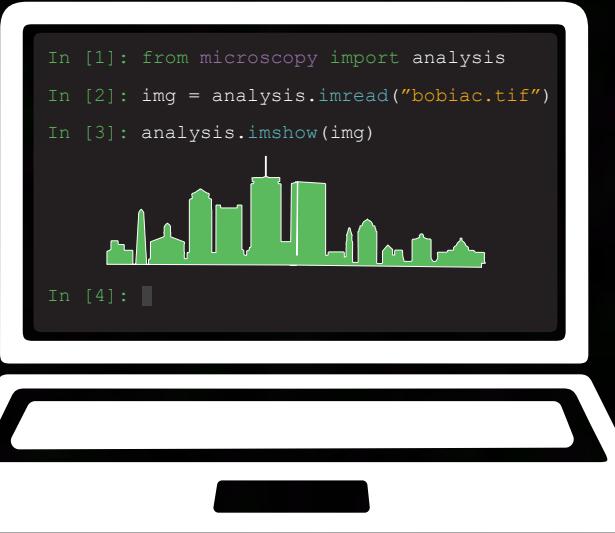


Bo BiAC

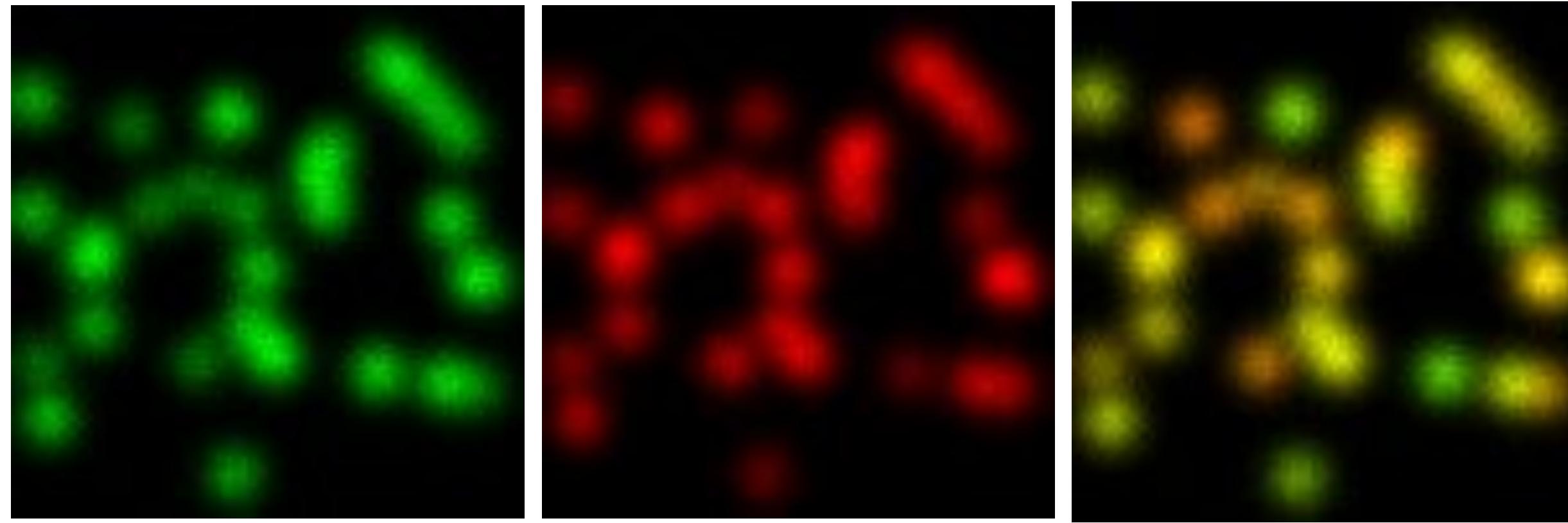
Boston Bioimage Analysis Course | 2025



Introduction to Colocalization in Fluorescence Microscopy



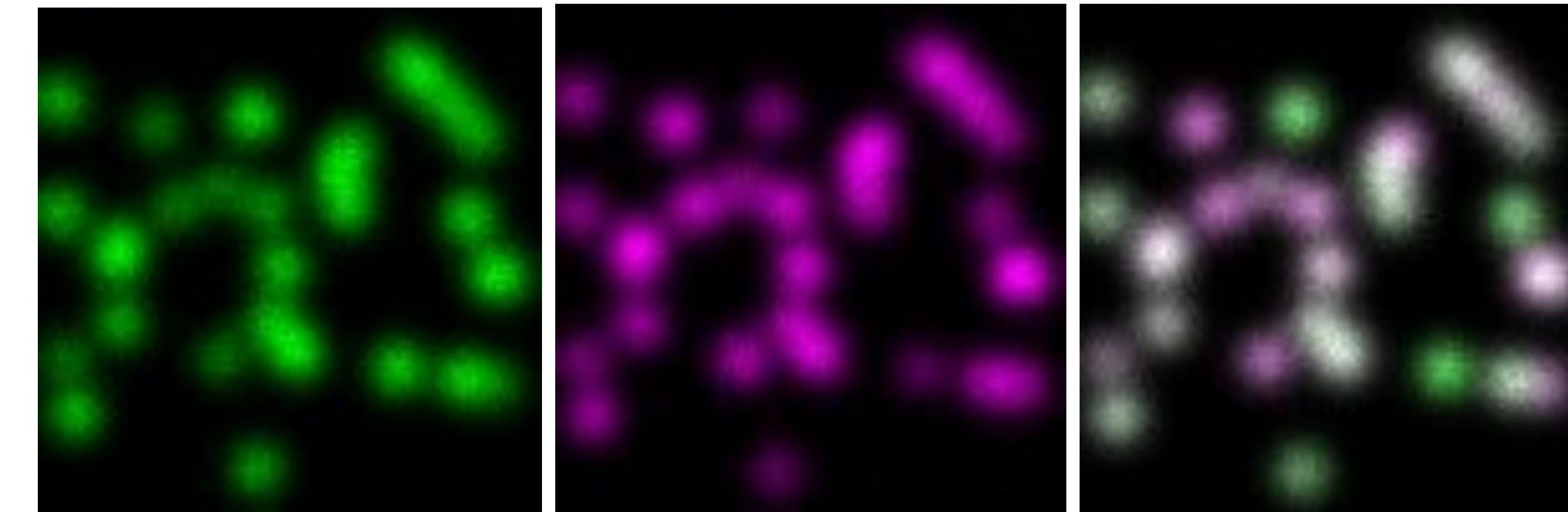
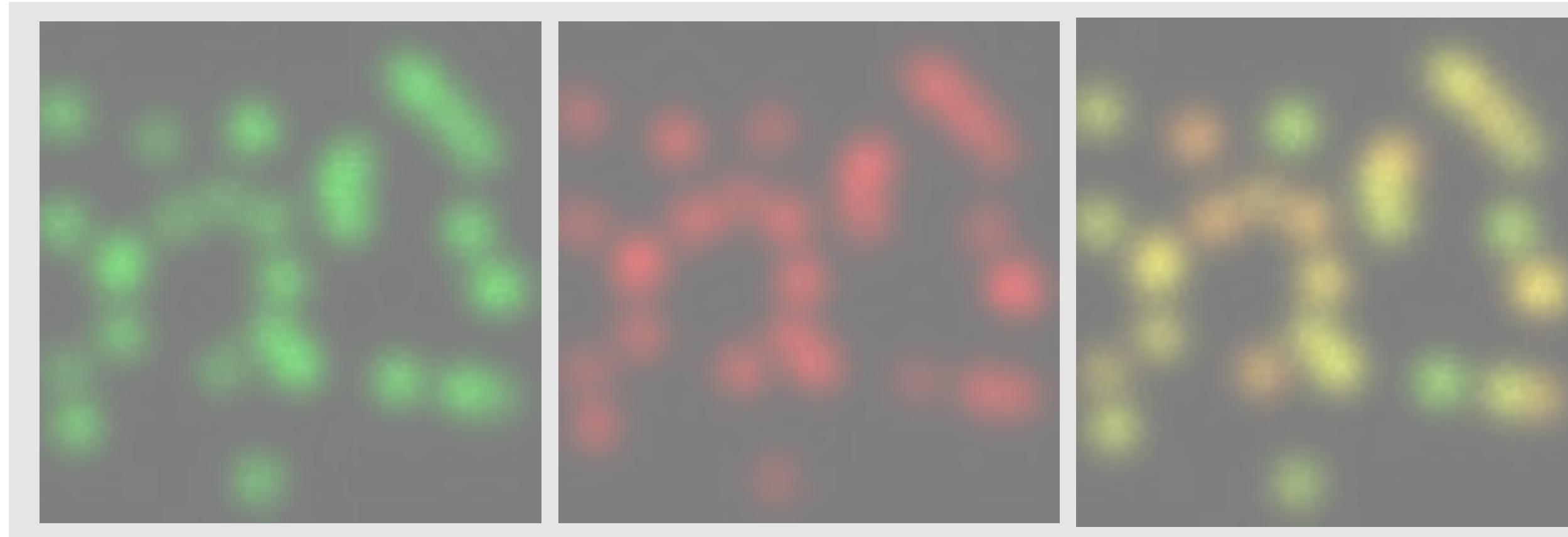
What Colocalization **IS NOT** in Fluorescence Microscopy



“Yellow” is **not** colocalization
Why?



What Colocalization **IS NOT** in Fluorescence Microscopy



“Yellow” is **not** colocalization

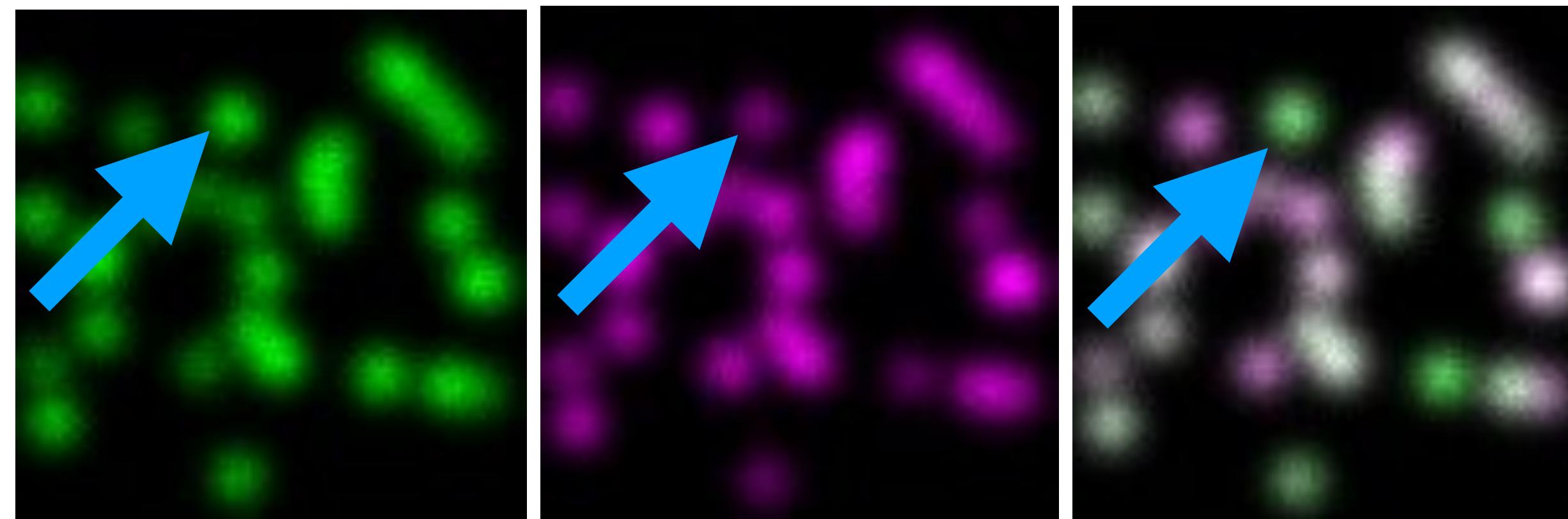
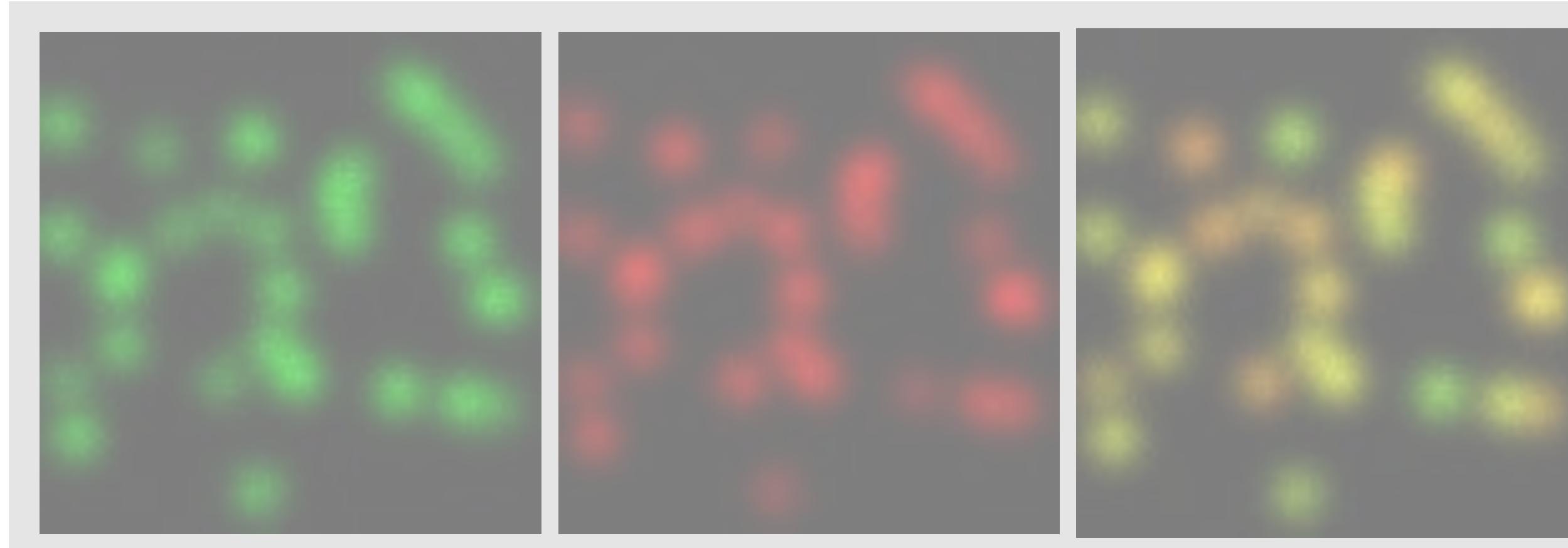
Why?

1. you should never see yellow because you should **not use red and green together.**

In Fiji: *Image > Color > Dichromacy* or *Image > Color > Simulate Color Blindness*



What Colocalization **IS NOT** in Fluorescence Microscopy



“Yellow” is **not** colocalization

Why?

1. you should never see yellow because you should **not use red and green** together.
2. You can visualize overlap only if the signal is high in both channels.
3. How to quantify?

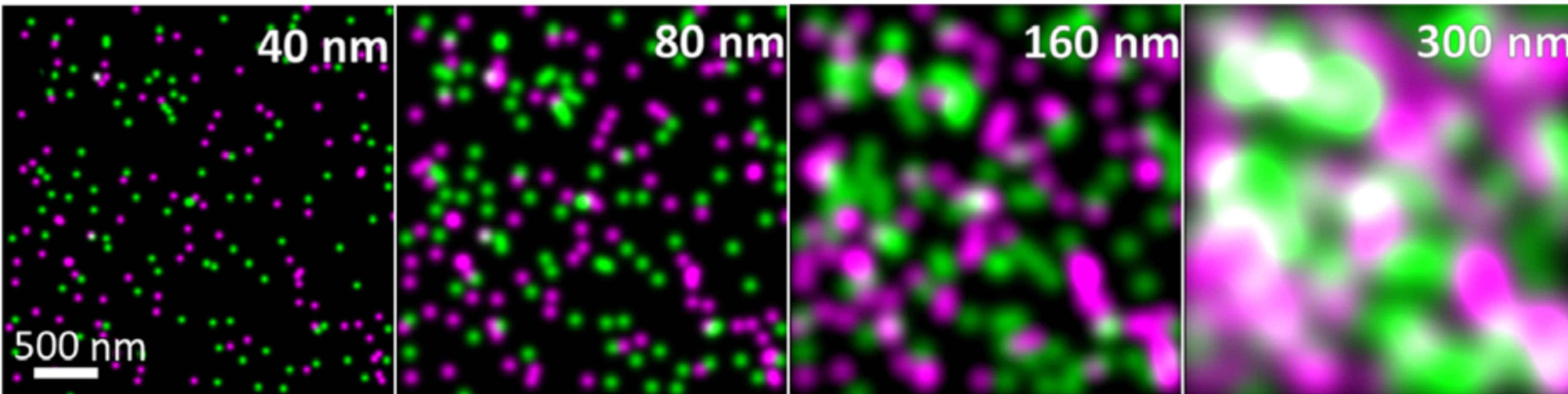
In Fiji: *Image > Color > Dichromacy* or *Image > Color > Simulate Color Blindness*



What Colocalization **IS NOT** in Fluorescence Microscopy

cannot prove information about protein/molecules **interaction** or **binding**
(but may provide evidence for)

