sizes were set to 256 kB and 98% data reduction was done during acquisition. Spatial resolution and SmartWalk sampling pattern were set to 100 µm and the final image contained 6,763 pixels with a file size of 7.75 GB.

Principal component analysis

PCA was calculated using SCiLS Lab pro by Bruker Daltonics with no normalization, scaling, nor denoising.

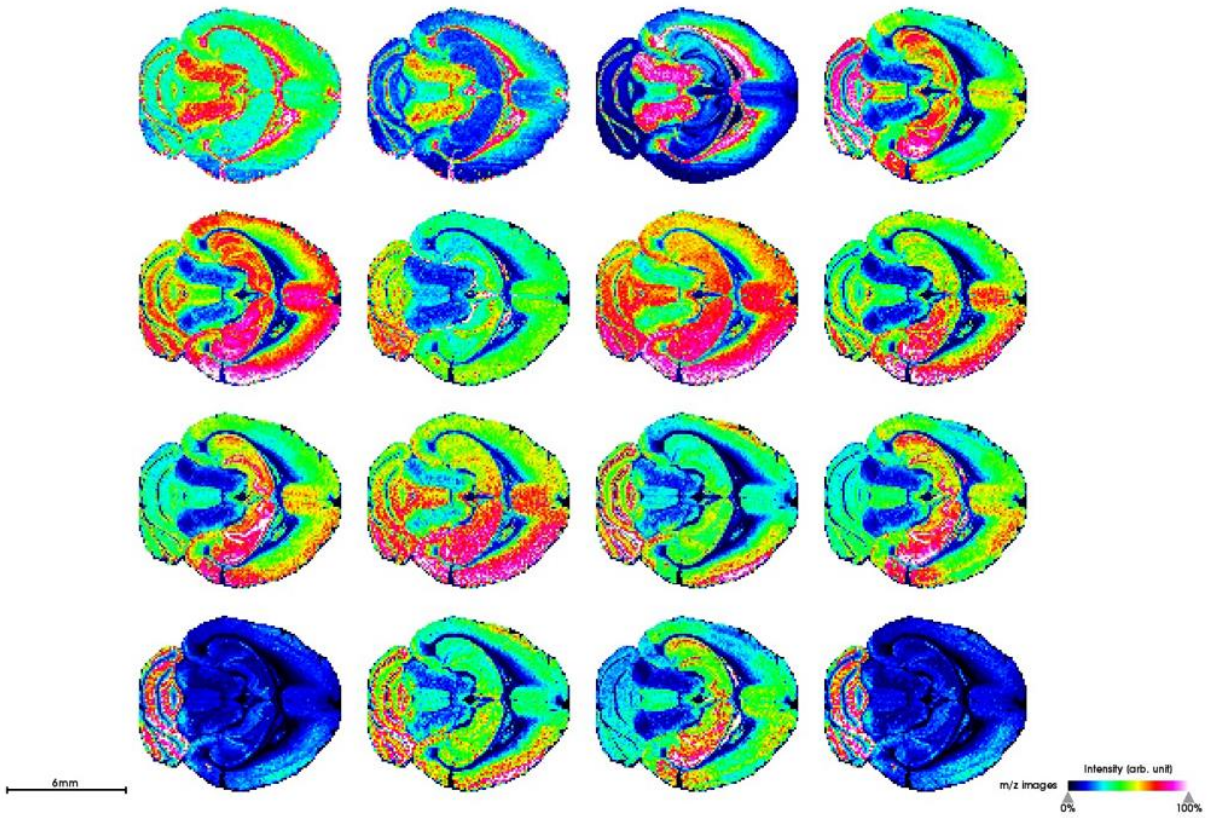


Figure S37: Ion images for *m/z* used in PCA. From top left to bottom right the *m/z* are as follows: 456.132, 473.195, 555.922, 731.278, 734.199, 758.568, 760.584, 772.460, 782.564, 798.542, 806.467, 810.454, 834.455, 844.468, 848.515, and 872.556.

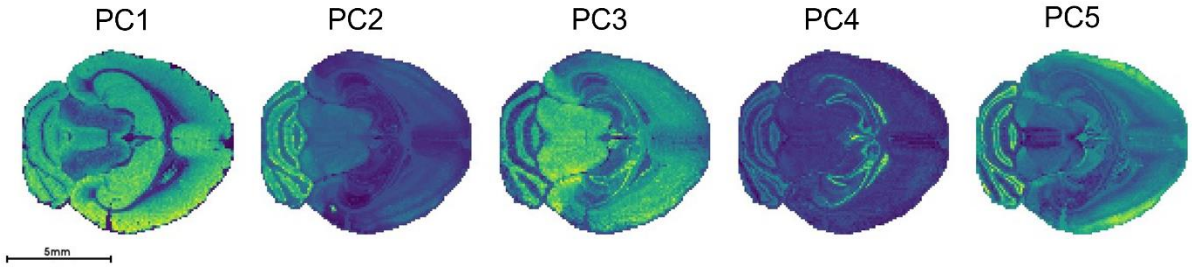


Figure S38: Spatial-expression images for PC 1-5.