We can detect **where** the fluorescence signal is

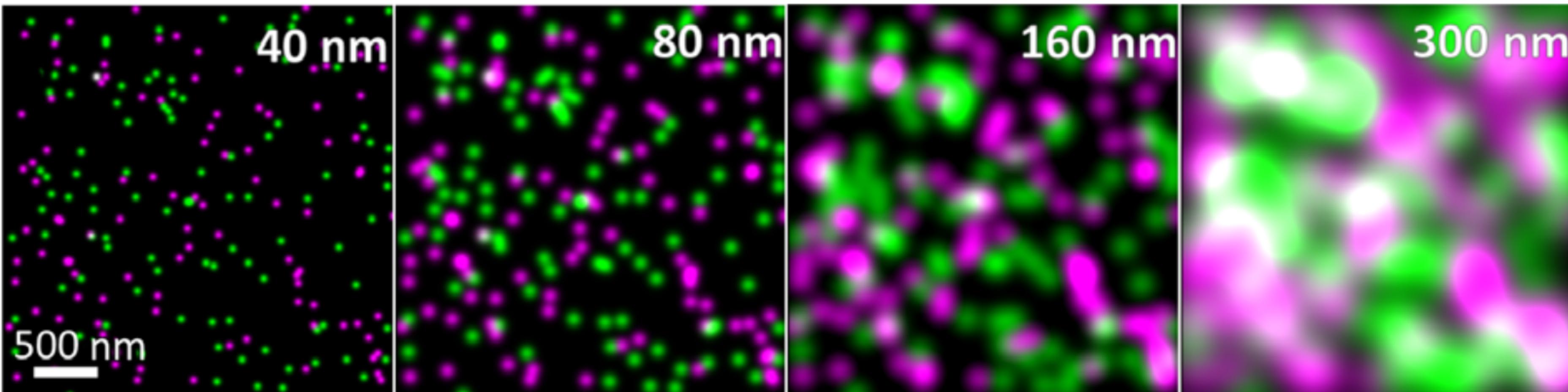




What Colocalization **IS NOT** in Fluorescence Microscopy

cannot prove information about protein/molecules **interaction** or **binding**
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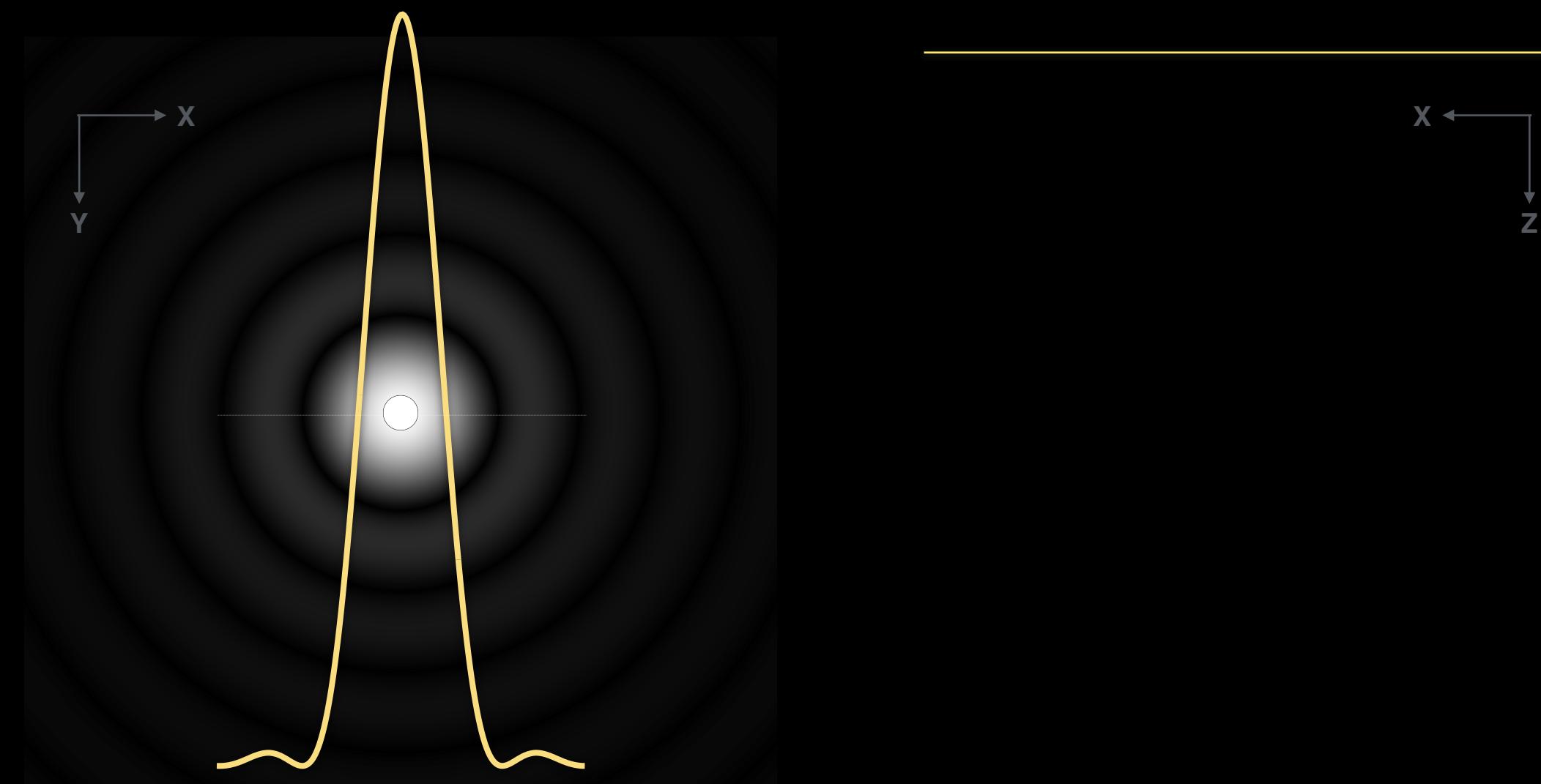


Resolution

resolution: the ability to distinguish objects that are separate in the sample as separate from one another in the image of the sample



The Point Spread Function (PSF)

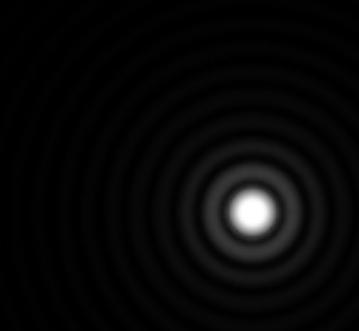


lateral

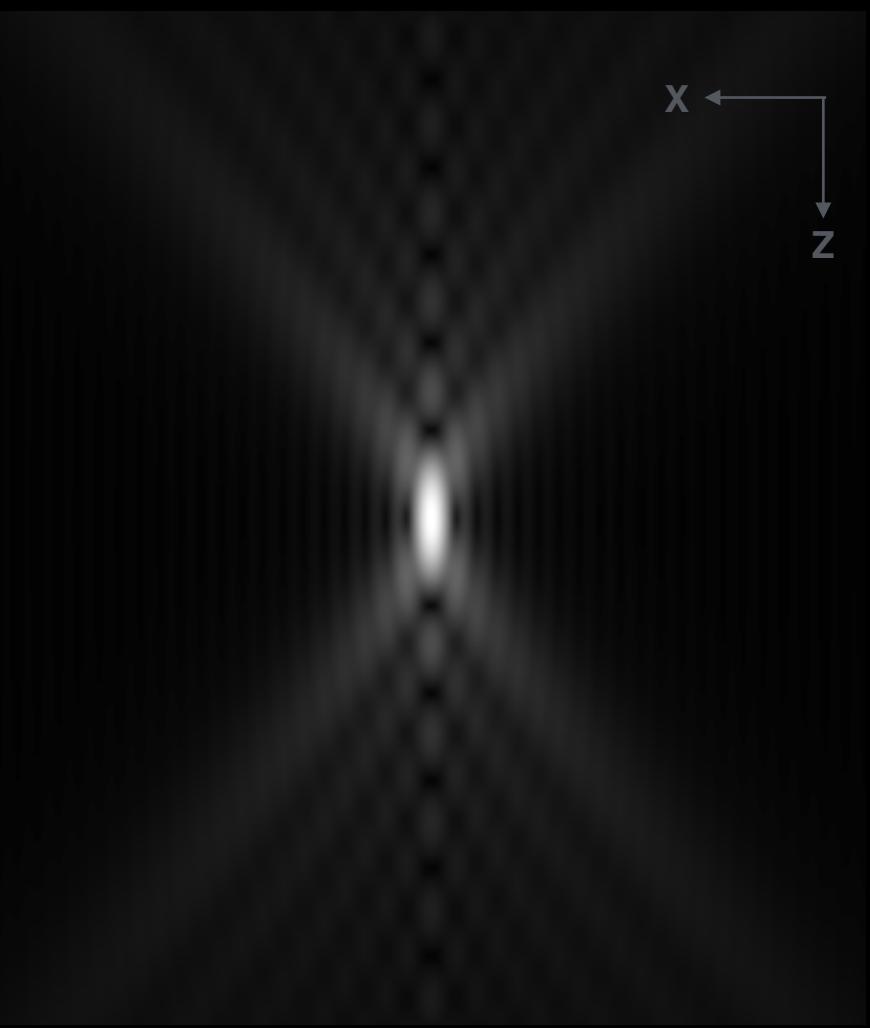
axial



The Point Spread Function (PSF)



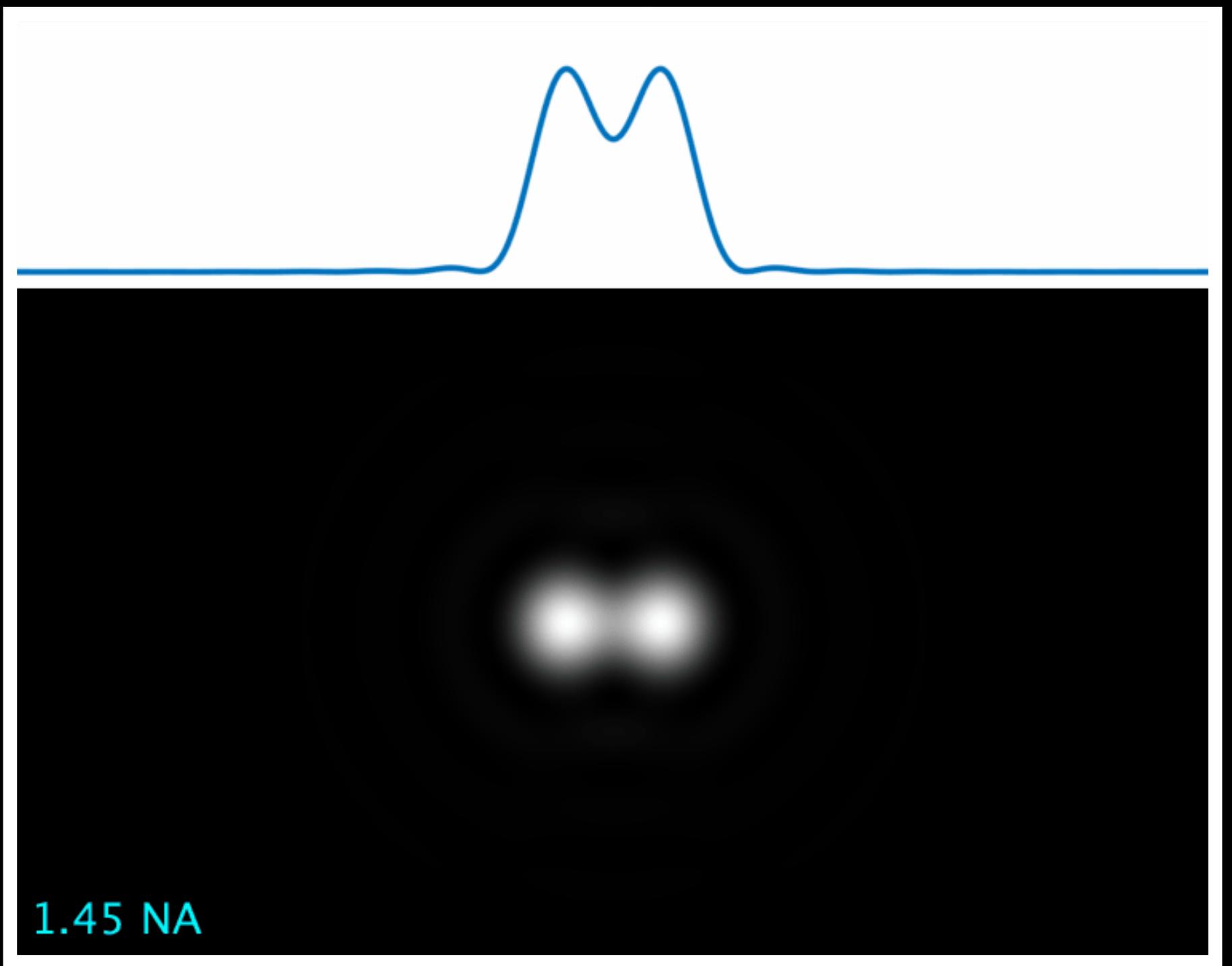
lateral



axial



Resolution is limited by the size of the PSF



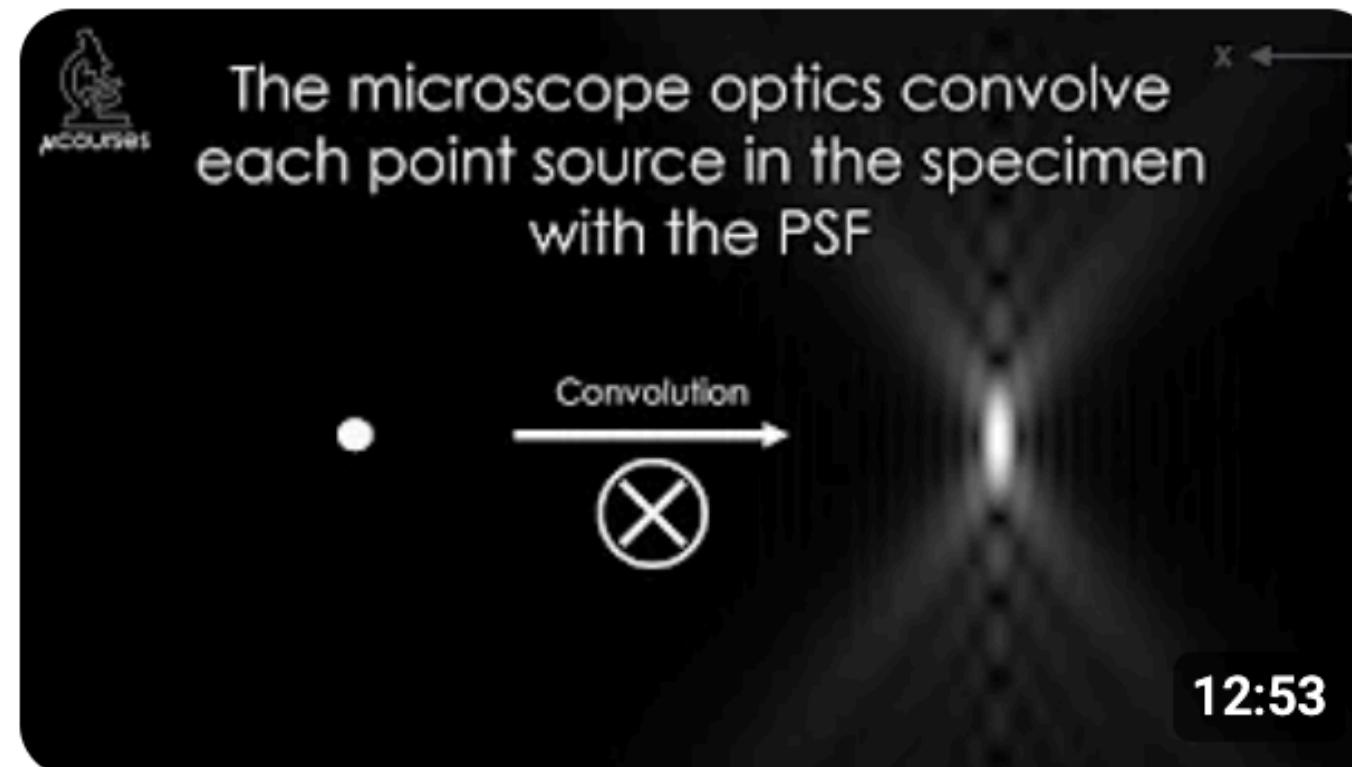


Microcourses

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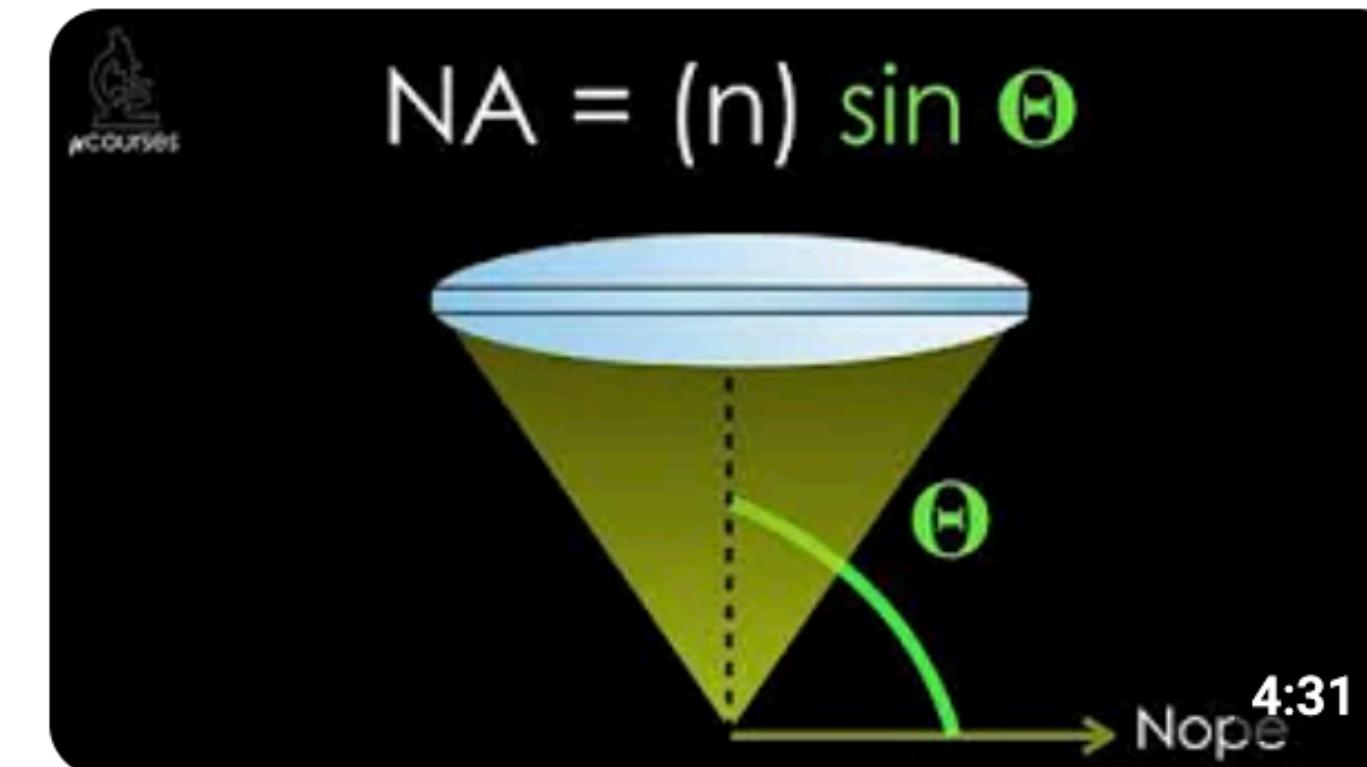
We are a team of light microscopists from core facilities at Harvard Medical School. We te...[more](#)
nic.med.harvard.edu and 5 more links

Subscribed ▾



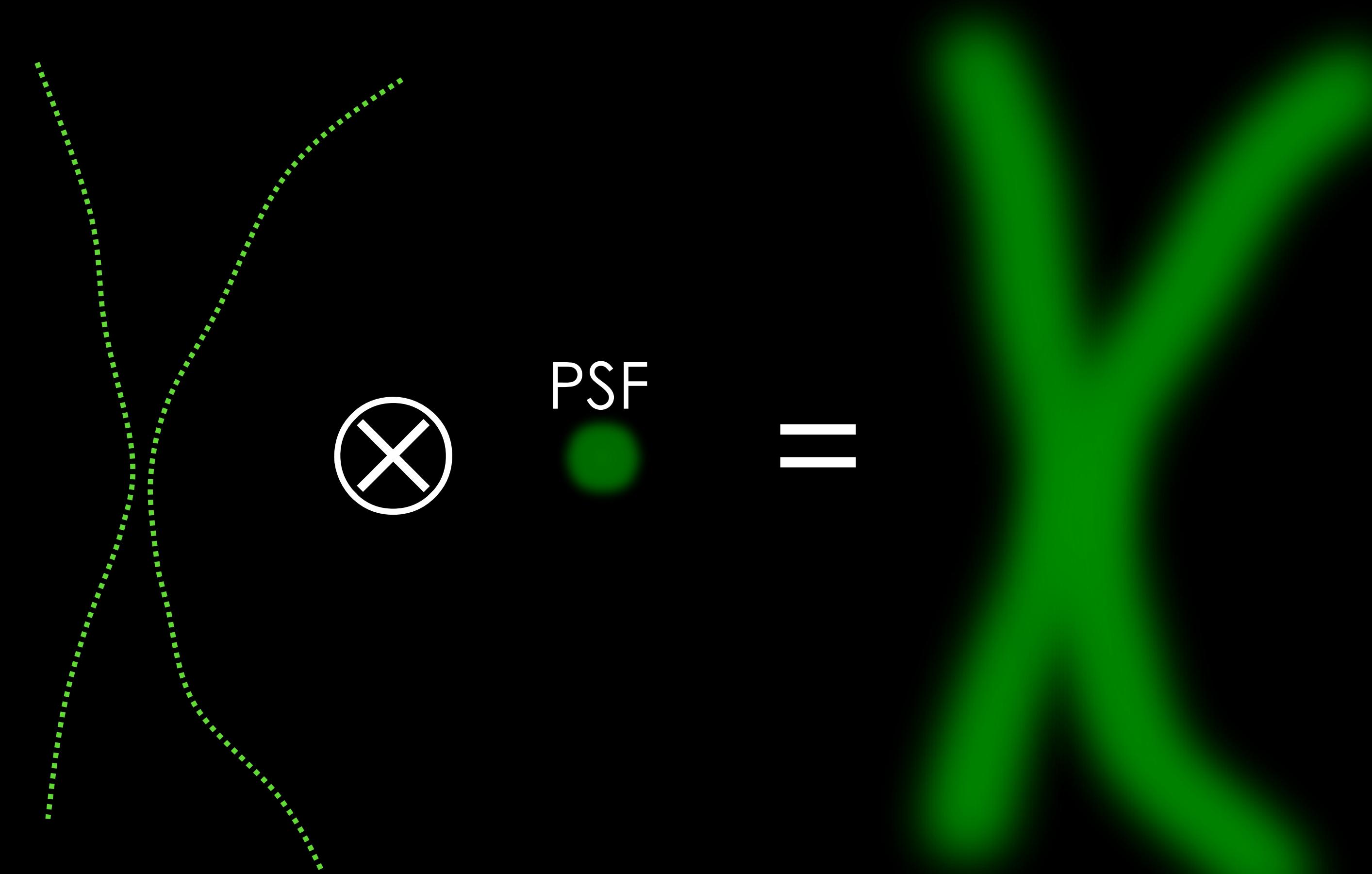
The Point Spread Function

70K views · 5 years ago



Numerical Aperture

82K views · 5 years ago



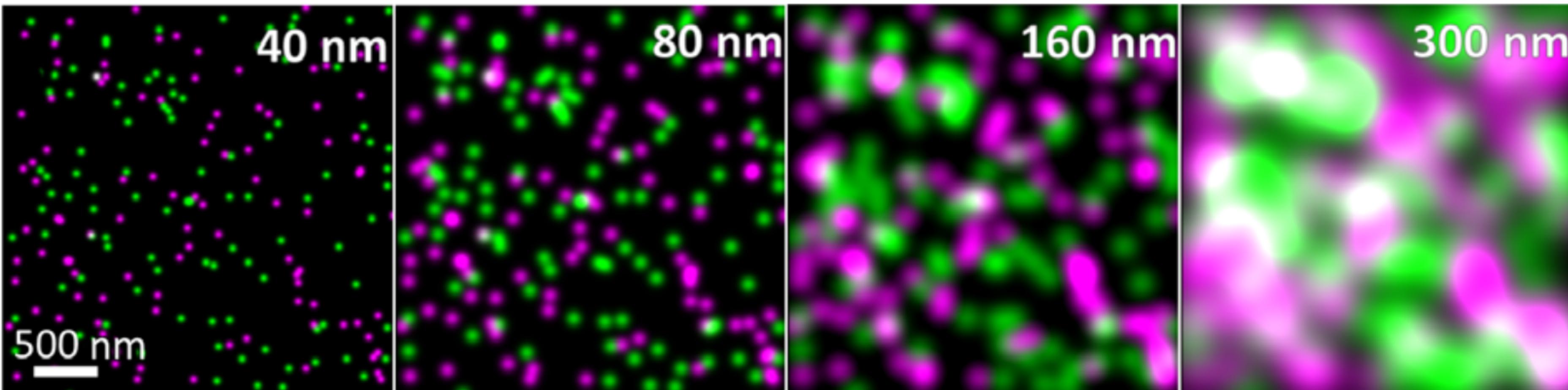
Adapted from Jennifer Waters



What Colocalization **IS NOT** in Fluorescence Microscopy

cannot prove information about protein/molecules **interaction** or **binding**
(but may provide evidence for)

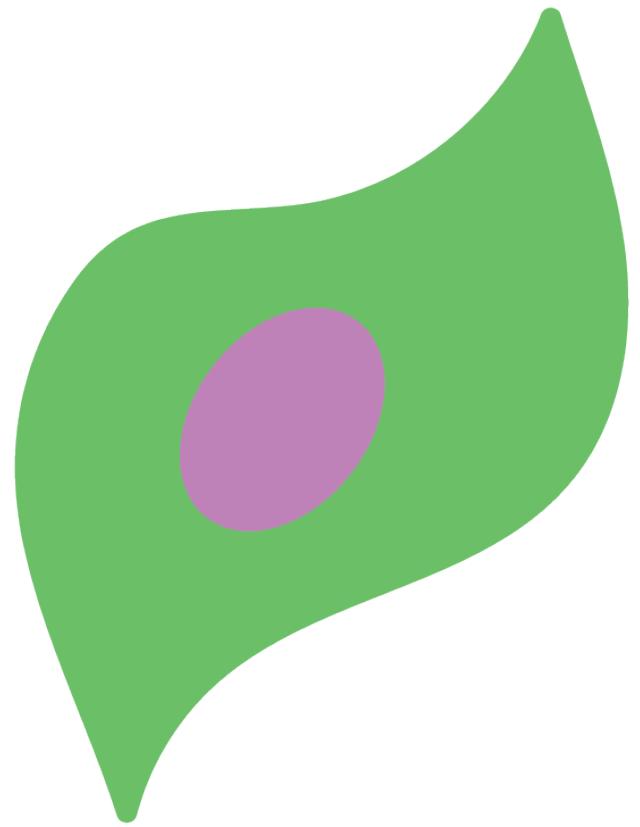
We can detect **where** the fluorescence signal is



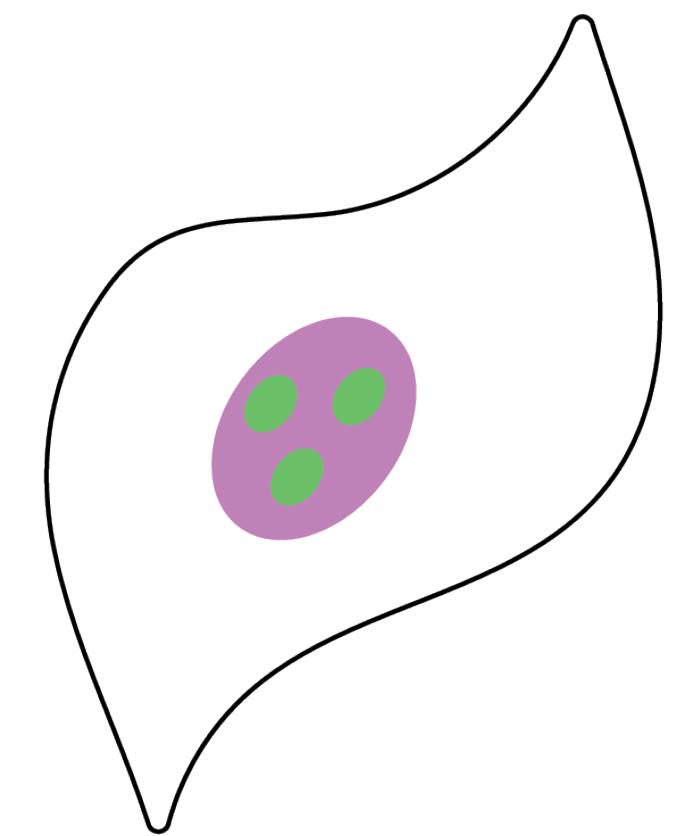
Resolution



What is Colocalization in Fluorescence Microscopy



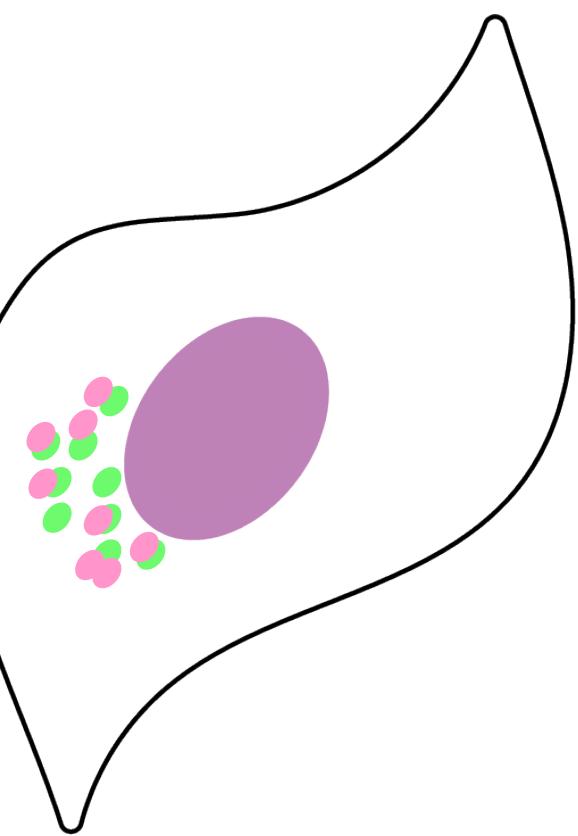
Co-expression: The presence of two or more fluorescent signals in the same cell, indicating that the corresponding proteins or molecules are expressed in the same biological sample.



Co-occurrence: The spatial overlap between fluorescent signals, suggesting that two or more molecules or structures are present in the same region of the cell.

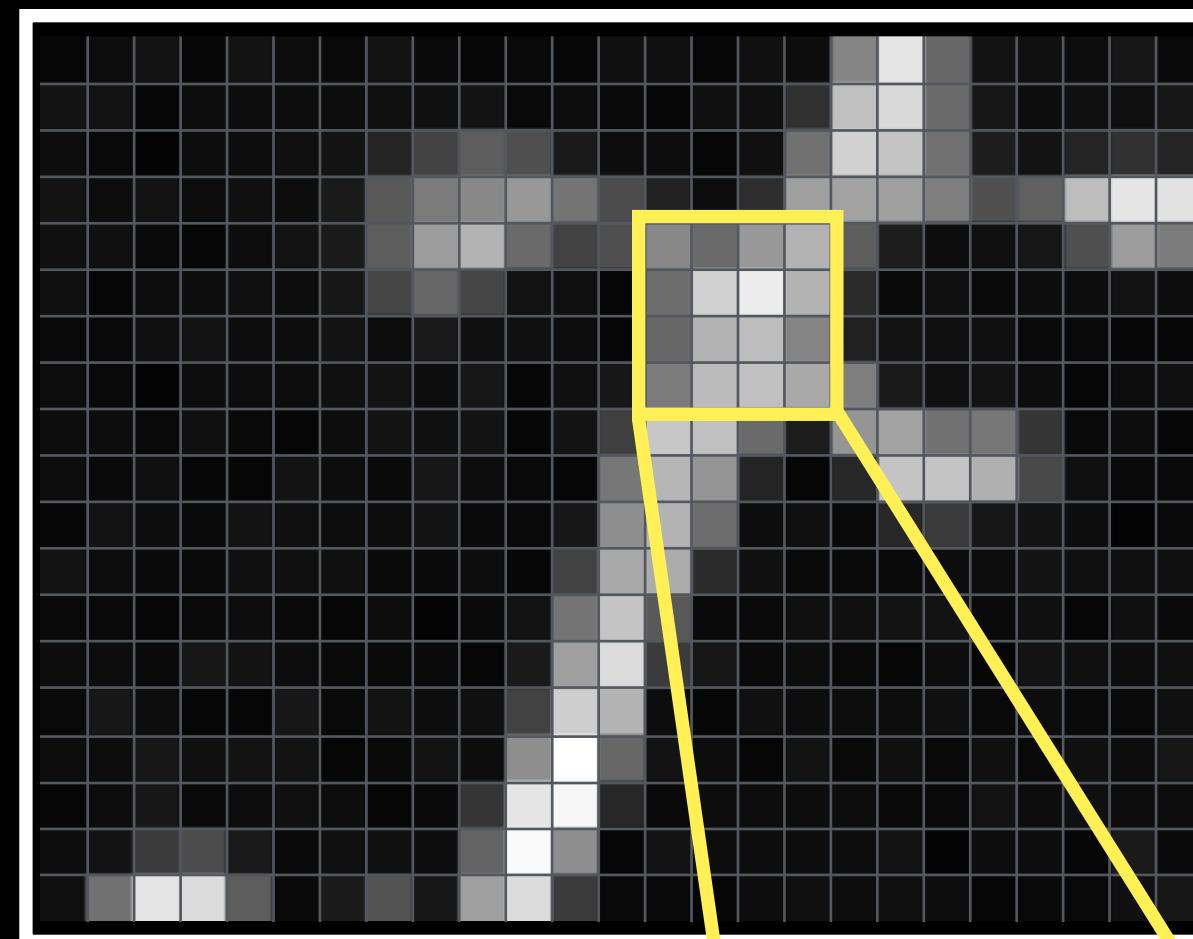


Correlation: A quantitative measure of how the intensity of two fluorescent signals changes together across the sample, helping to determine if their distributions are related.



Co-distribution: The extent to which two or more fluorescent signals are distributed similarly across different regions of the cell.

A digital image is a matrix of numbers!



=

6	13	19	6	19	13	9	19	9	6	9	6	16	16	6	16	13	132	229	103	19	16	13	23	9	9
19	19	6	13	13	13	13	16	16	19	9	13	9	6	16	16	49	192	216	106	23	13	16	16	23	13
13	9	4	13	13	16	19	36	66	93	79	26	13	13	6	16	113	209	196	113	29	19	36	49	36	33
19	13	19	13	16	13	26	89	123	136	152	116	76	33	13	46	159	162	159	126	79	96	189	229	226	212
16	16	9	6	13	19	26	93	156	179	106	66	79	136	106	152	179	93	29	13	16	23	79	156	123	49
16	6	13	13	16	13	23	69	103	69	19	16	6	109	209	236	179	43	9	16	9	13	13	19	13	13
9	9	16	19	13	13	19	13	26	16	16	13	6	103	179	189	132	33	19	16	16	9	9	6	6	6
13	9	4	13	13	13	16	19	13	23	6	16	23	123	186	192	169	126	26	16	19	13	6	13	16	13
13	13	9	16	9	6	13	19	16	19	6	19	63	199	192	106	29	149	162	113	119	53	9	13	6	13
13	9	16	6	6	19	13	9	23	13	9	6	119	182	149	36	6	39	196	196	176	73	16	9	9	9
6	19	13	9	19	16	13	13	19	9	9	23	142	179	109	13	16	9	39	59	23	19	13	4	9	9
19	13	9	9	16	16	16	9	9	13	6	66	169	172	43	16	9	9	9	13	13	19	16	16	16	9
9	9	6	9	13	9	6	13	4	9	19	116	196	89	9	9	16	16	19	19	9	16	6	16	9	9
13	13	9	23	19	13	9	9	9	6	26	159	219	59	23	9	13	9	6	13	6	19	16	13	16	13
9	23	13	6	6	23	9	19	13	16	66	206	179	13	6	16	13	13	13	16	9	13	9	9	16	13
13	13	23	16	19	19	6	9	19	13	142	255	103	19	13	6	19	9	16	9	16	9	16	13	23	9
6	13	23	9	13	16	13	6	9	53	229	246	39	9	13	13	13	13	9	9	19	13	16	13	13	13
13	19	59	76	26	9	16	16	13	99	249	142	6	19	13	13	13	13	19	4	13	13	6	26	9	13
16	113	229	219	93	9	26	83	23	159	219	59	9	9	6	13	16	13	16	13	6	9	9	16	23	9

=

136	106	152	179
109	209	236	179
103	179	189	132
123	186	192	169

Pixel = Picture Element



How can we Measure Colocalization?

- Pixel Intensity-based methods for co-occurrence & correlation
- Object-based methods for co-expression & co-distribution (spatial statistics)



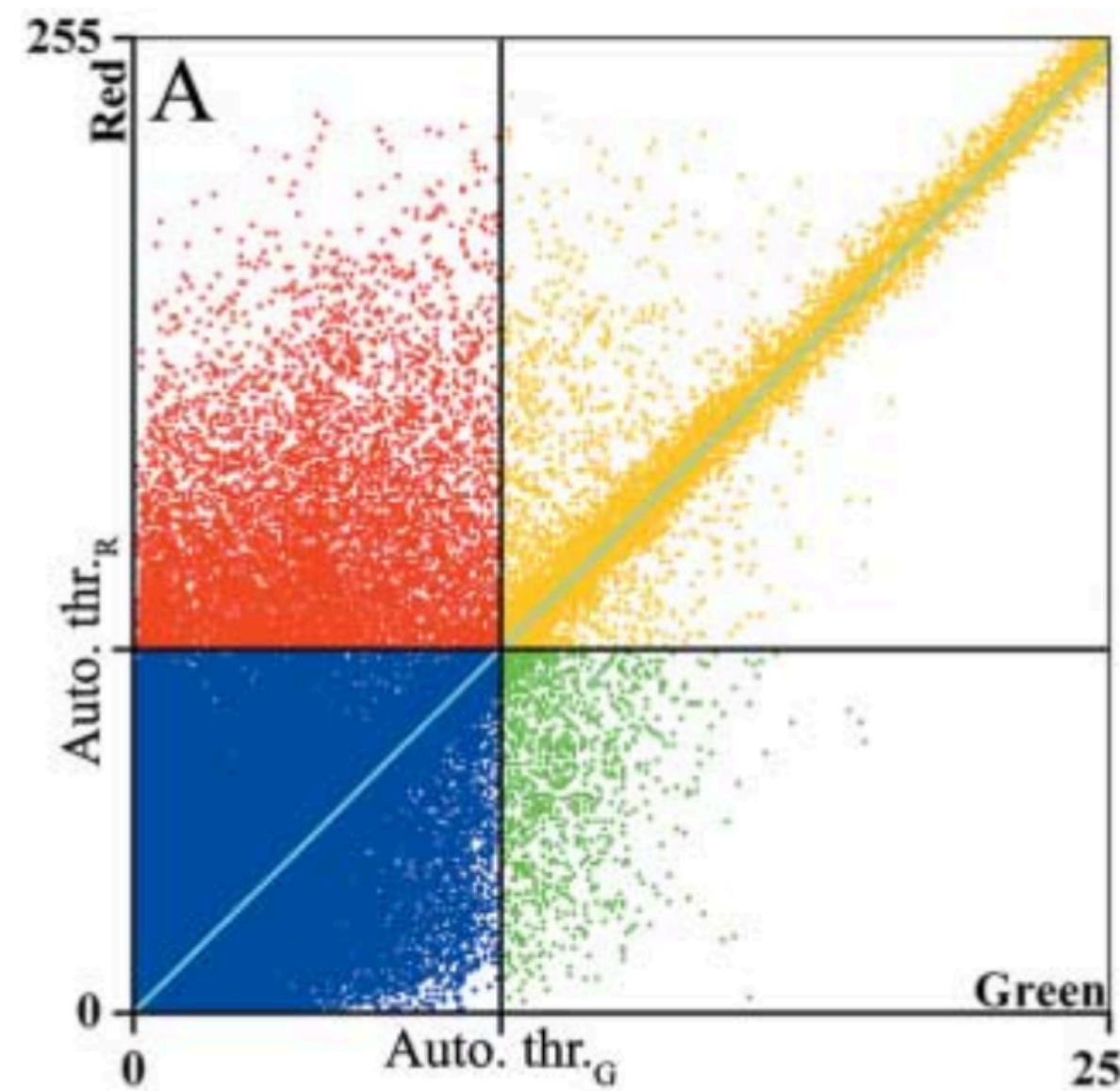
Pixel Intensity-based methods for Co-occurrence and Correlation

- The pixel values in the image are directly used in the evaluation of the correlation
- Can require thresholding/segmentation



Pixel Intensity-based methods for Co-occurrence and Correlation

- The pixel values in the image are directly used in the evaluation of spatial correlation
- Can require thresholding/segmentation
- Fraction of overlap (e.g. Manders' correlation coefficients)
- Intensity correlation (e.g. Pearson's or Spearman's correlation coefficients)
- Cross-correlation