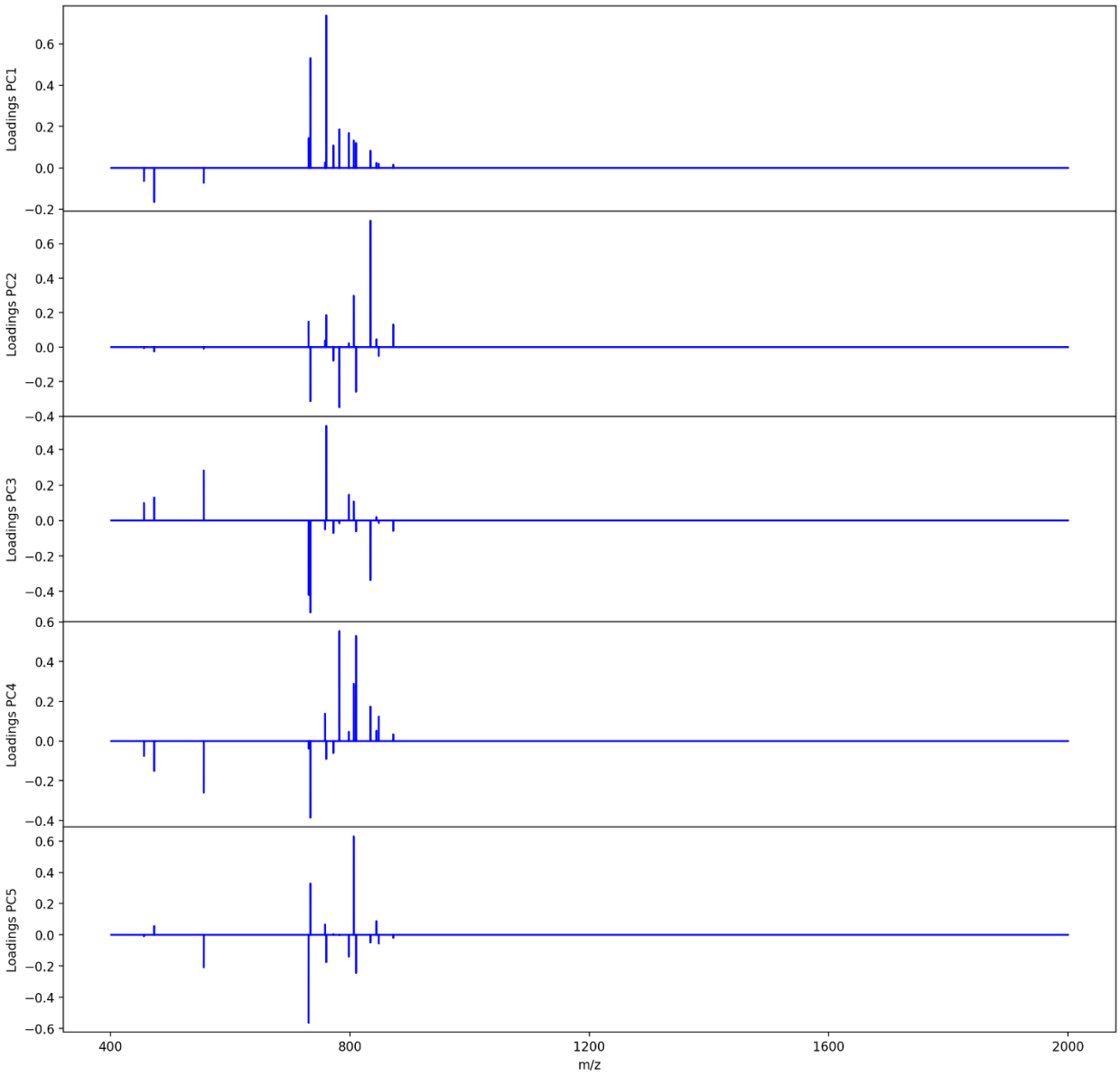


Figure S39: Pseudo-spectra for PC 1-5 from top to bottom, respectively.

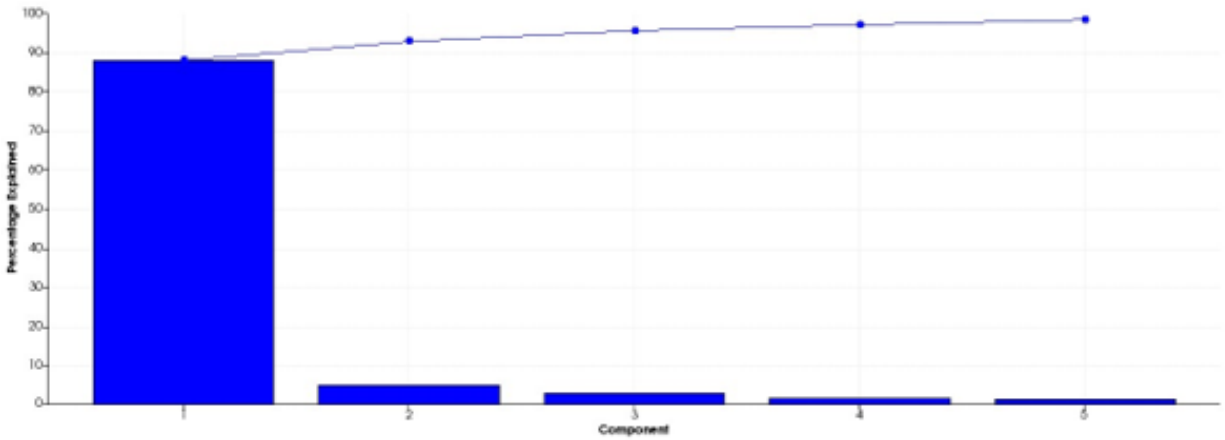
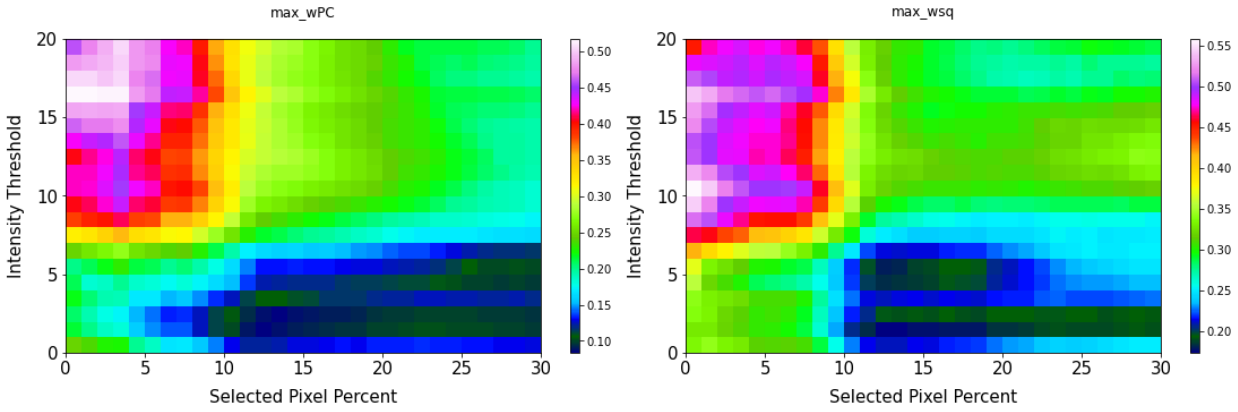


Figure S40: Pareto plot for the explained variance of each PC. Explained variance for PC 1-5 are 87.97%, 4.867%, 2.782%, 1.508%, and 1.286%, respectively. The cumulative sum of the explained variance is as follows: 87.97%, 92.84%, 95.62%, 97.13%, and 98.41%.