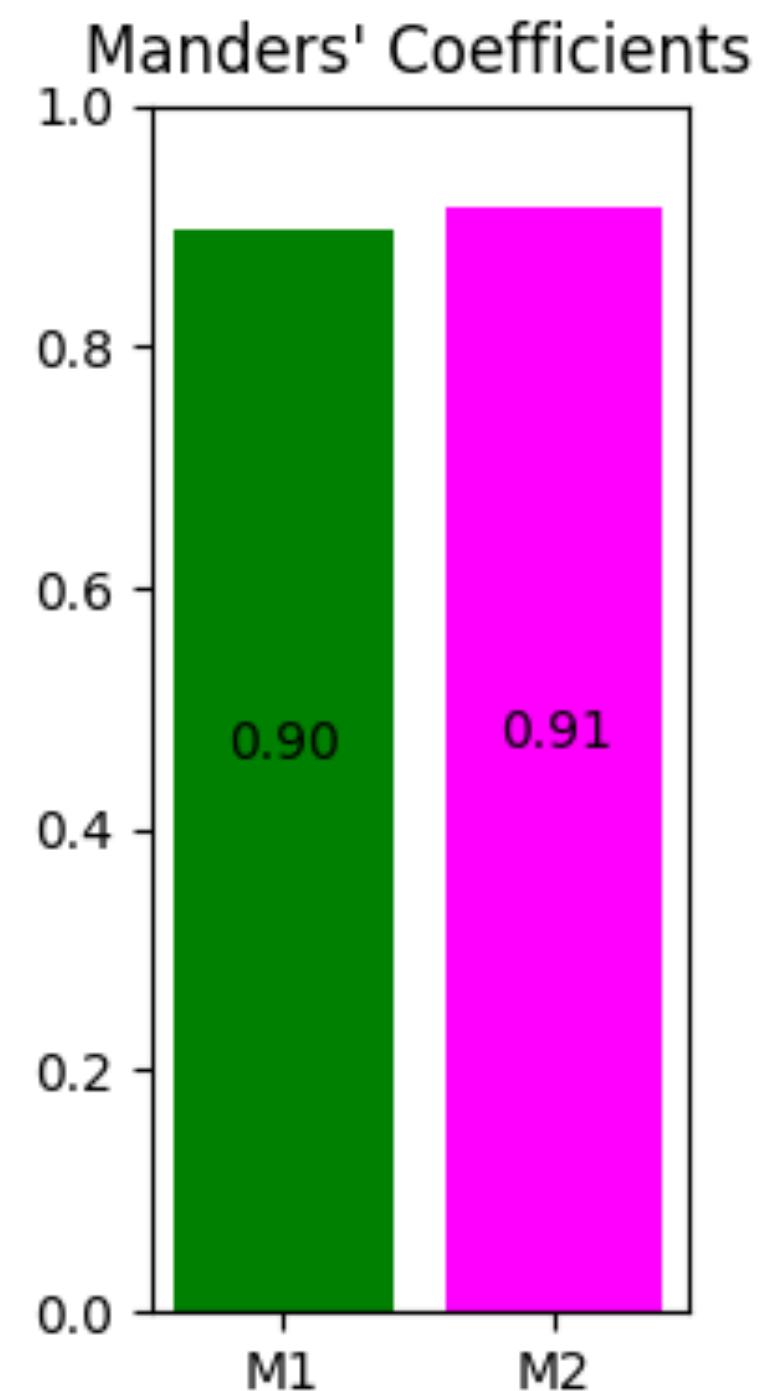


Manders' correlation coefficients

$$M_1 = \frac{\sum_i R_i^{coloc}}{\sum_i R_i} \text{ and } M_2 = \frac{\sum_i G_i^{coloc}}{\sum_i G_i}$$

Pearson's correlation coefficient

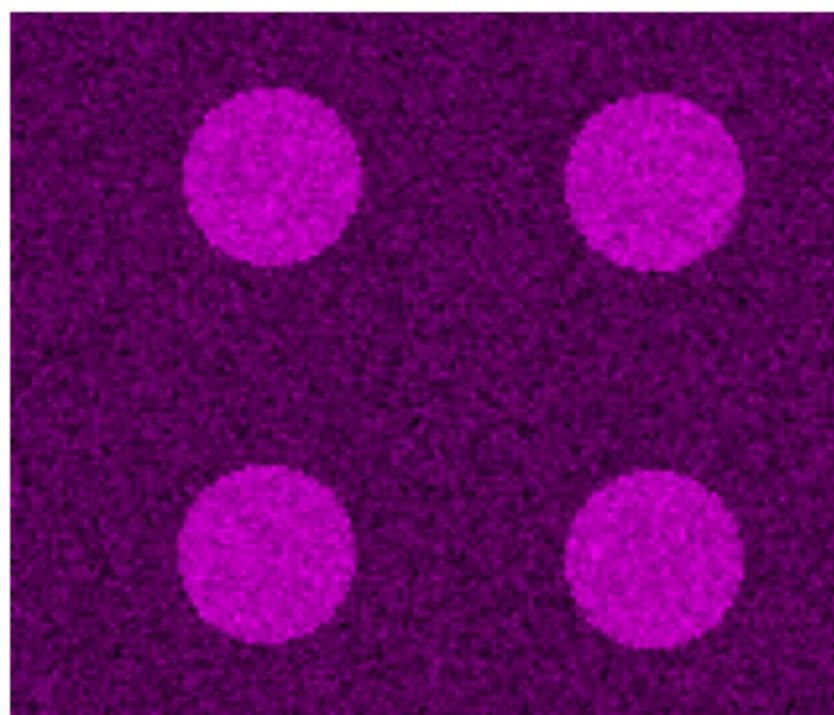
$$r_P = \frac{\sum_i (R_i - R_{avg})(G_i - G_{avg})}{\sqrt{\sum_i (R_i - R_{avg})^2 \sum_i (G_i - G_{avg})^2}}$$



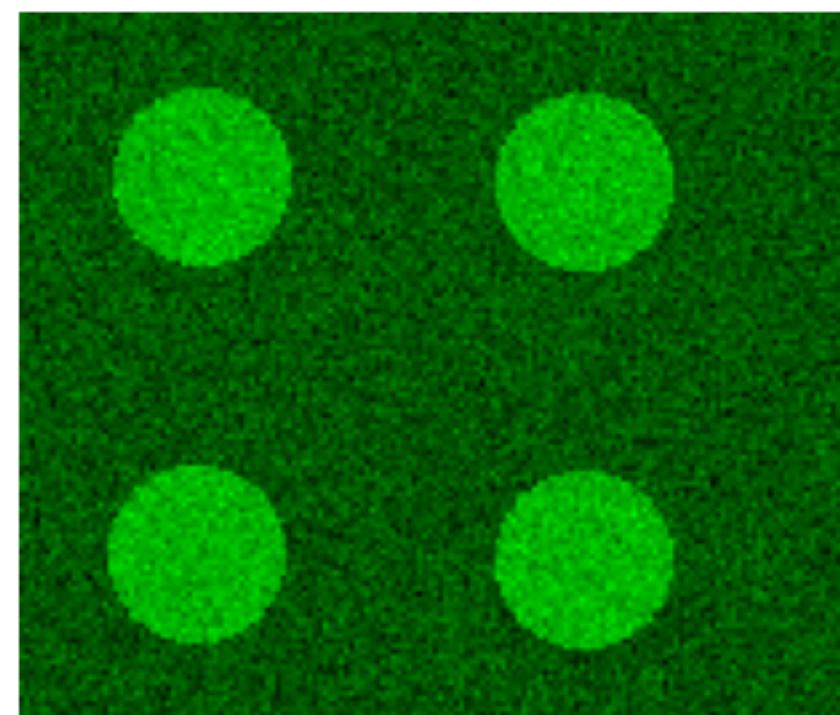


Scatter Plot

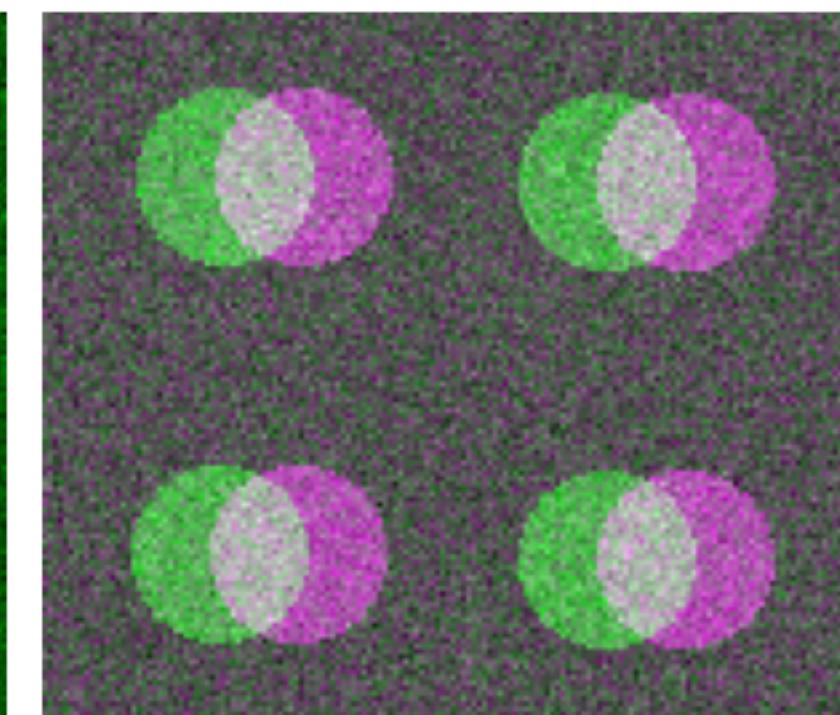
Channel 1



Channel 2

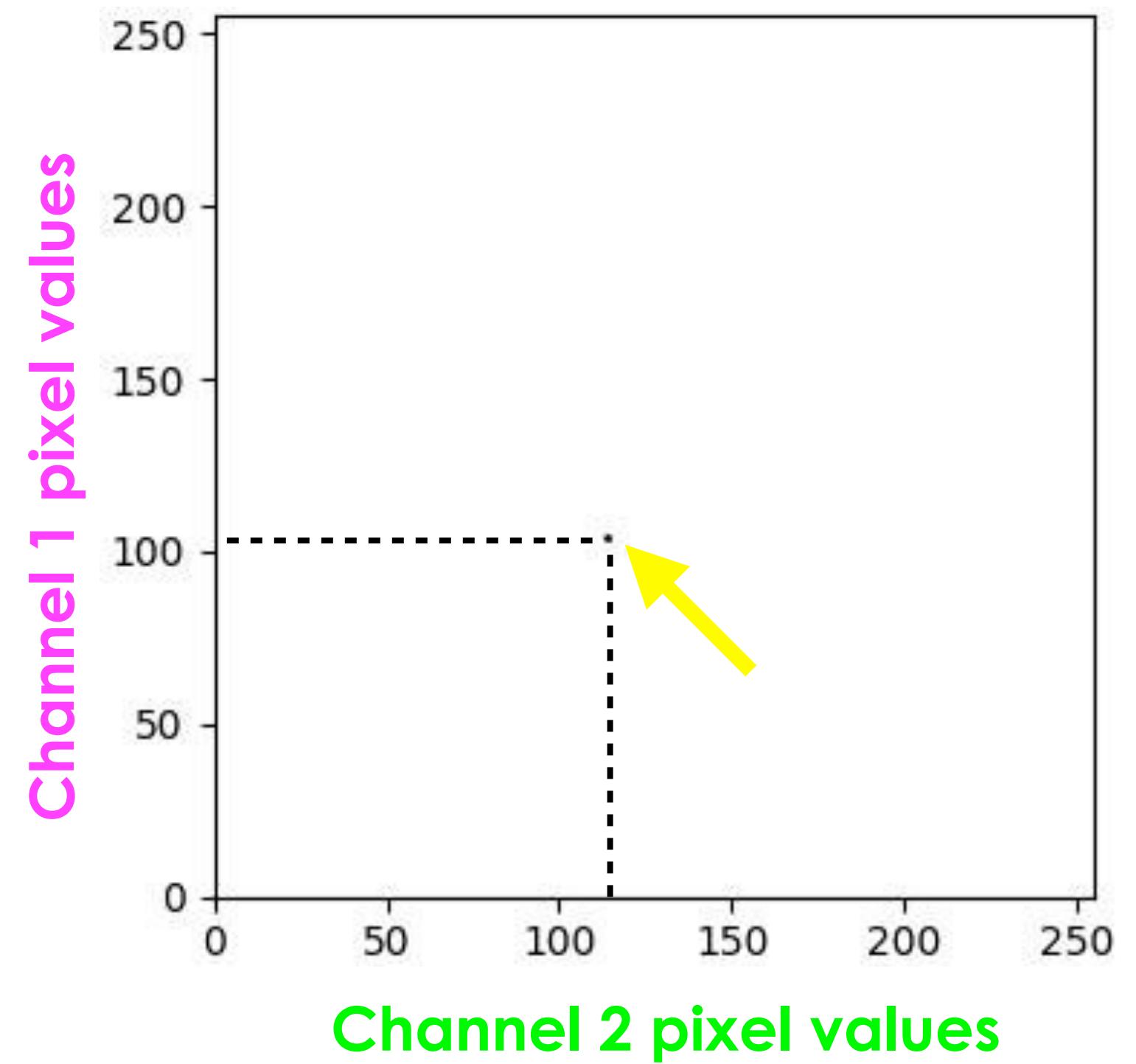
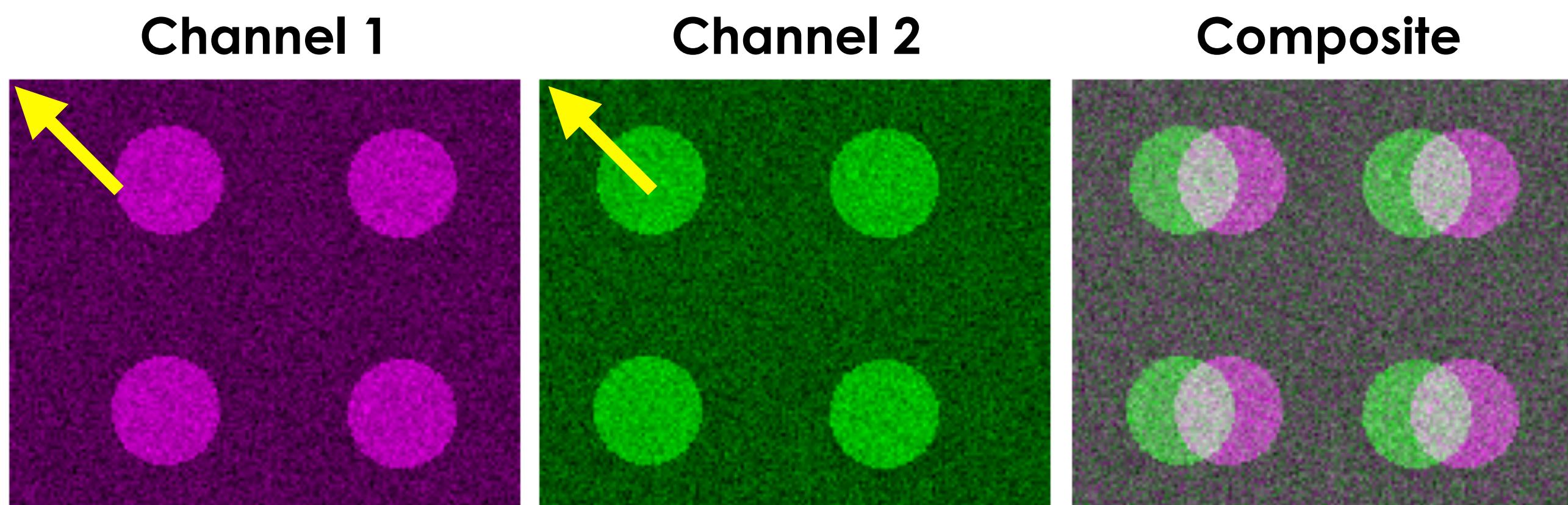


Composite





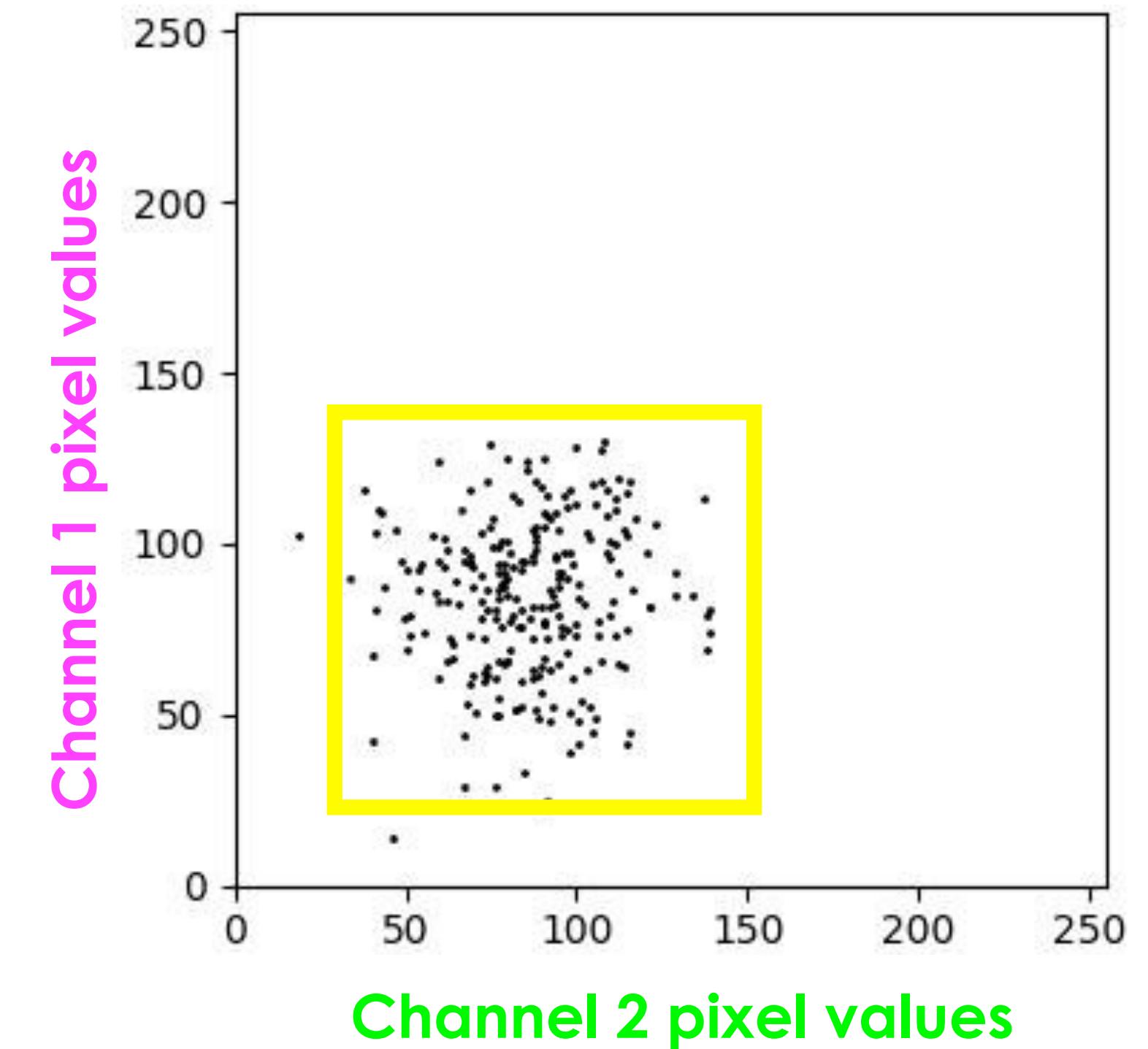
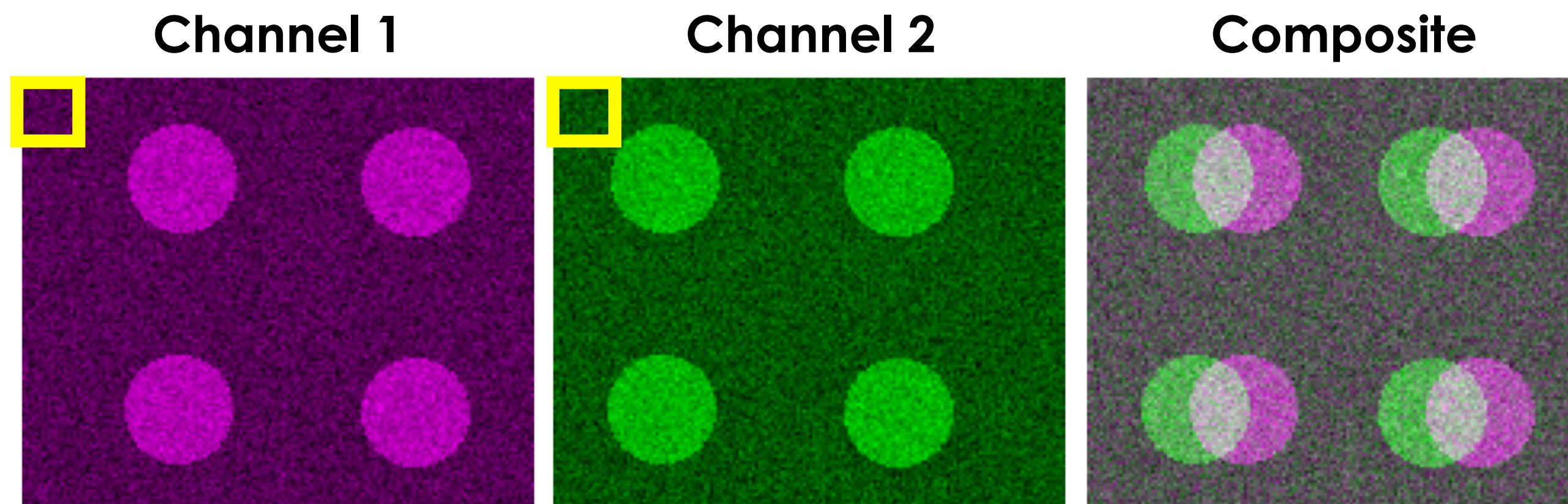
Scatter Plot