E-index Plots

local normalization from non-normalized PCA results



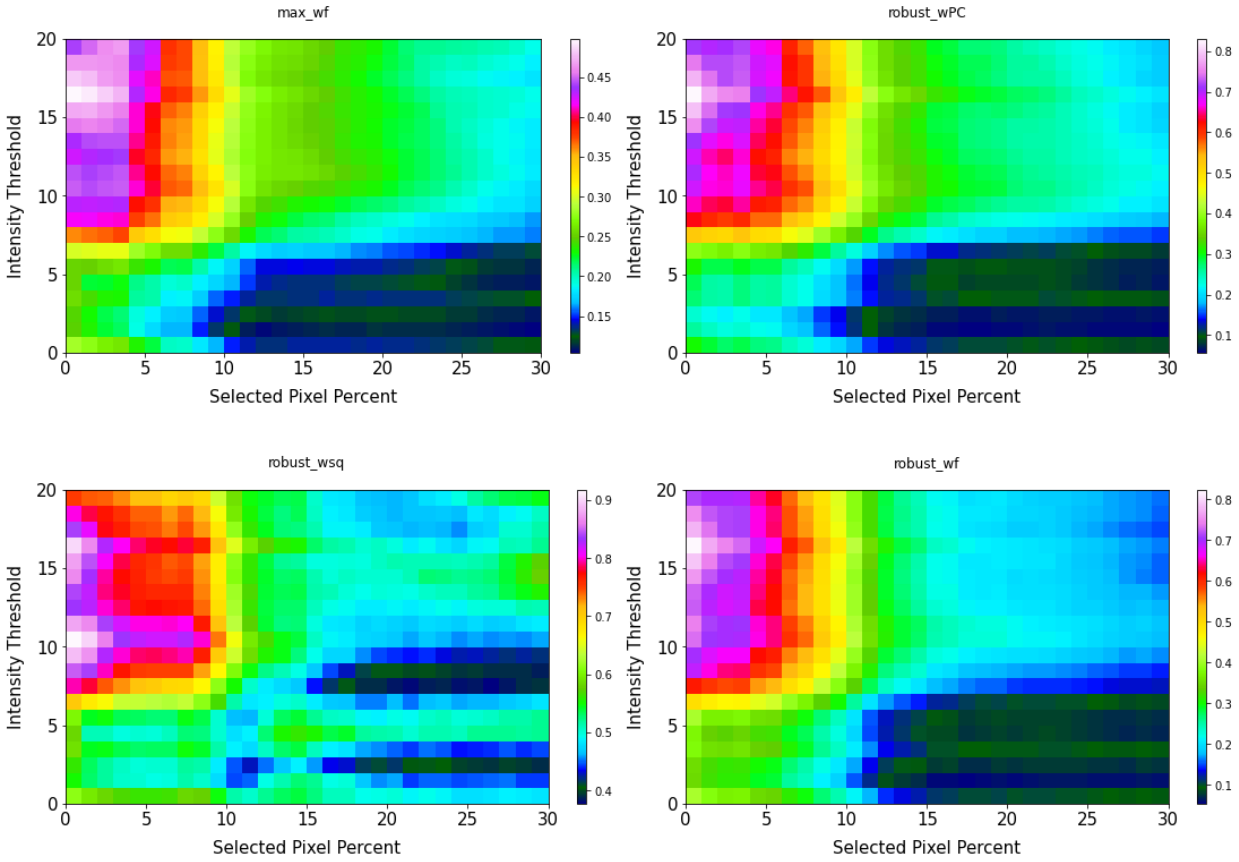


Figure S41: E-index results for local normalization from non-normalized PCA. Both functions of the E-index and all three types of weighted functions result in a local maximum tend. The local maximum is typically with 5-10% selected pixels. The largest local maximum from the E_{m_wsq} (intensity threshold = 0.17 and selected pixel percent = 7) resulted in the optimal set of parameters for calculation.

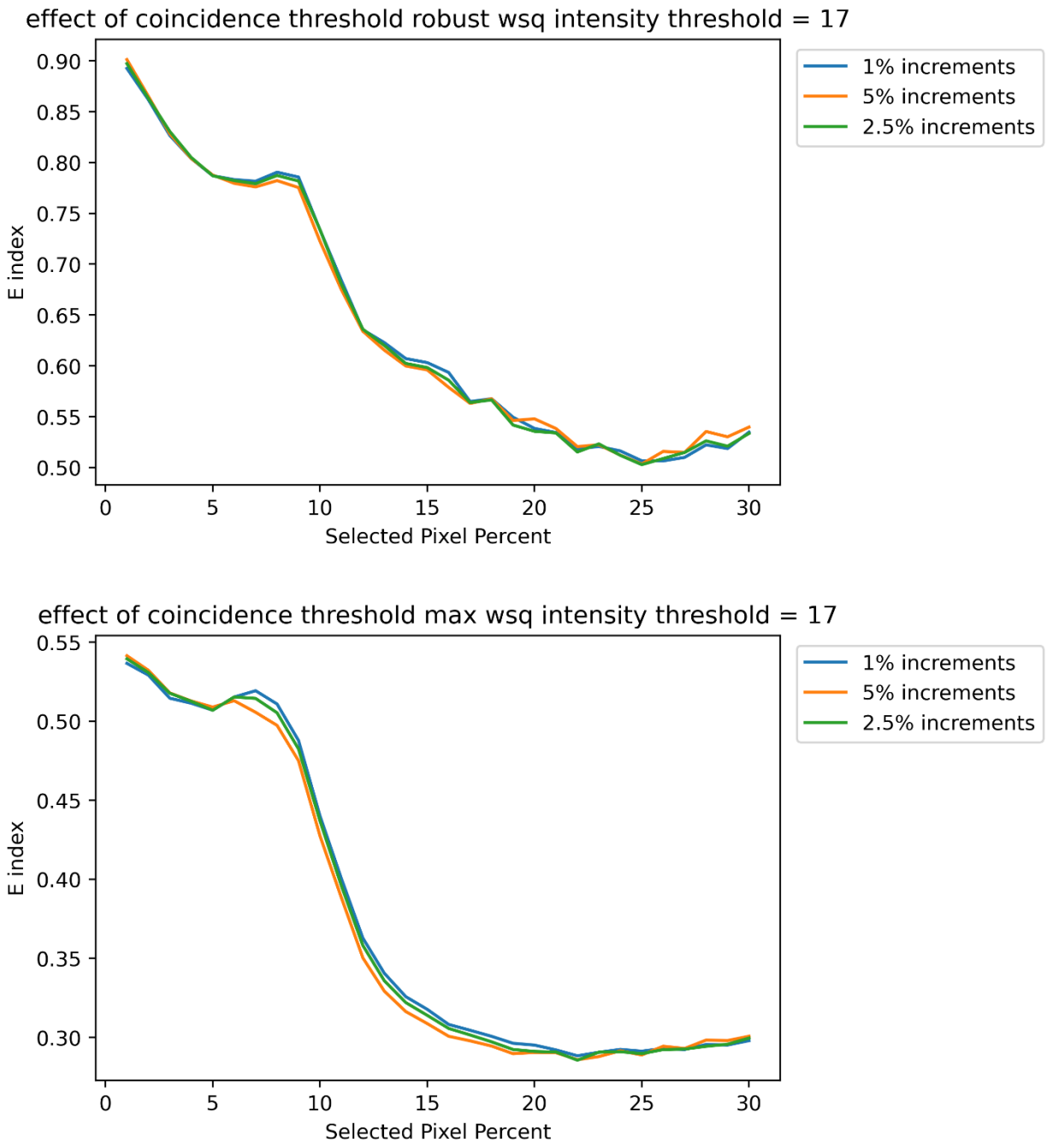


Figure S42: The coincidence threshold was tested to see its effect on the E-index. Originally 5% increments from [*n*mod2, *n*-1] was chosen. As the increments are shortened the local maximum becomes more apparent, potentially indicating a better estimate of the optimal parameters when more coincidence thresholds are used. Local maximum for 5% increments was found to be at 6% and the local maximum for 1% increments was found to be 7%.

Russel-Rao 2-D V Plots

2-D V plots for local normalization with non-normalized PCA results

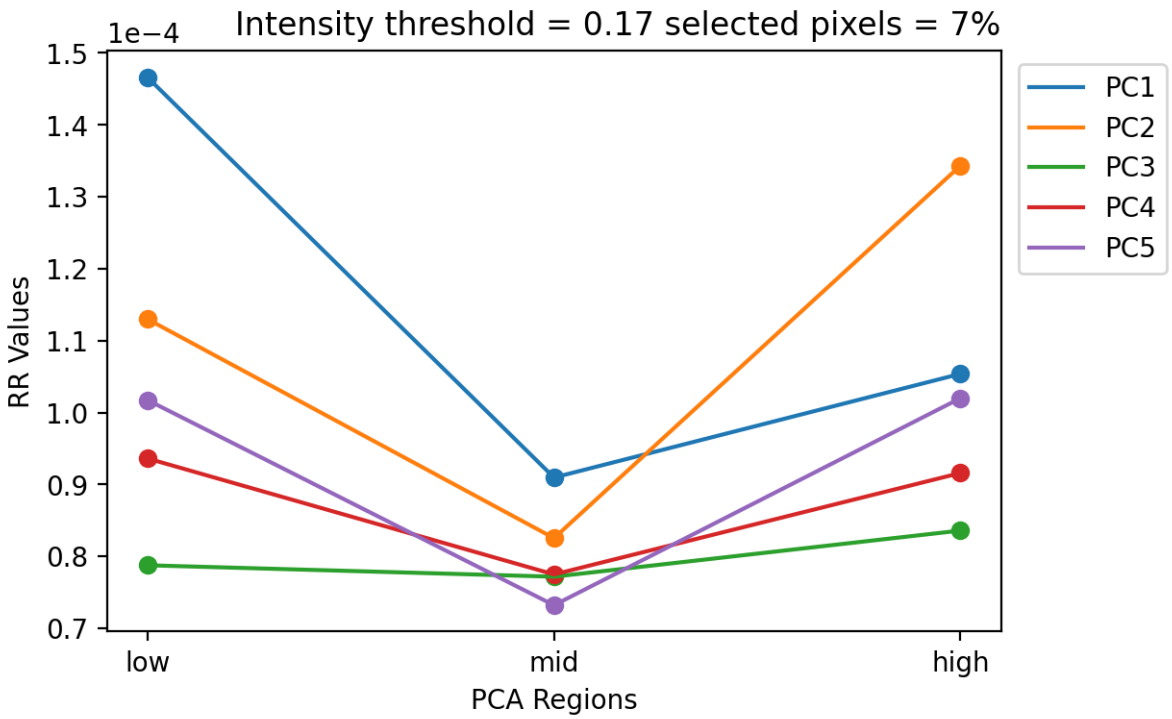


Figure S43: 2-D V plot of optimal parameters from the E-index calculations.