Scatter Plot

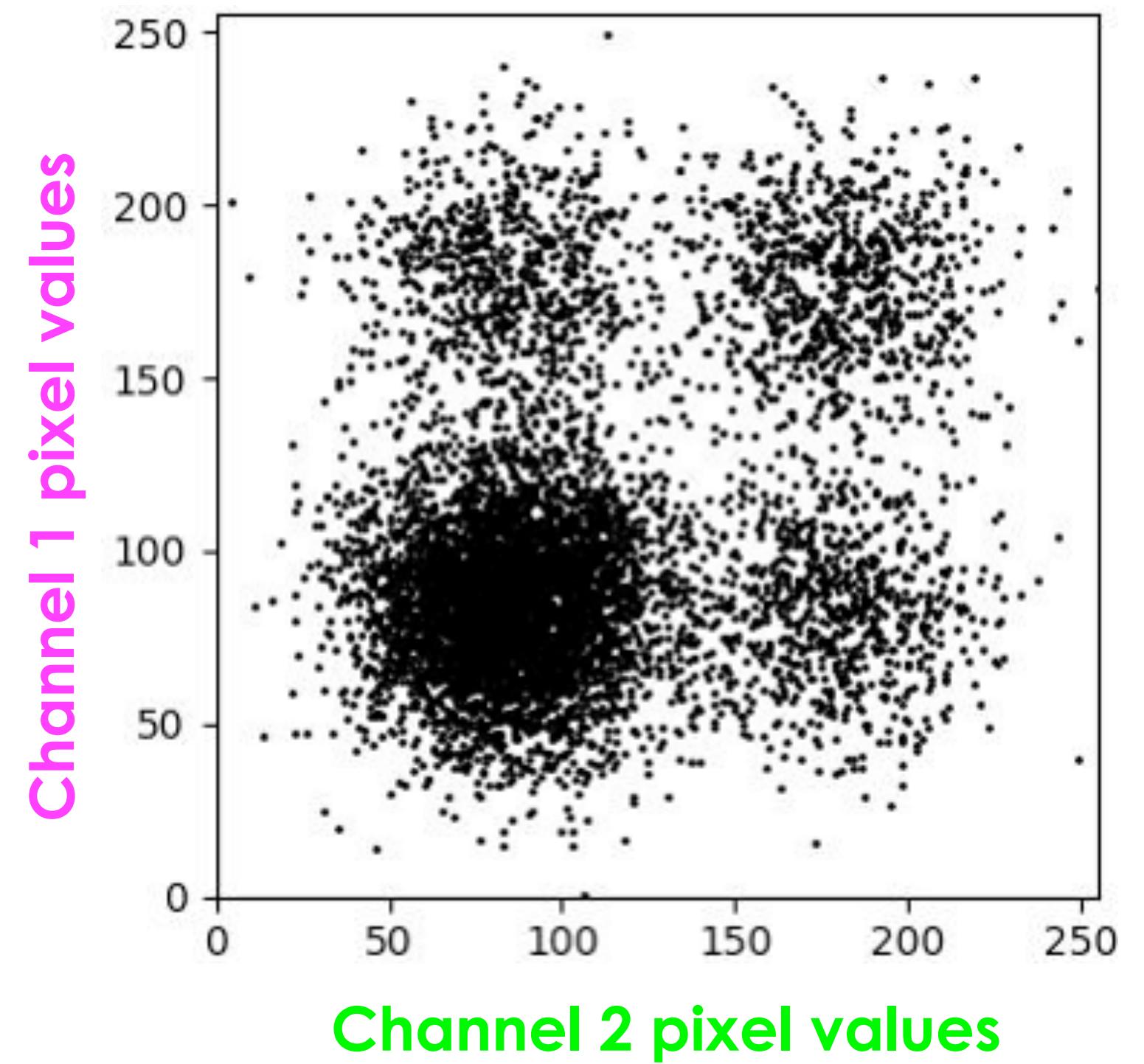
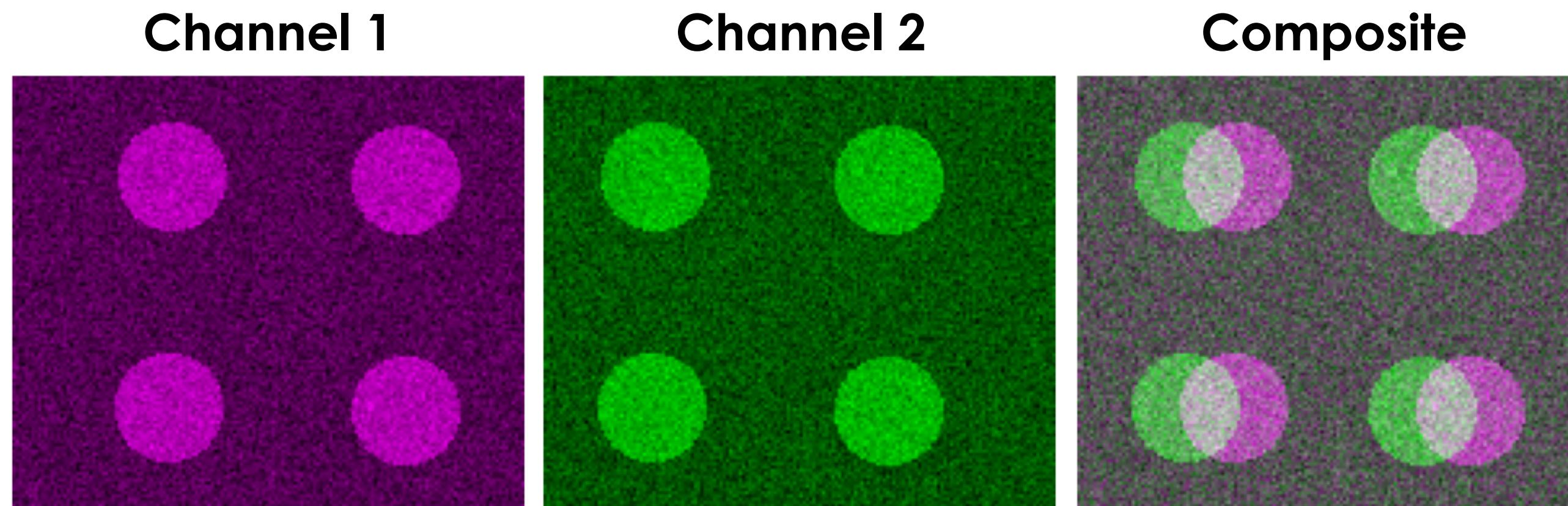
more pixels





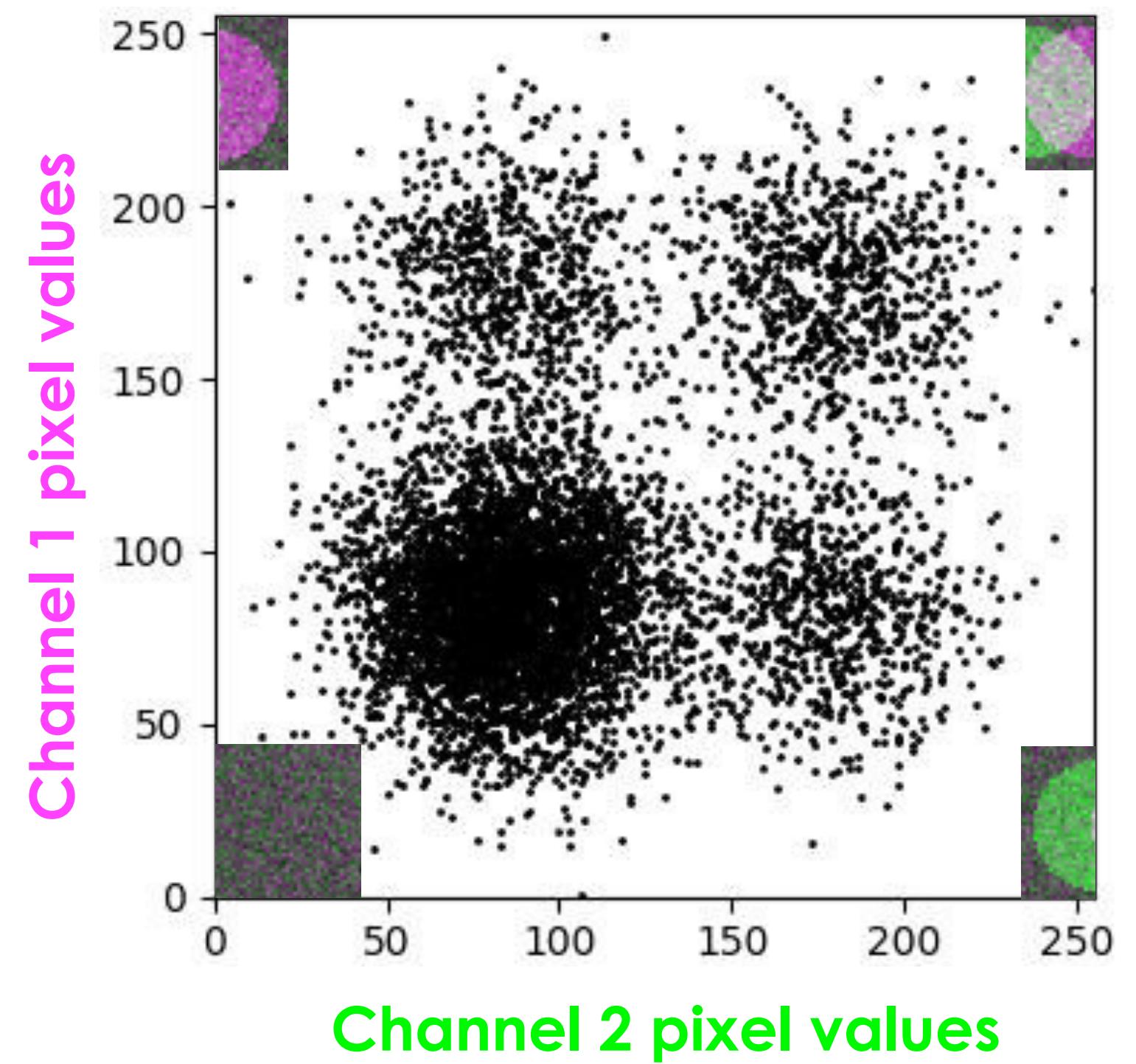
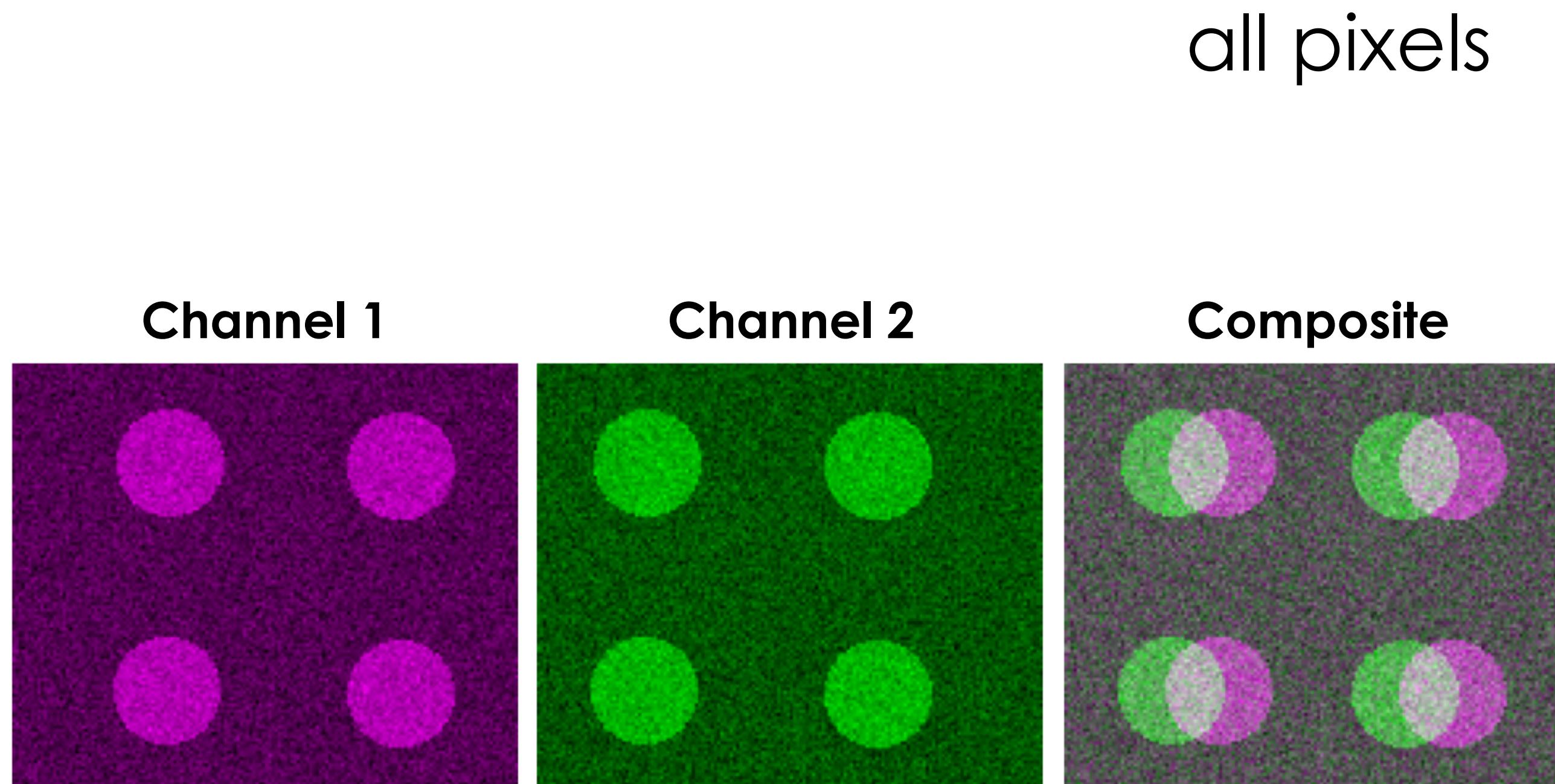
Scatter Plot