Russel-Rao 3-D V Plots

3-D V plots for local normalization with non-normalized PCA results

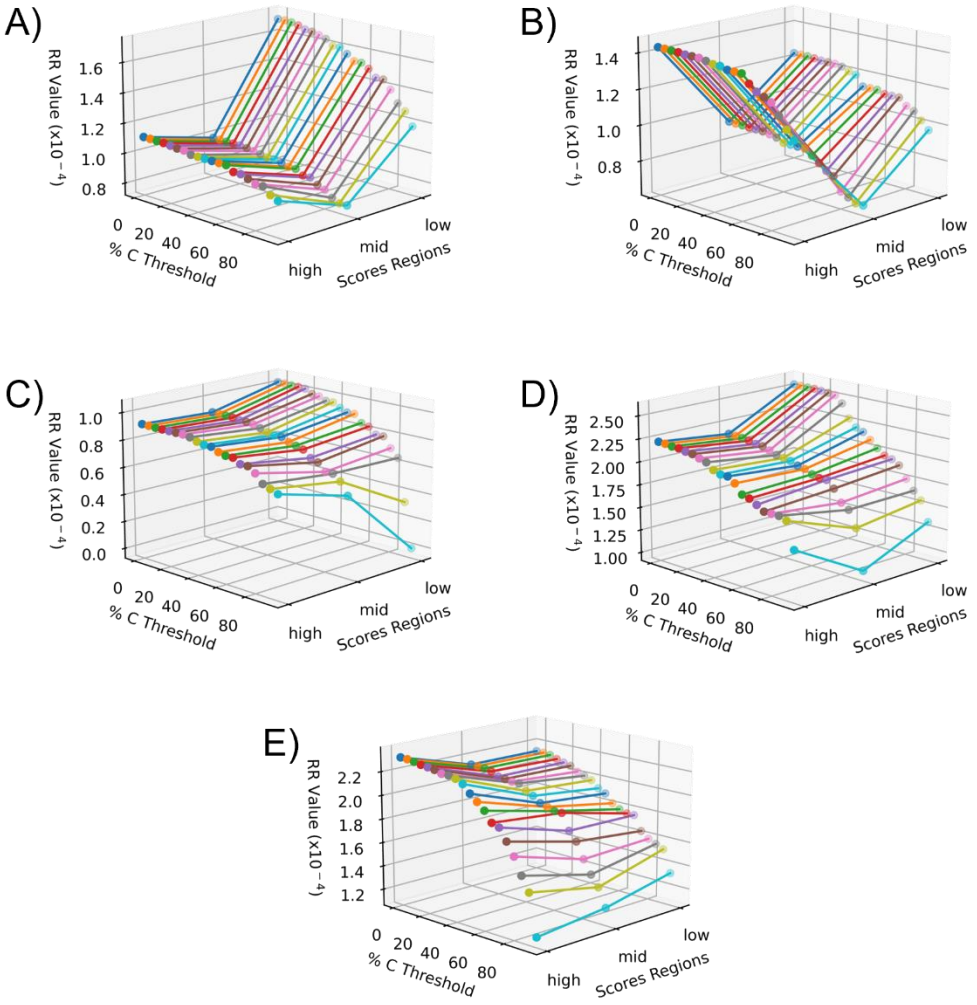


Figure S44: 3-D V plots for optimal parameters intensity threshold of 0.17 and selected pixels percentage of 7%.

Rat kidney image dataset

Imaging conditions

Ten micrometer thick transverse rat kidney sections were prepared using CM 3050S cryostat (Leica Biosystems, Vista, CA) and stored in a -80°C freezer. The tissue sections were placed in the desiccator for 30 min prior to MALDI matrix application. A 1,5- diaminonapthalene (DAN) MALDI matrix layer was applied using a TM Sprayer (HTX Technologies, Chapel Hill, NC). Spray conditions were as follows: 10 mg/mL of DAN in 9/1 acetonitrile/water, 30°C nozzle temperature, 6 passes, 0.1 mL/min flow rate, and 25 mm track spacing. The rat kidney sections were then stored in the desiccator for another 30 min before matrix-assisted laser desorption/ionization (MALDI) imaging with a 7T solariX Fourier transform ion cyclotron resonance mass spectrometer (FTICR) (Bruker Daltonics, Billerica, MA). The kidney images were acquired using a pixel spacing of 60 µm in both the x- and y-dimensions using a laser power of 50% with 100 shots per pixel. Imaging parameters were collected in negative ion mode between *m/z* 400-2000. Spectra file sizes were set to 256 kB and 98% data reduction was done during acquisition. The final image contained 29,255 pixels with a file size of 2.34 GB.

Principal component analysis

PCA was calculated using SCiLS Lab pro by Bruker Daltonics with no normalization, scaling, nor denoising.

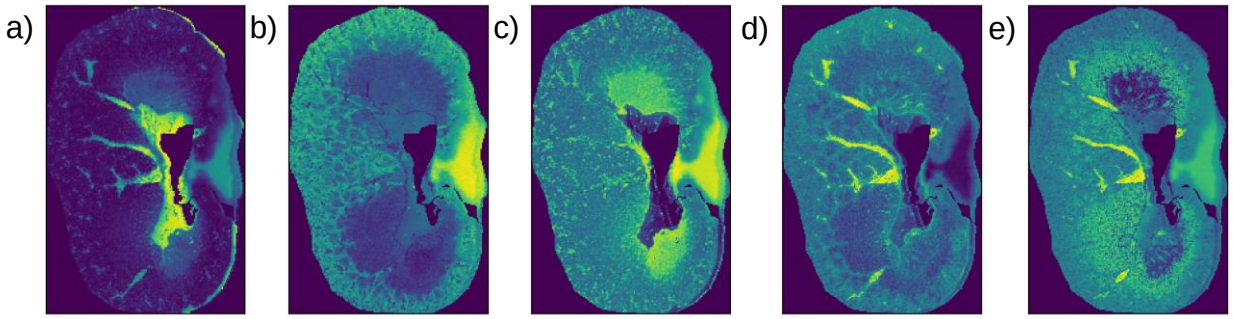


Figure S45: PCA spatial expression images of first five PCs. Spatial expression images for a) PC 1, b) PC2, c) PC3, d) PC4, e) PC5 of a rat kidney. Brighter regions in the spatial expression images correspond to more positive PCA score values and darker regions correspond to more negative score values.