all pixels





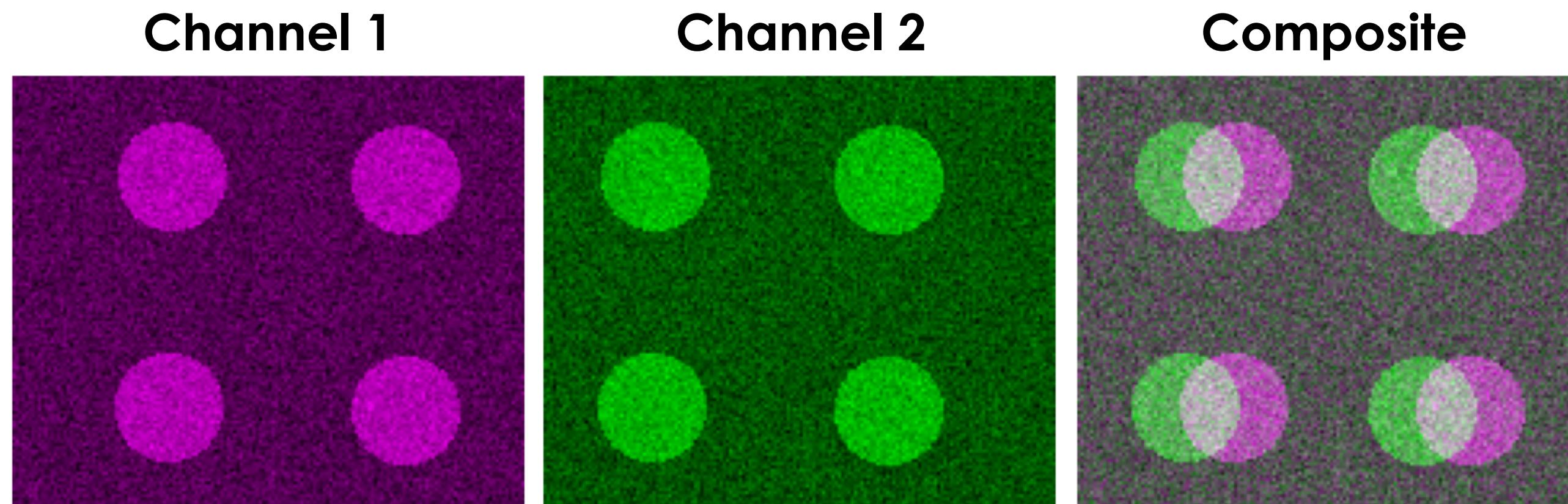
Scatter Plot



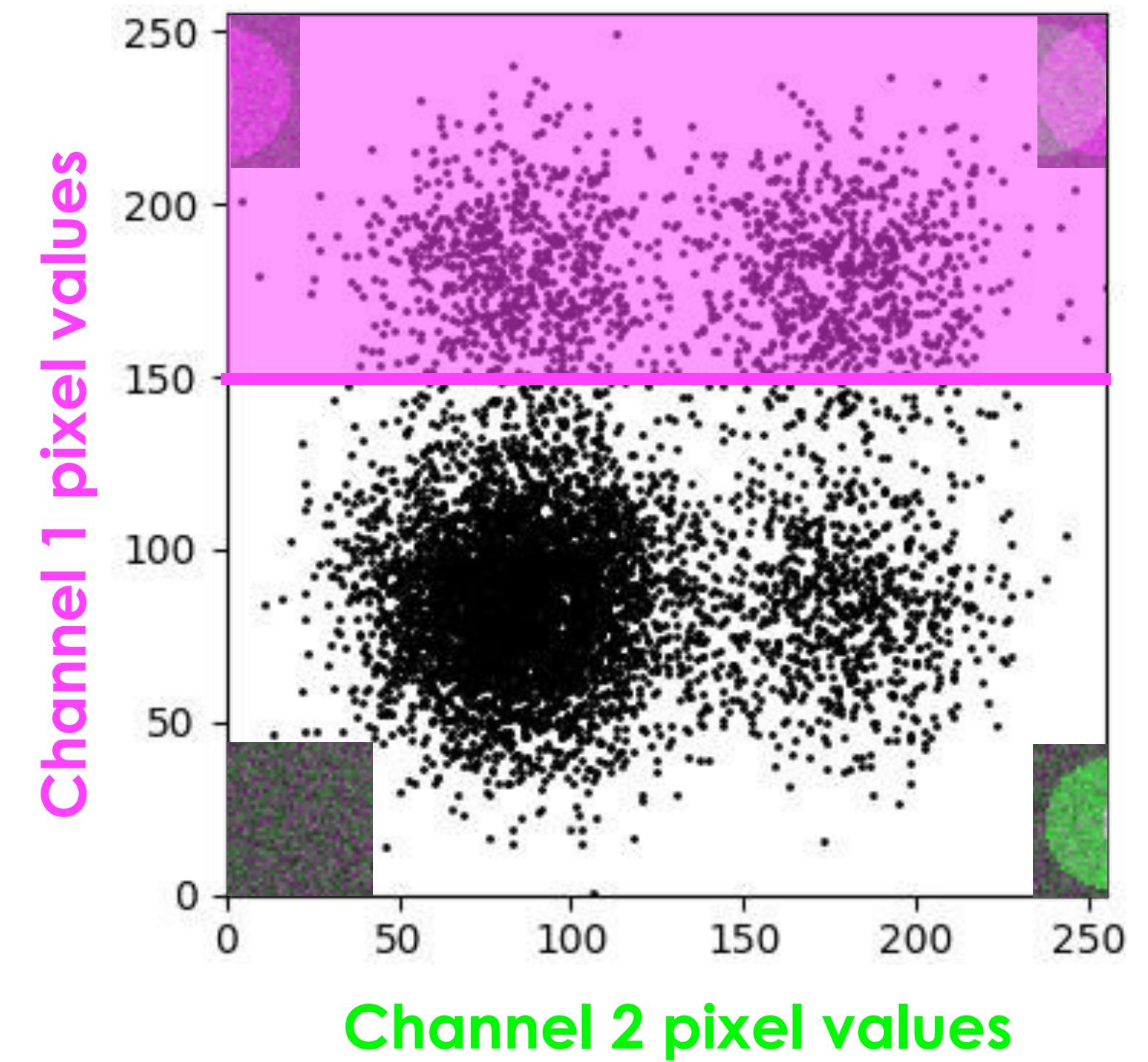


Scatter Plot

visualize thresholds



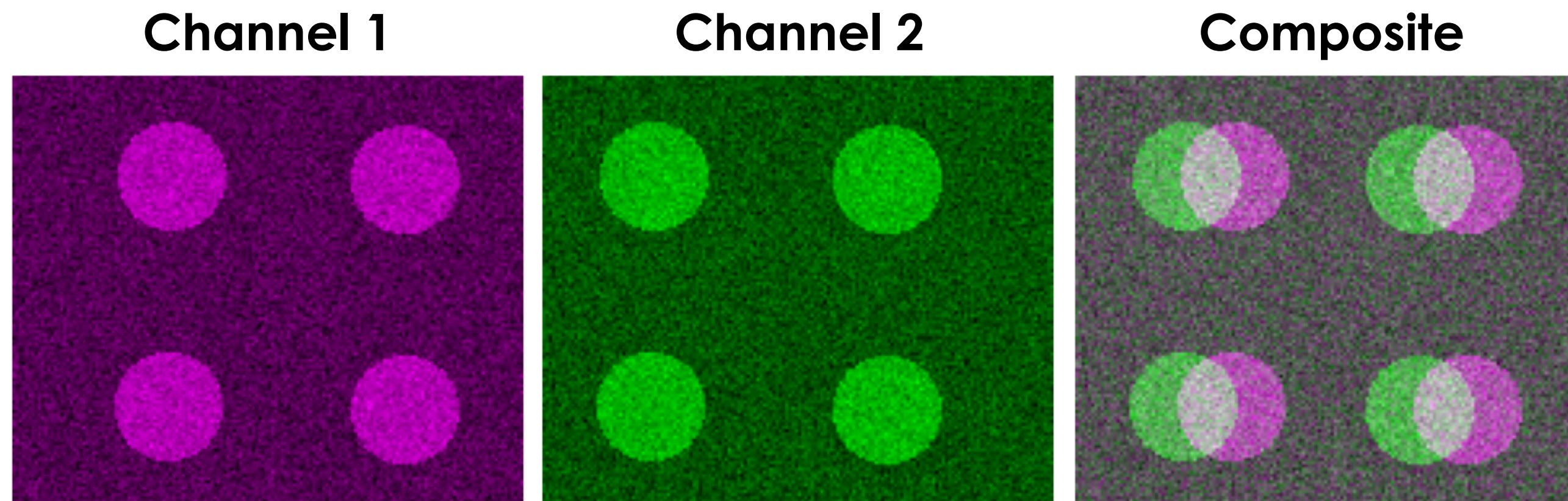
Channel 1 threshold = 150





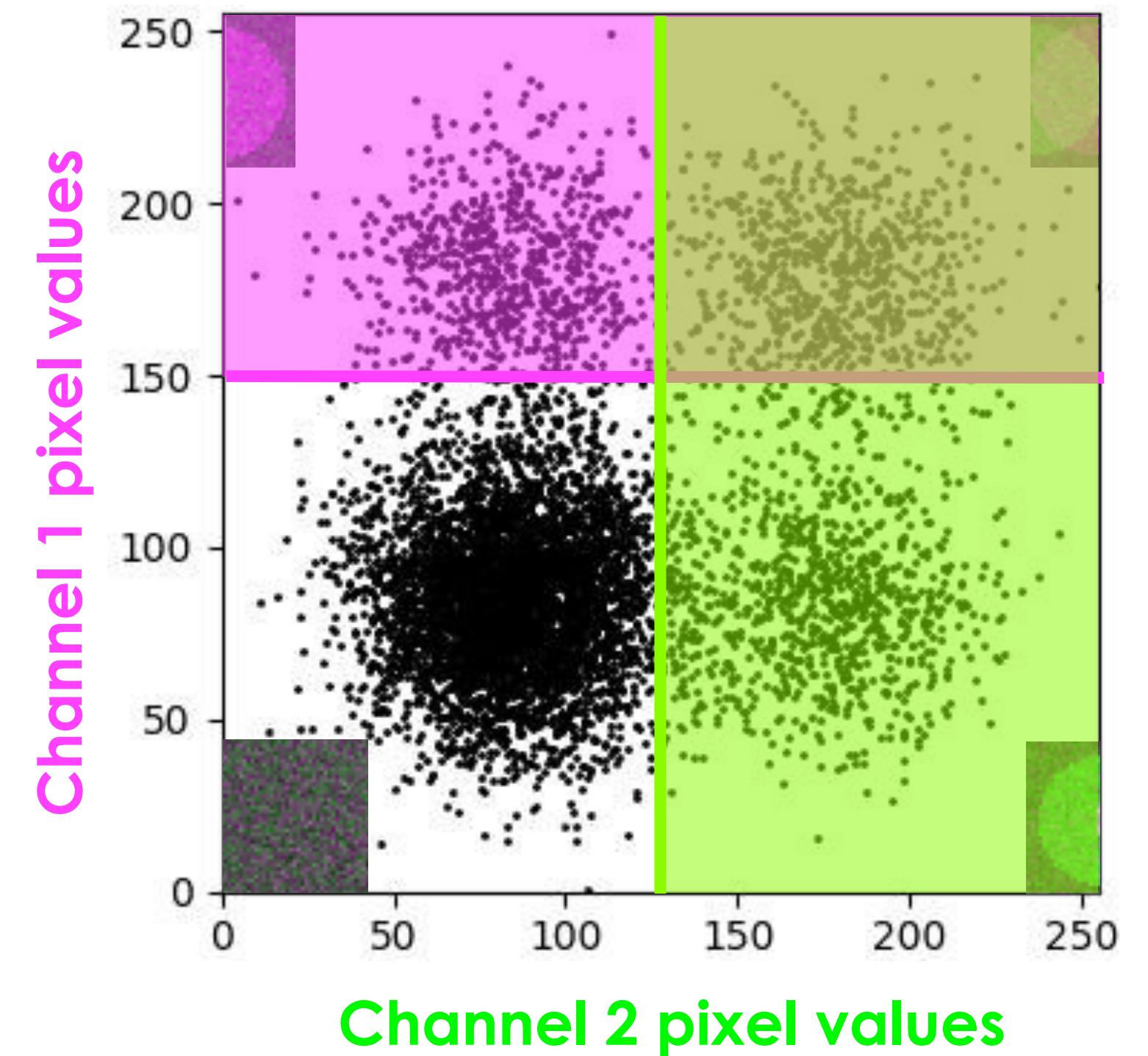
Scatter Plot

visualize thresholds



Channel 1 threshold = 150

Channel 2 threshold = 140

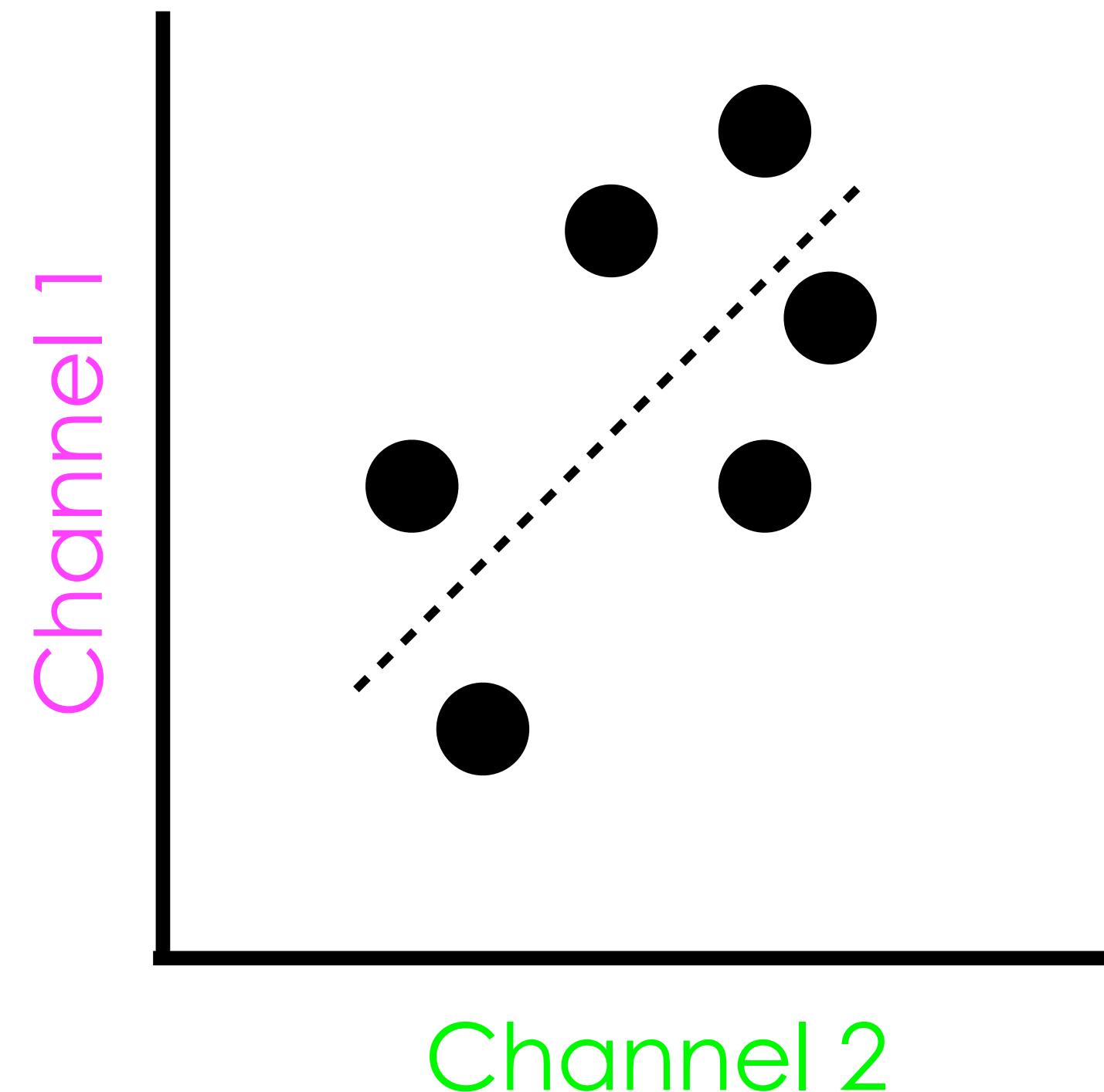




Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

$$r_P = \frac{\sum_i (R_i - R_{avg})(G_i - G_{avg})}{\sqrt{\sum_i (R_i - R_{avg})^2 \sum_i (G_i - G_{avg})^2}}$$

To measure the **degree of linear correlation between the intensities** of two signals across the entire image, pixel by pixel (no spatial).



How well are the points fit to a line (linear correlation)?

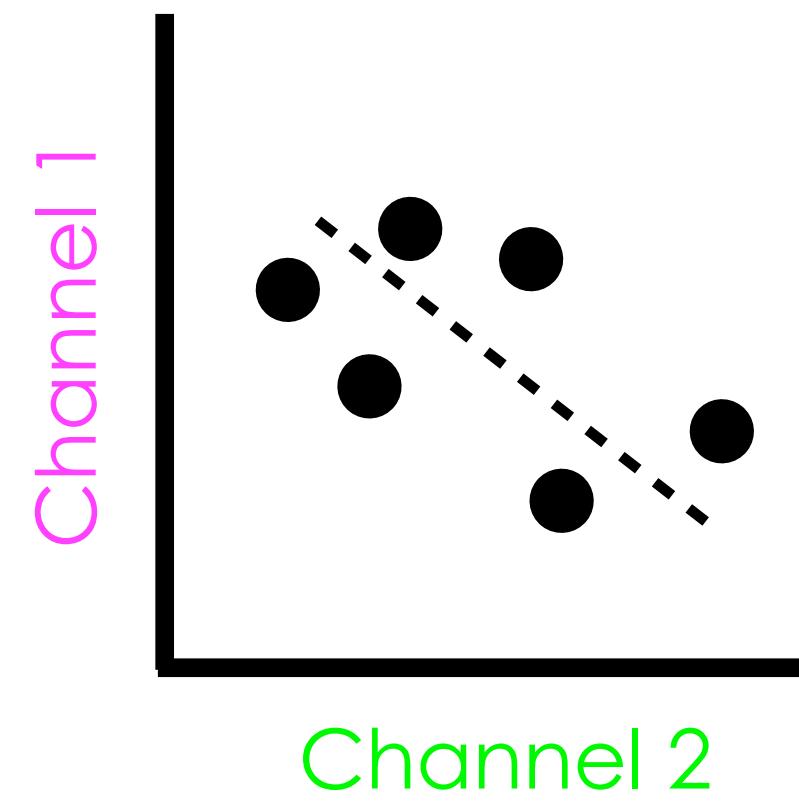
How well can I predict the intensity change of channel 1 (y) based on the intensity change of channel 2 (x)?



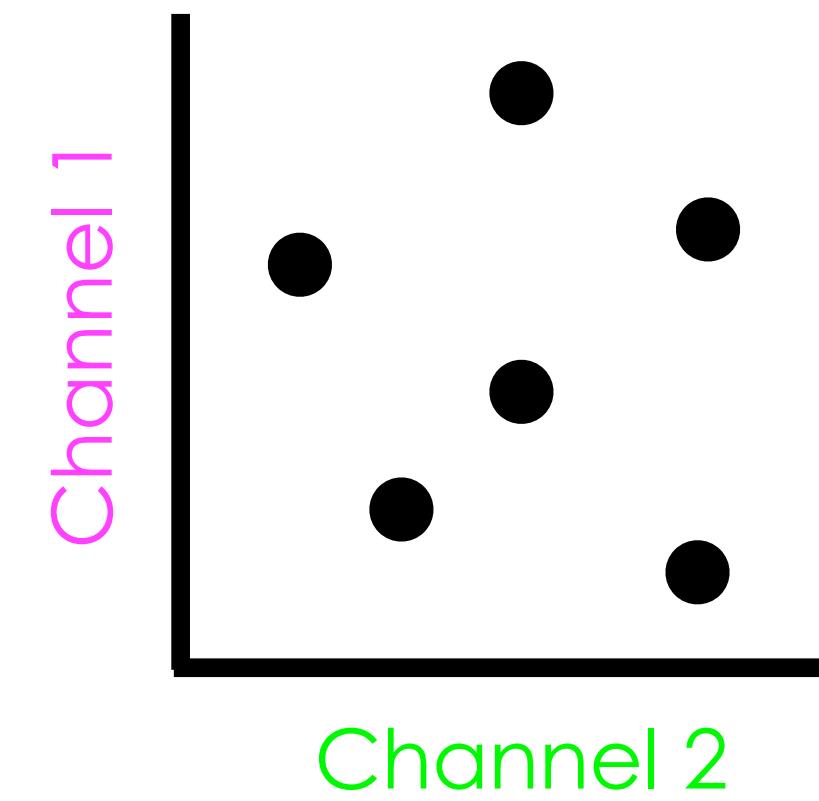
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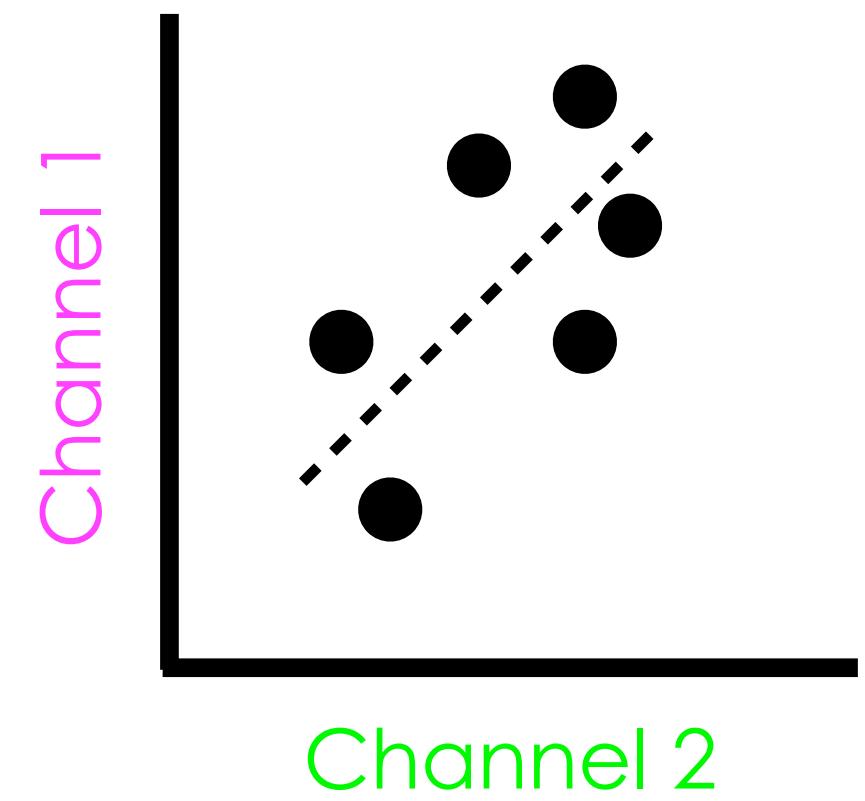
To measure the **degree of linear correlation between the intensities** of two signals across the entire image, pixel by pixel.



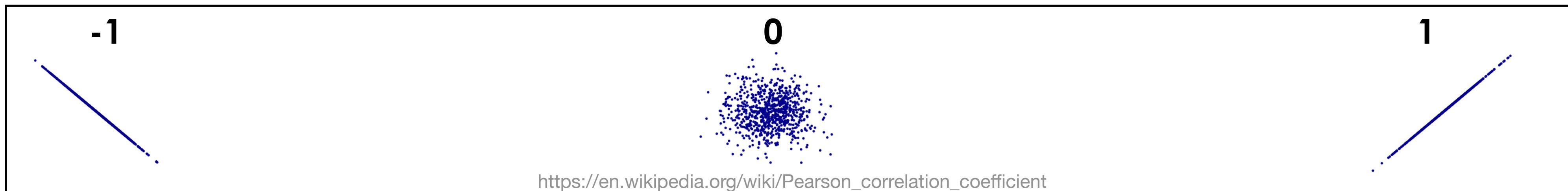
$$r_P \sim -1$$



$$r_P \sim 0$$



$$r_P \sim 1$$

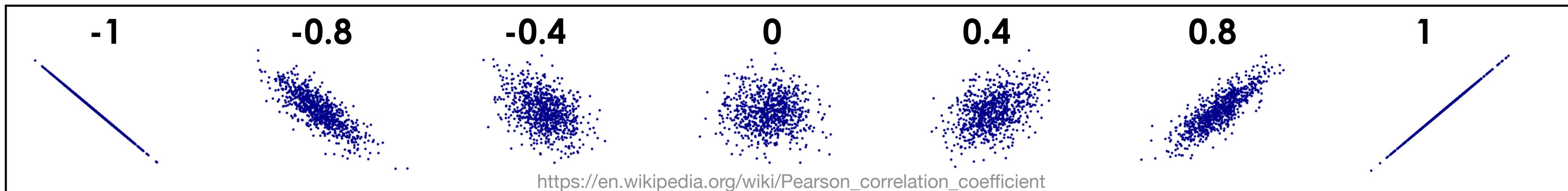
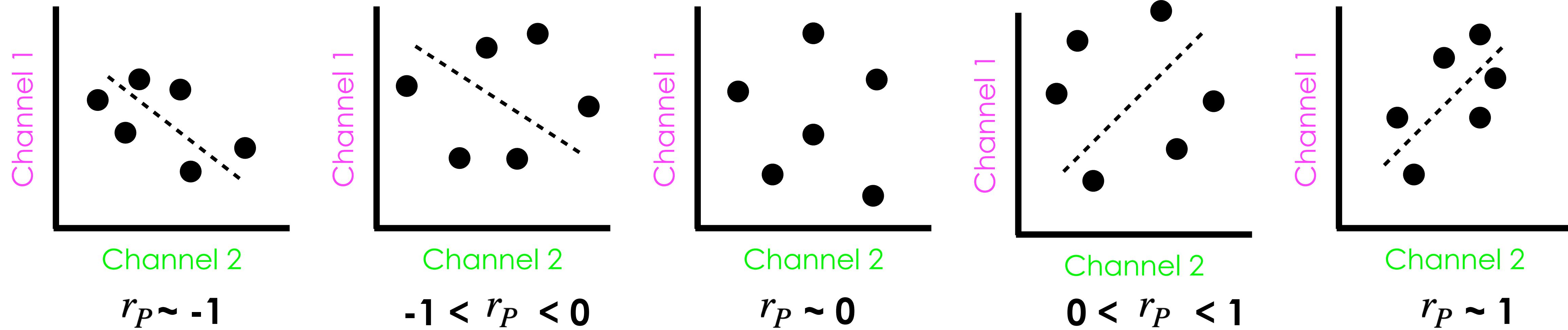




Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

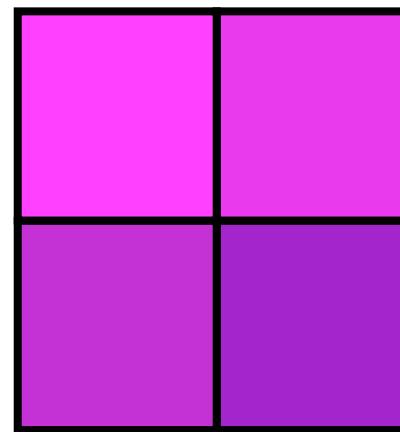
$$r_P = \frac{\sum_i (R_i - R_{avg})(G_i - G_{avg})}{\sqrt{\sum_i (R_i - R_{avg})^2 \sum_i (G_i - G_{avg})^2}}$$

To measure the **degree of linear correlation between the intensities** of two signals across the entire image, pixel by pixel.