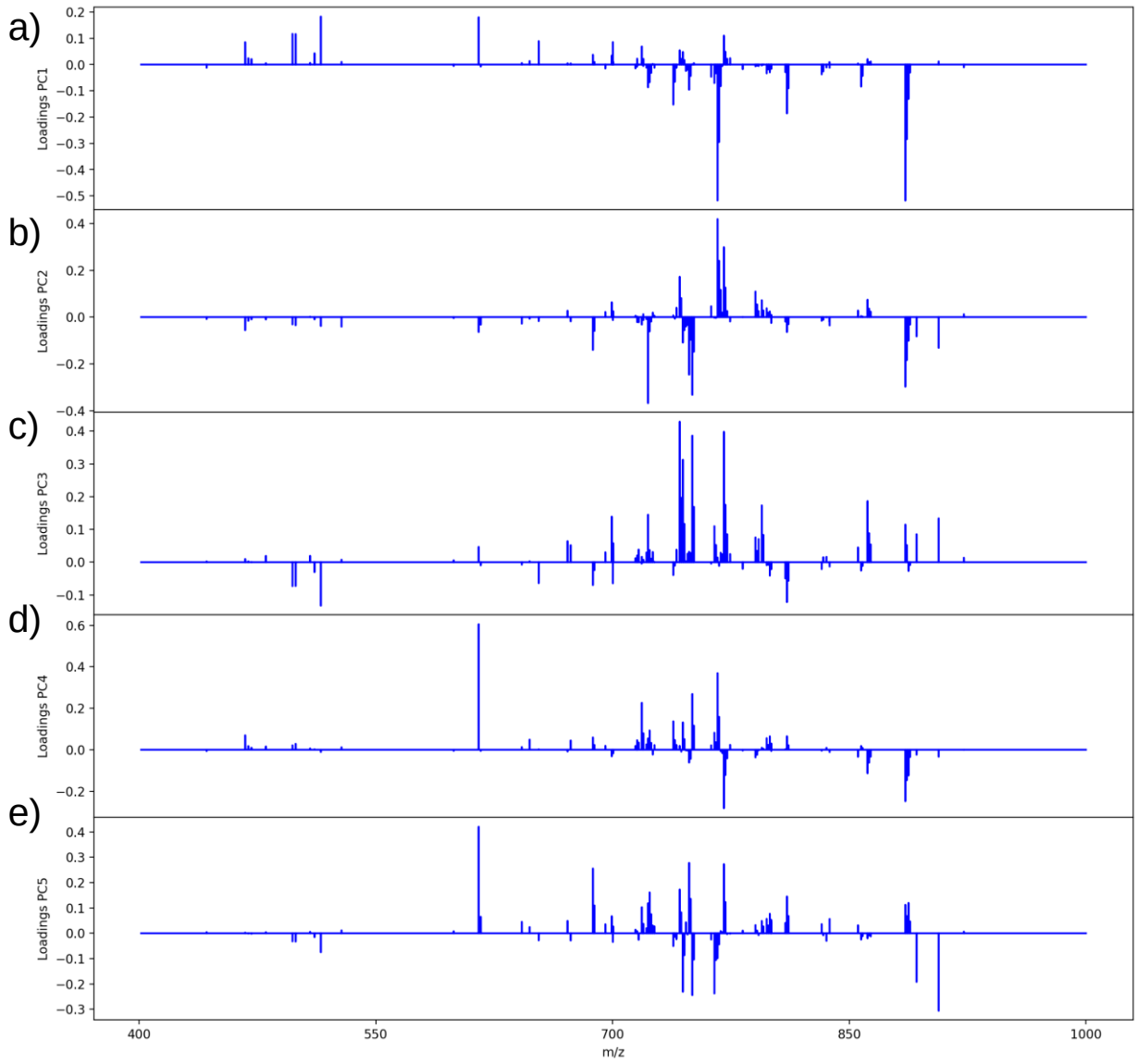


Figure S46: PCA pseudo-spectra of first five PCs. Pseudo-spectra are shown for a) PC 1, b) PC2, c) PC3, d) PC4, e) PC5. Loadings of the same sign correspond to greater positive correlation within the PC and loadings of opposite signs correspond to greater negative correlation within the PC.

E-index Plots

local normalization from RMS pre-processed PCA results

In this rat kidney IMS dataset, the first 1% of selected pixels always gave the largest E-index value for a particular intensity threshold, regardless of which combination of functions were used.

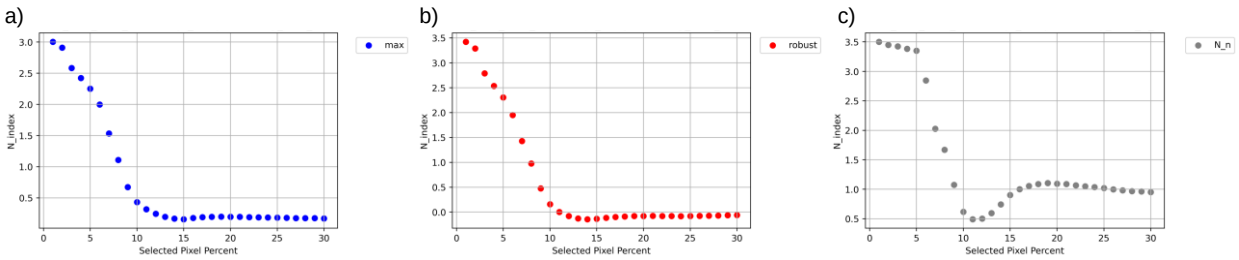


Figure S47: E-index of a) the maximum, b) the robust, and c) weight function were tested for relative comparison.

Russel-Rao 2-D V Plots

2-D V plots for local normalization with RMS pre-processed PCA results

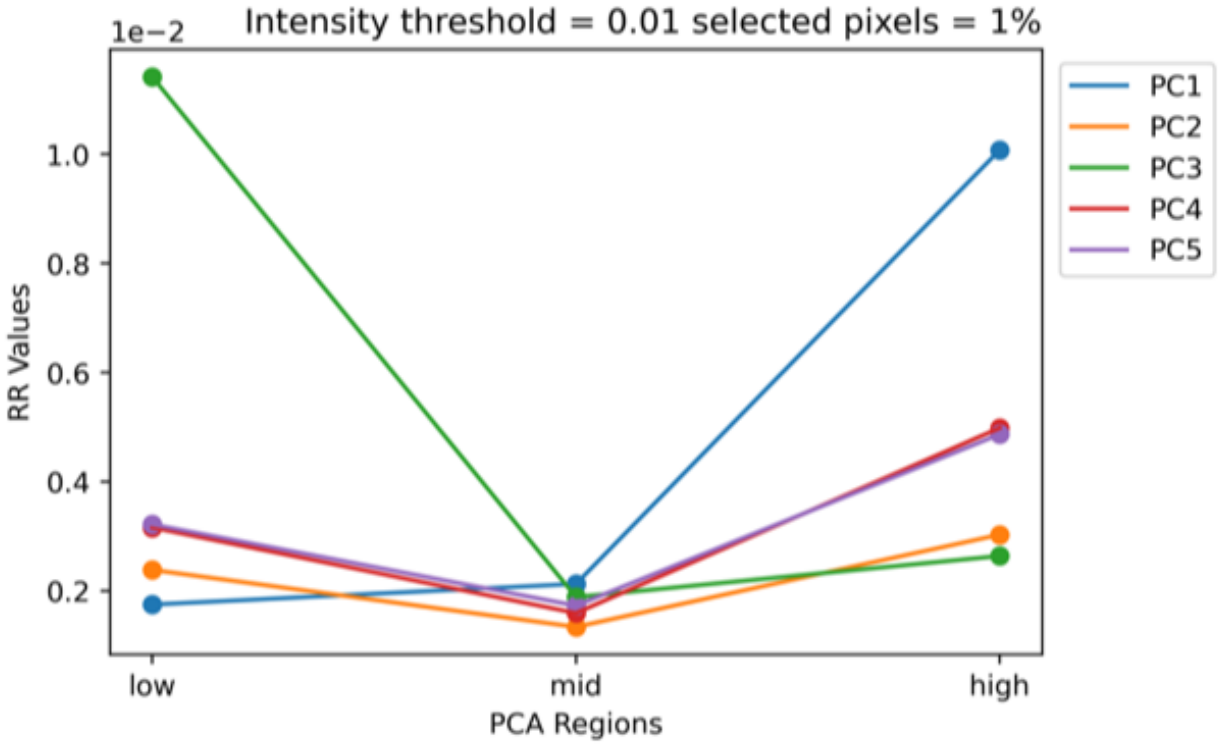


Figure S48: 2-D V plot of averaged extended similarity coefficients as a function of group for each PC.

Russel-Rao 3-D V Plots

3-D V plots for local normalization with RMS pre-processed PCA results

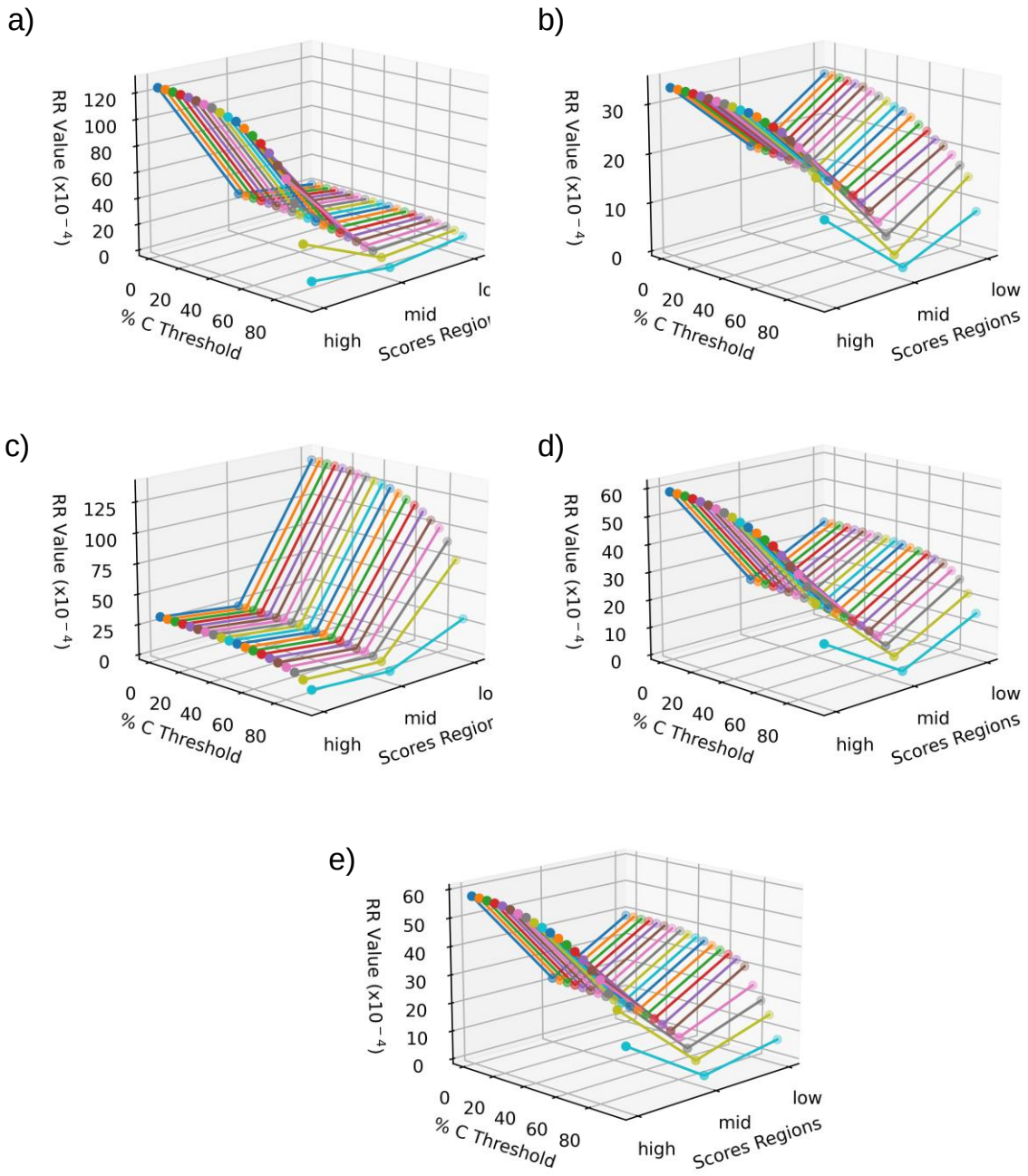


Figure S49: 3-D V plots for optimal parameters intensity threshold of 0.01 and selected pixels percentage of 1%. Extended similarity indices as a function of region and percent coincidence threshold for a) PC 1, b) PC2, c) PC3, d) PC4, e) PC5.

Spatial Distribution of Selected Pixels

The spatial distribution patterns for all the score groups confirm the extended similarity indices' ability to discern biological regions of PCA correlated spectra.

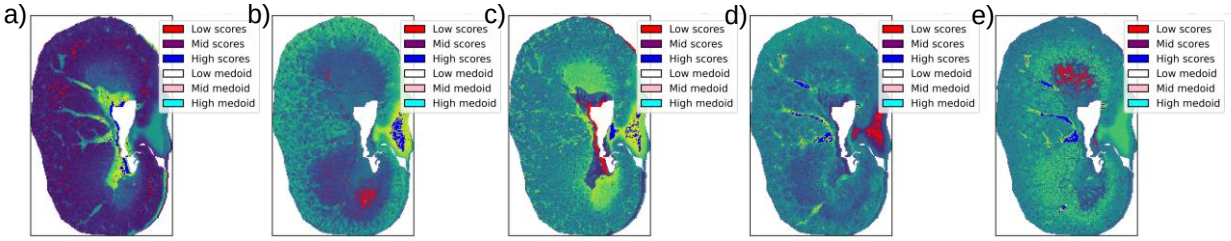


Figure S50: Selected pixels of optimal extended similarity indices parameters for a) PC 1, b) PC2, c) PC3, d) PC4, e) PC5.

Medoid Spectra

Local normalization with 1% selected pixels, Intensity threshold 0.01, and RMS normalizes PCA results

Many of the loadings from PCA are properly correlated with their respective peaks in the binary fingerprints, such as those seen in PC 1.

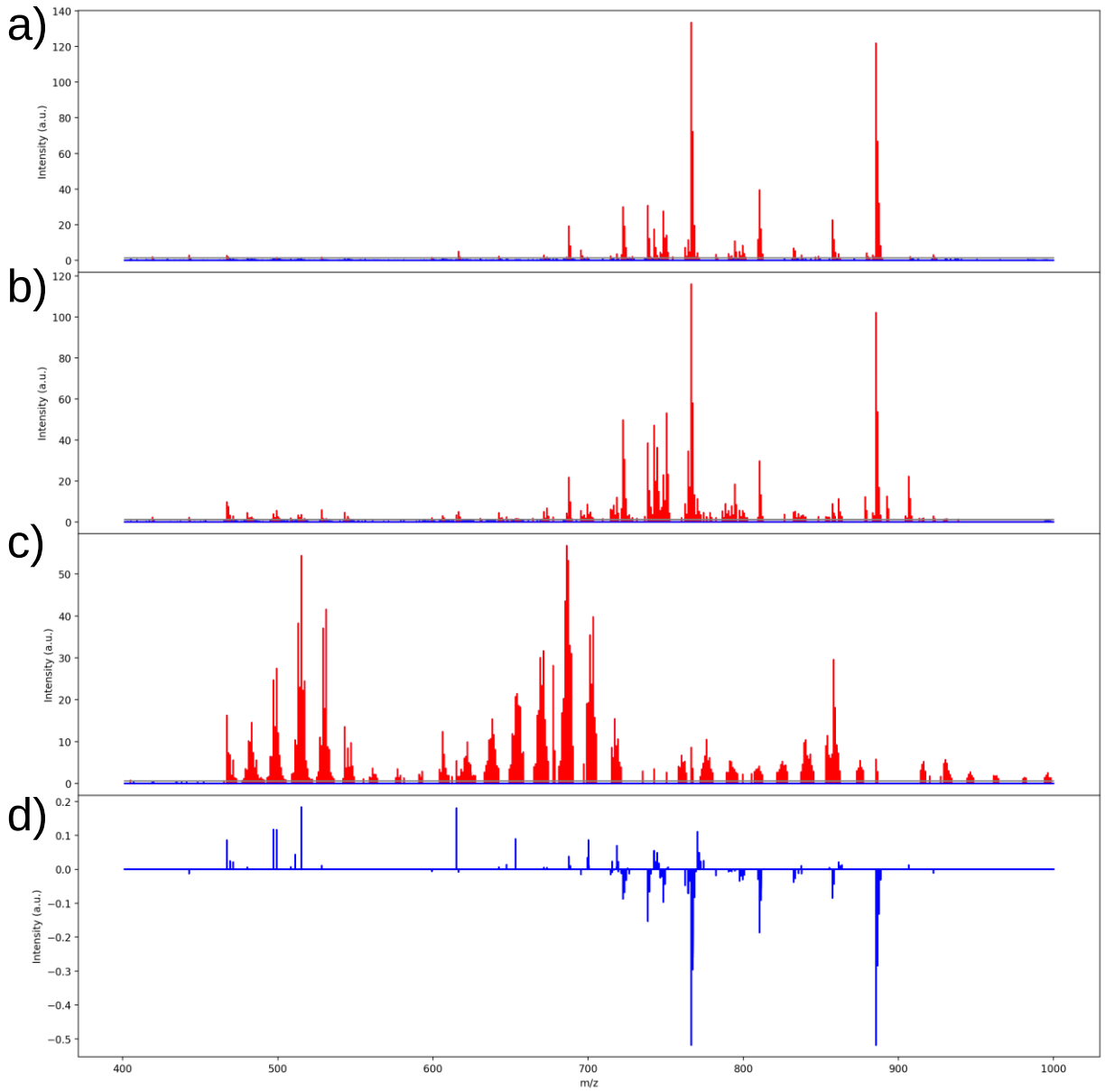


Figure S51: Medoid spectra of the three score groups for PC 1. a) Low group medoid spectrum for PC 1, b) mid group medoid spectrum for PC 1, c) high group medoid spectrum for PC 1, d) pseudo-spectrum for PC 1.

Rat brain image dataset

Imaging conditions

Ten micrometer thick transverse rat brain sections were prepared using CM 3050S cryostat (Leica Biosystems, Vista, CA) and stored in a -80°C freezer. The tissue sections were placed in the desiccator for 30 min prior to MALDI matrix application. A 1,5- diaminonapthalene (DAN) MALDI matrix layer was applied using a TM Sprayer (HTX Technologies, Chapel Hill, NC). Spray conditions were as follows: 10 mg/mL of DAN in 9/1 acetonitrile/water, 30°C nozzle temperature, 6 passes, 0.1 mL/min flow rate, and 25 mm track spacing. The rat brain sections were then stored in the desiccator for another 30 min before matrix-assisted laser desorption/ionization (MALDI) imaging with a 7T solariX Fourier transform ion cyclotron resonance mass spectrometer (FTICR) (Bruker Daltonics, Billerica, MA). The rat brain images were acquired using a pixel spacing of 43 µm in both the x- and y-dimensions using a laser power of 60% with 200 shots per pixel. Imaging parameters were collected in positive ion mode between *m/z* 400-1000. Spectra file sizes were set to 128 kB and 98% data reduction was done during acquisition. The final image contained 103,427 pixels with a file size of 126 GB.

Principal component analysis

PCA was calculated using SCiLS Lab pro by Bruker Daltonics with no normalization, scaling, nor denoising.

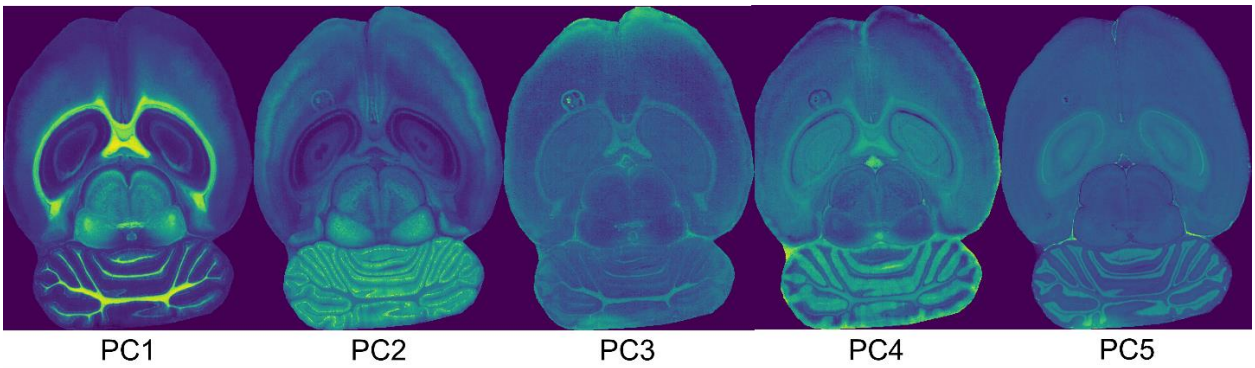


Figure S52: PCA spatial expression images of first five PCs. Spatial expression images for PC 1, PC2, PC3, PC4, PC5 of a rat brain. Brighter regions in the spatial expression images correspond to more positive PCA score values and darker regions correspond to more negative score values.