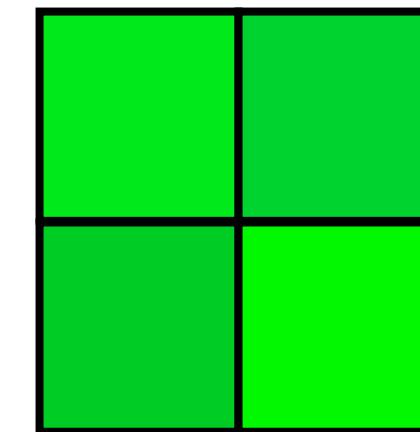




Intensity/Pixel-based: Pearson's correlation coefficient (correlation)



200	190
90	80



100	90
70	152

$$r_P = \frac{\sum_i (R_i - R_{avg})(G_i - G_{avg})}{\sqrt{\sum_i (R_i - R_{avg})^2 \sum_i (G_i - G_{avg})^2}}$$

$$R_{avg} = \frac{200+190+90+80}{4} = 140$$

$$(R_i - R_{avg}) = \begin{array}{|c|c|} \hline 200-140 & 190-140 \\ \hline 90-140 & 80-140 \\ \hline \end{array} = \begin{array}{|c|c|} \hline 60 & 50 \\ \hline -50 & -60 \\ \hline \end{array}$$

$$(R_i - R_{avg})(G_i - G_{avg}) = \begin{array}{|c|c|} \hline 60 & 50 \\ \hline -50 & -60 \\ \hline \end{array} \times \begin{array}{|c|c|} \hline -3 & -13 \\ \hline -33 & 49 \\ \hline \end{array} = \begin{array}{|c|c|} \hline -180 & -650 \\ \hline 1650 & -2940 \\ \hline \end{array}$$

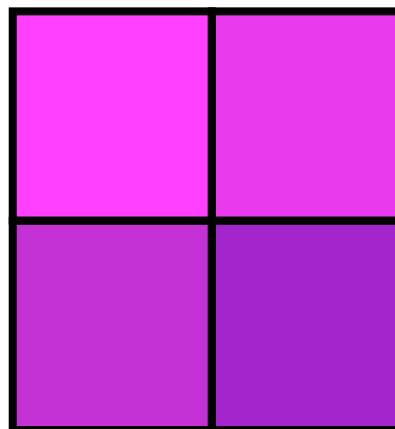
$$G_{avg} = \frac{100+90+70+152}{4} = 103$$

$$\sum_i (R_i - R_{avg})(G_i - G_{avg}) = \begin{array}{|c|c|} \hline -180 & -650 \\ \hline 1650 & -2940 \\ \hline \end{array} = -2120$$

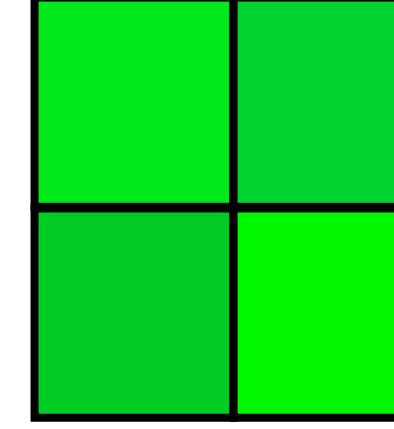
$$(G_i - G_{avg}) = \begin{array}{|c|c|} \hline 100-103 & 90-103 \\ \hline 70-103 & 152-103 \\ \hline \end{array} = \begin{array}{|c|c|} \hline -3 & -13 \\ \hline -33 & 49 \\ \hline \end{array}$$



Intensity/Pixel-based: Pearson's correlation coefficient (correlation)



200	190
90	80



100	90
70	152

$$r_P = \frac{\sum_i (R_i - R_{avg})(G_i - G_{avg})}{\sqrt{\sum_i (R_i - R_{avg})^2 \sum_i (G_i - G_{avg})^2}}$$

$$(R_i - R_{avg}) = \begin{array}{|c|c|} \hline 60 & 50 \\ \hline -50 & -60 \\ \hline \end{array}$$

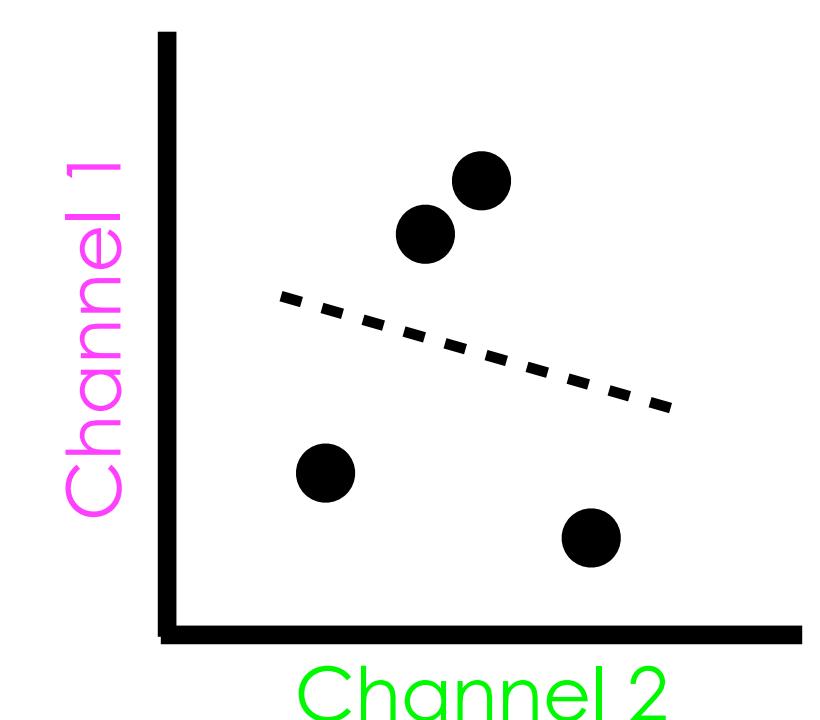
$$\sum_i (R_i - R_{avg})^2 = \begin{array}{|c|c|} \hline 60^2 & 50^2 \\ \hline (-50)^2 & (-60)^2 \\ \hline \end{array} = \begin{array}{|c|c|} \hline 3600 & 2500 \\ \hline 2500 & 3600 \\ \hline \end{array} = 12200$$

$$\sqrt{\sum_i (R_i - R_{avg})^2 \sum_i (G_i - G_{avg})^2} = \sqrt{12200 \times 3668} = 6689.51$$

$$(G_i - G_{avg}) = \begin{array}{|c|c|} \hline -3 & -13 \\ \hline -33 & 49 \\ \hline \end{array}$$

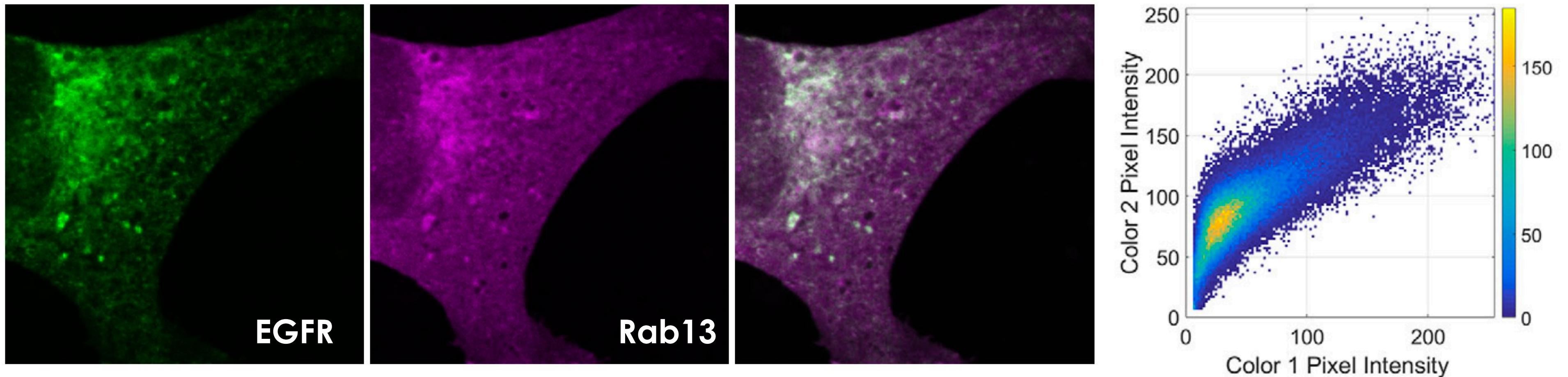
$$\sum_i (G_i - G_{avg})^2 = \begin{array}{|c|c|} \hline (-3)^2 & (-13)^2 \\ \hline (-33)^2 & 49^2 \\ \hline \end{array} = \begin{array}{|c|c|} \hline 9 & 169 \\ \hline 1089 & 2401 \\ \hline \end{array} = 3668$$

$$r_P = \frac{-2120}{6689.51} = -0.317$$





Intensity/Pixel-based: Pearson's correlation coefficient (correlation)



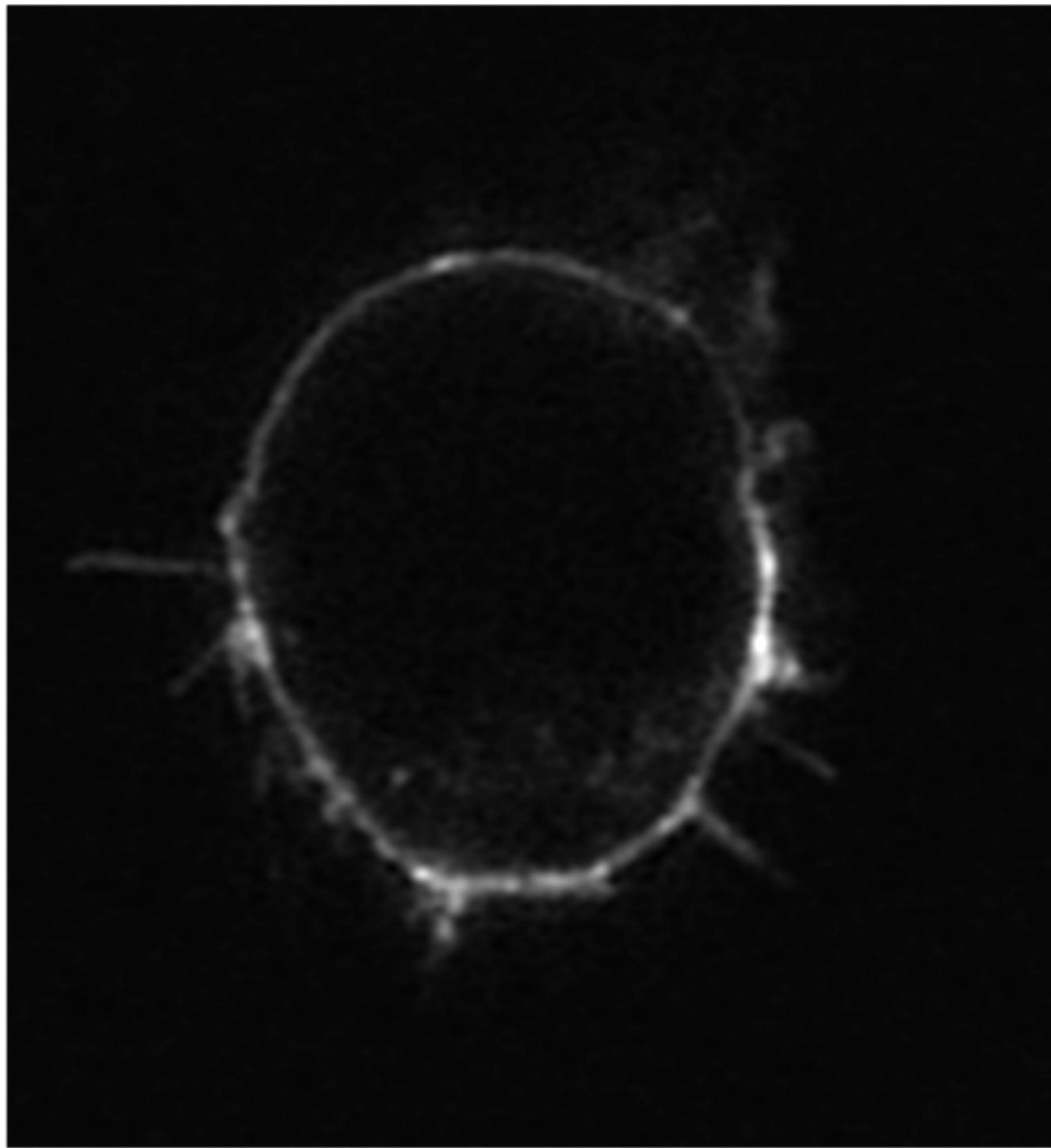
$r_P = 0.76$ EGFR and Rab13 concentrations predict each other relatively well, indicating a concentration-dependent relationship between these molecules.



Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

Pixel Randomization

Original Channel 2



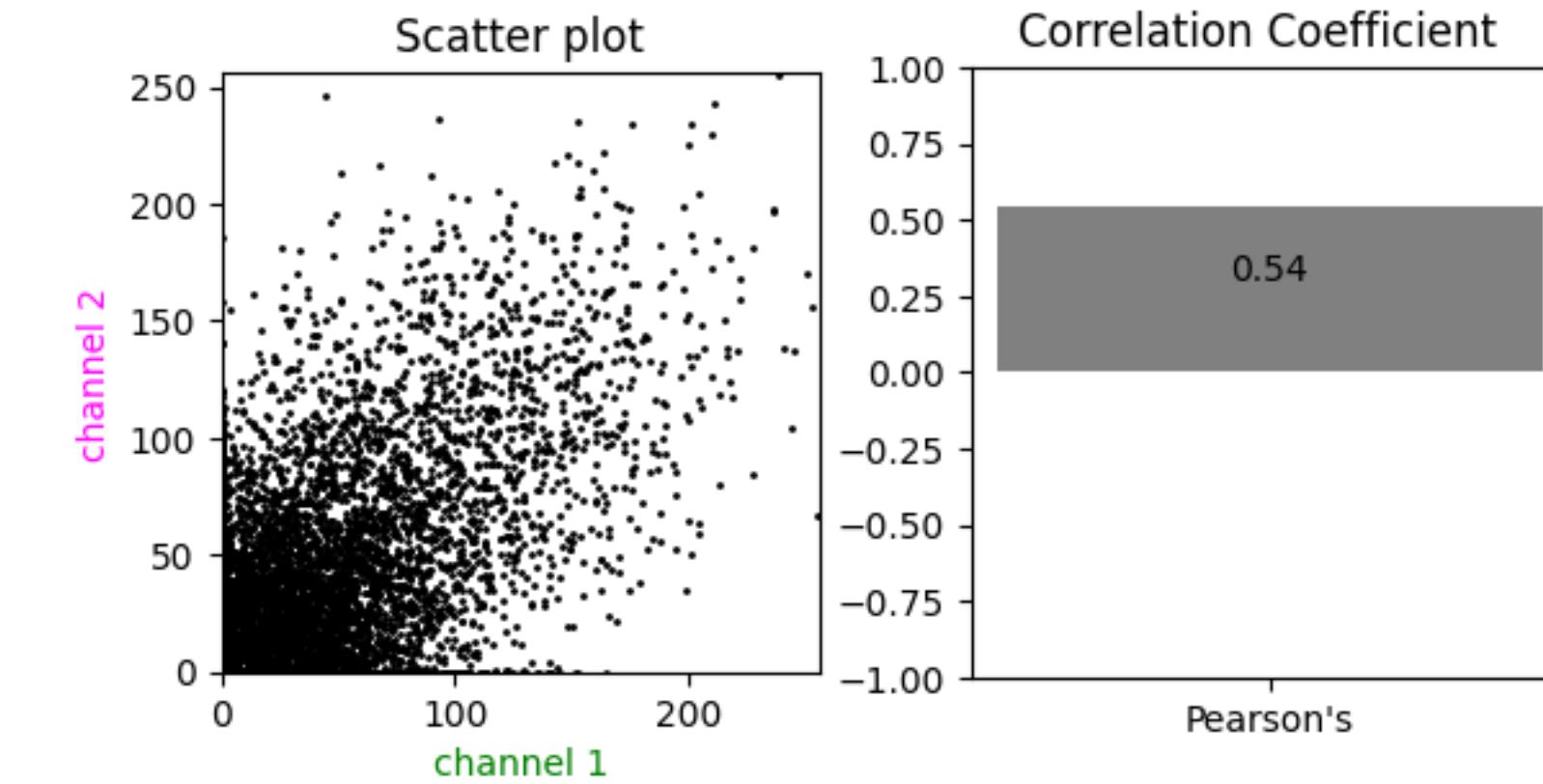
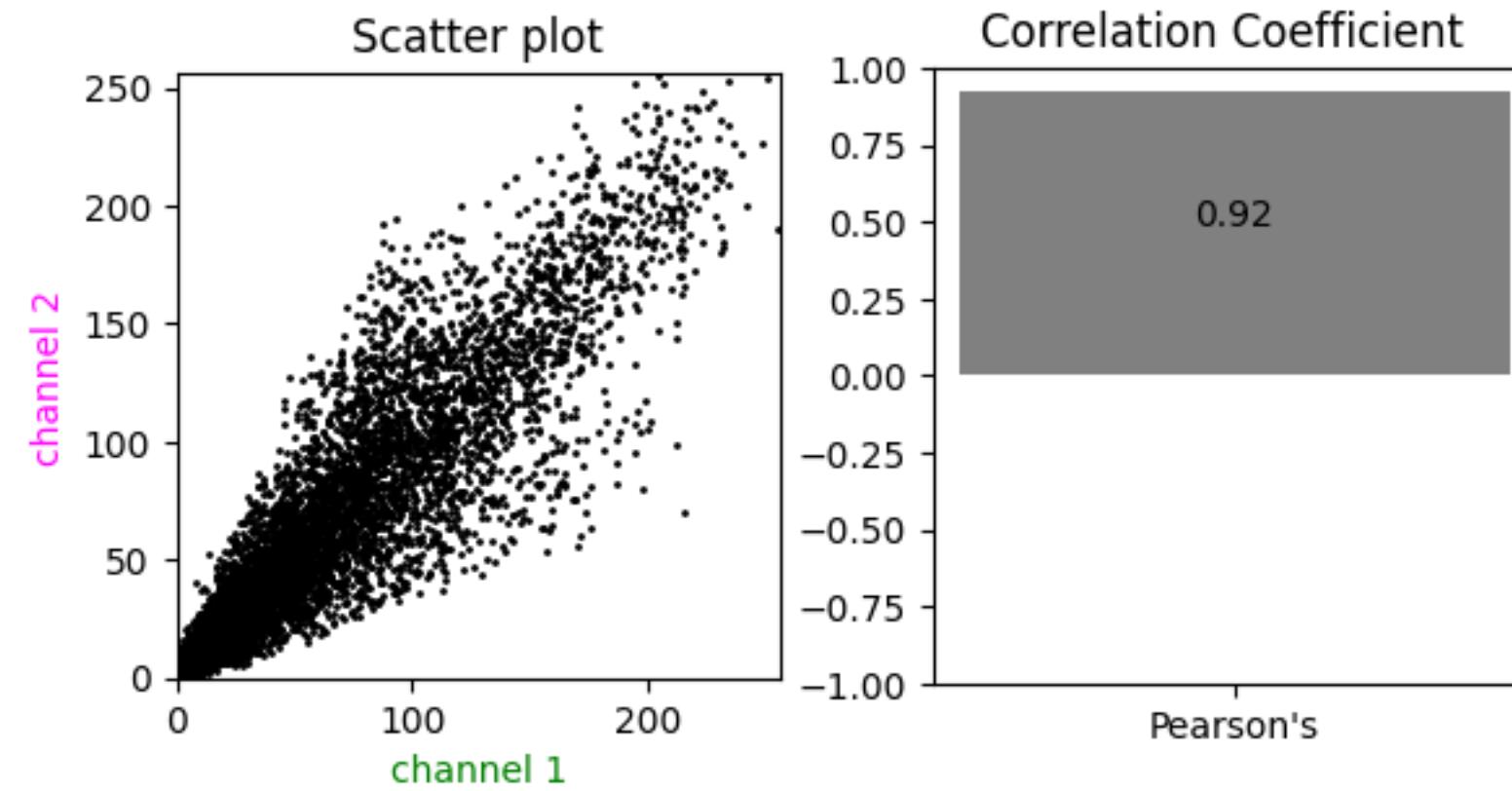
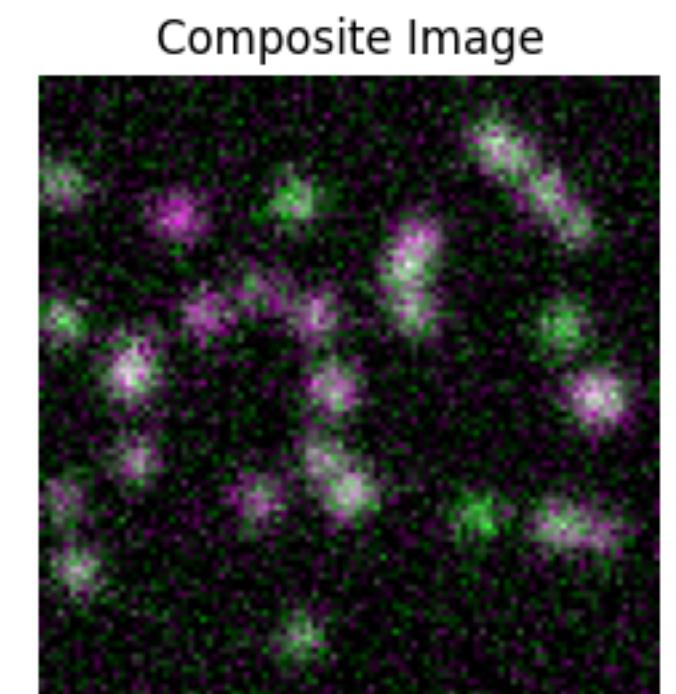
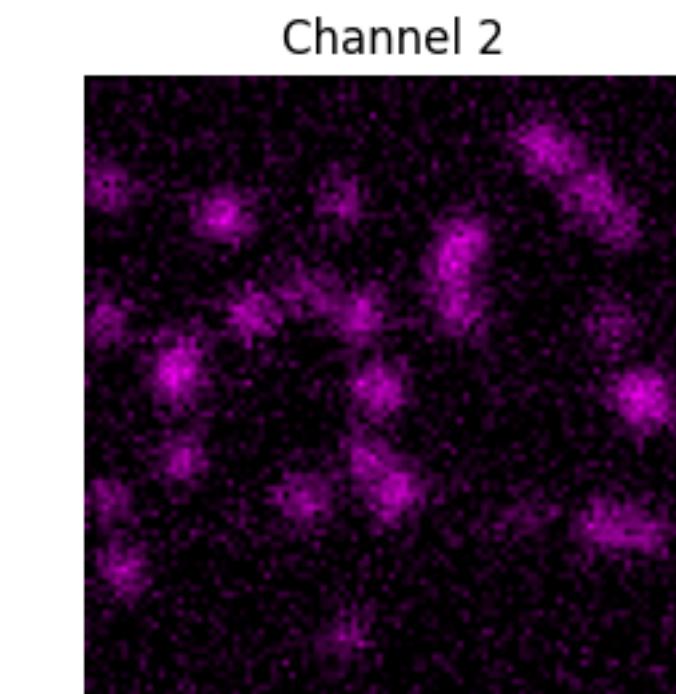
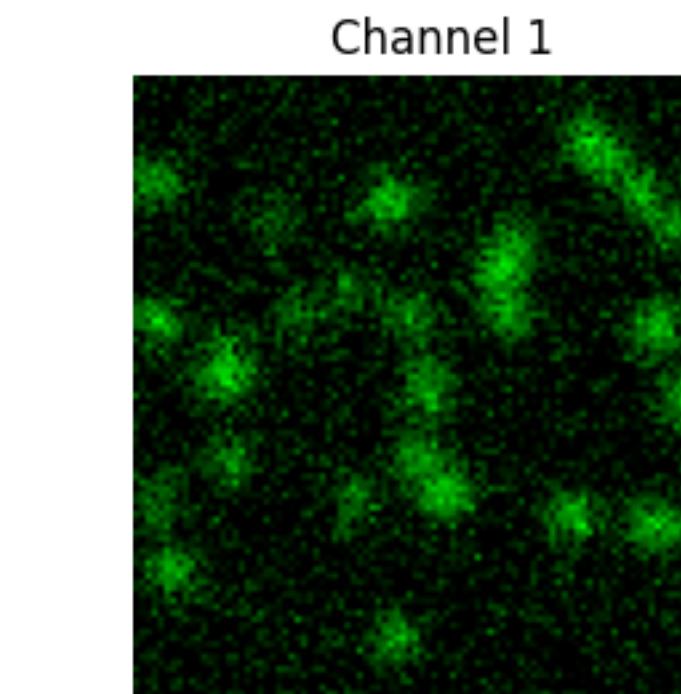
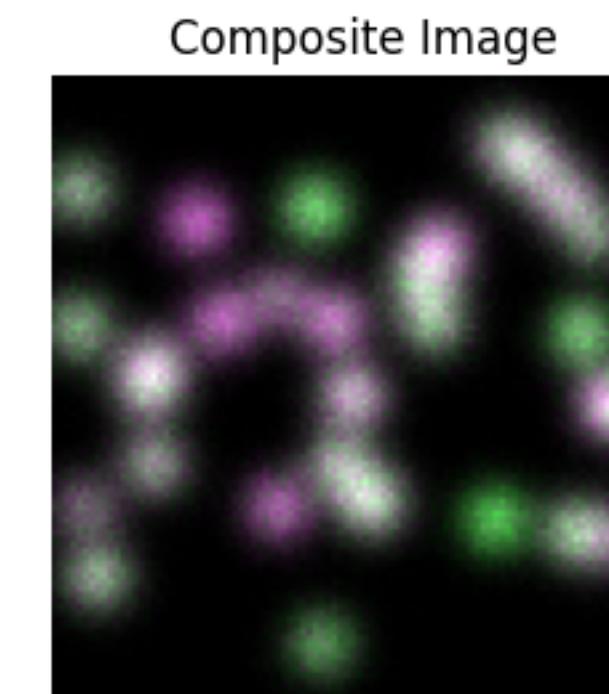
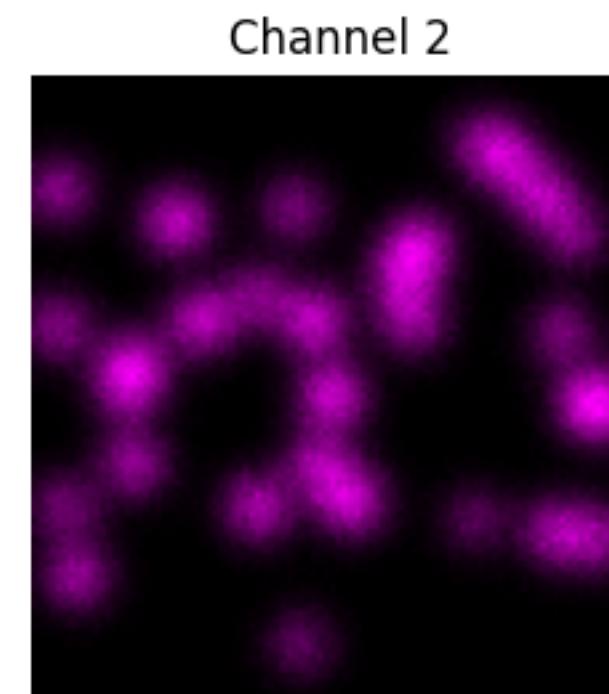
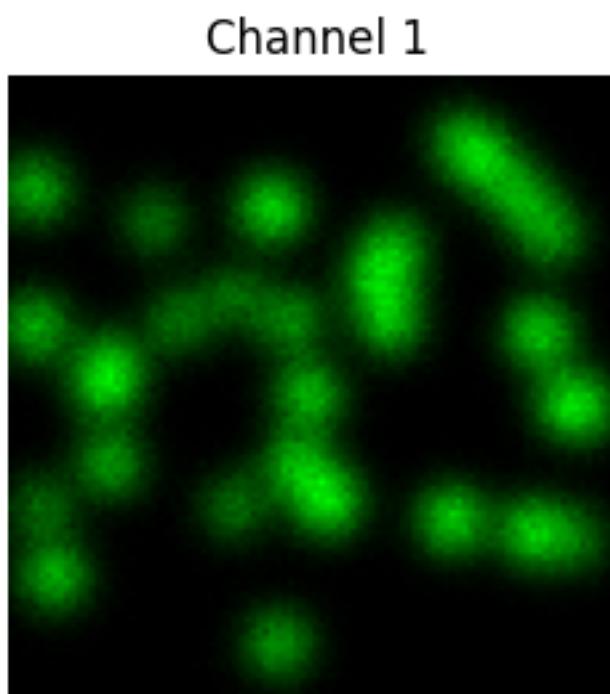
Randomized Channel 2 (Iteration 1)





Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

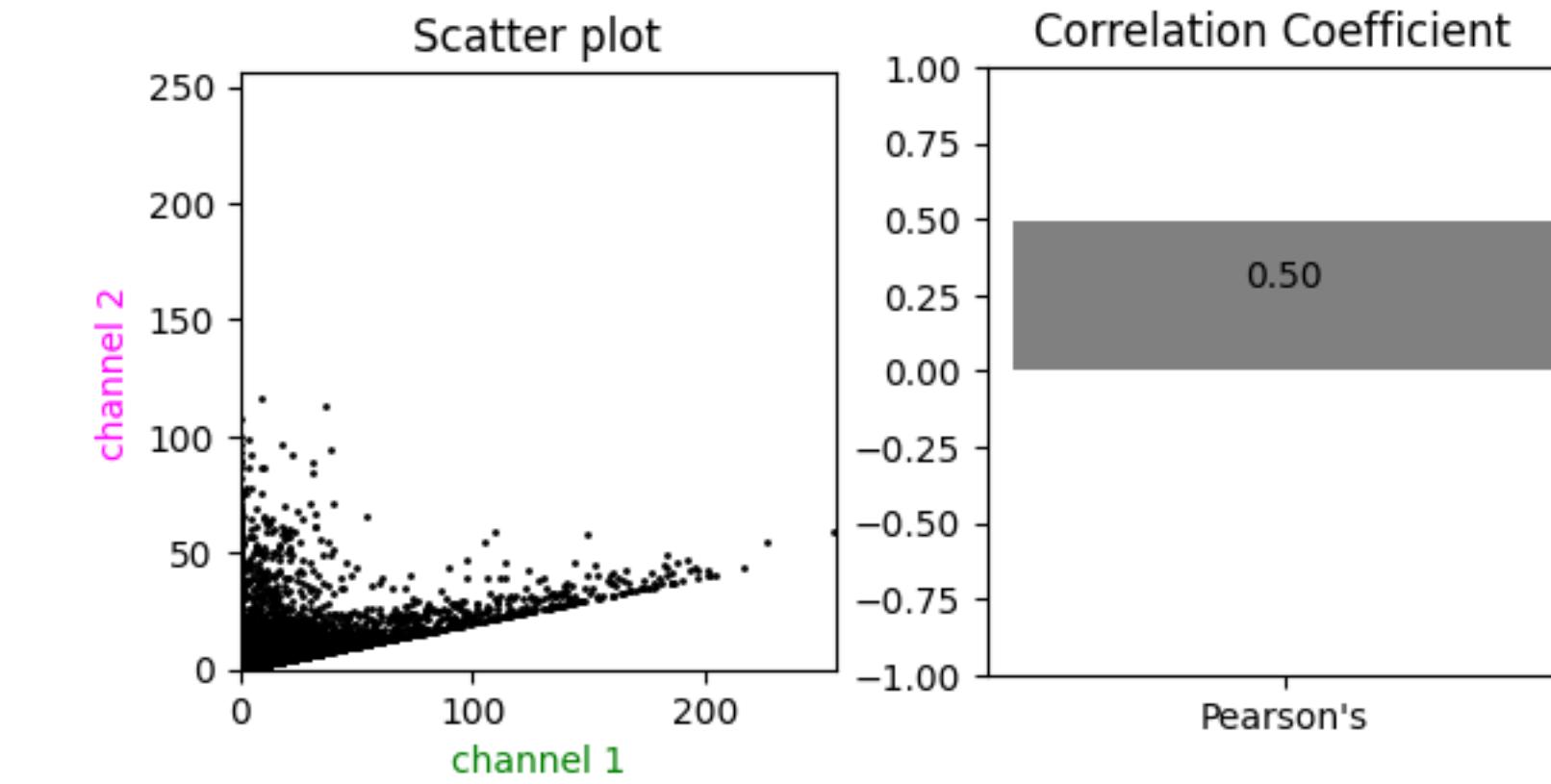
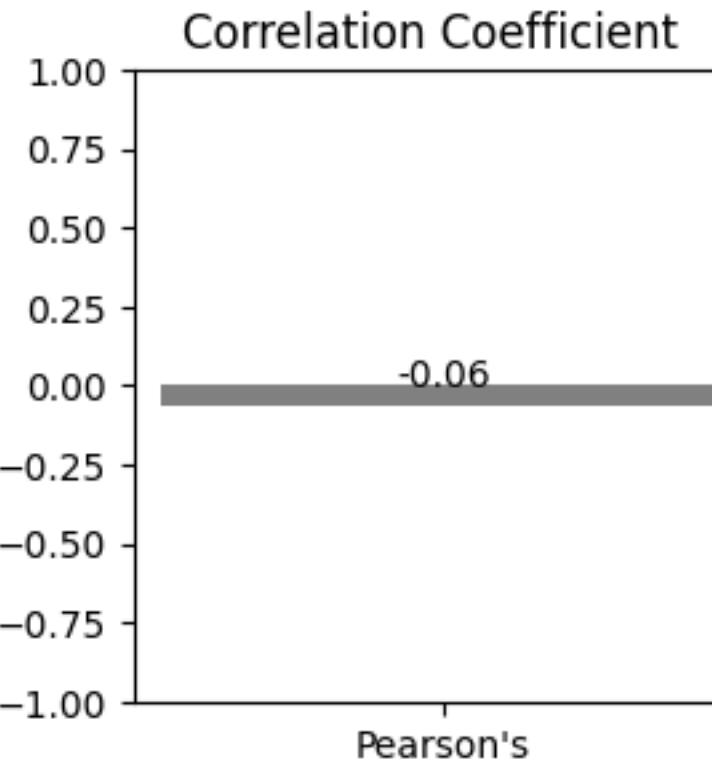
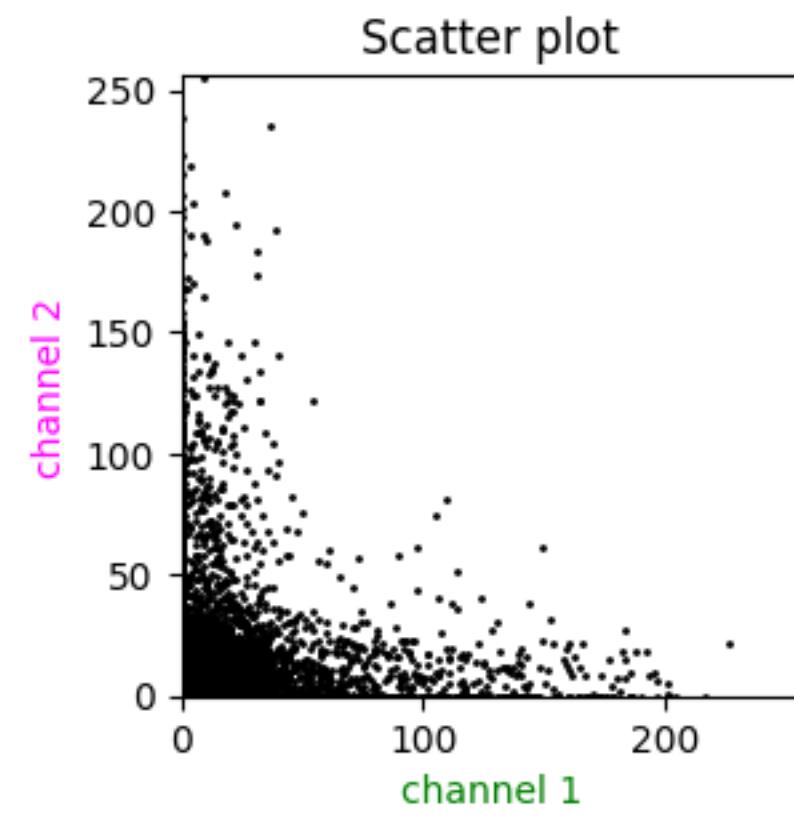
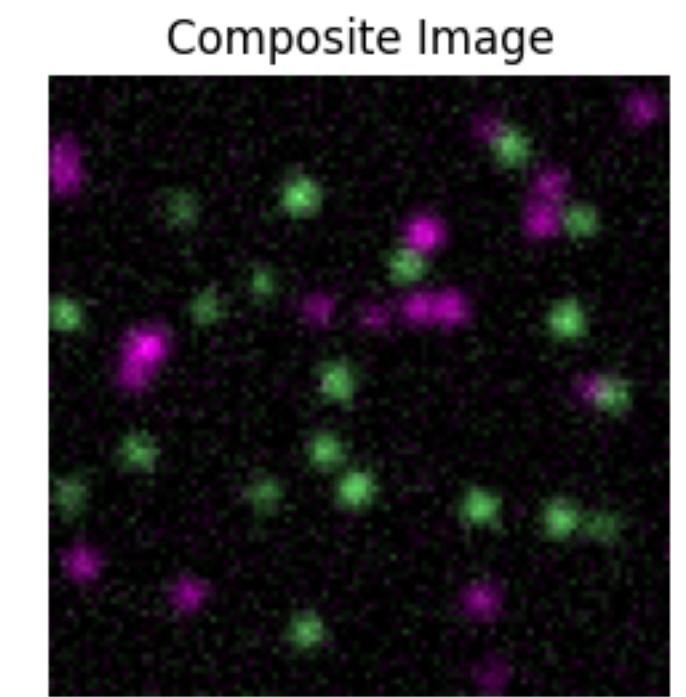
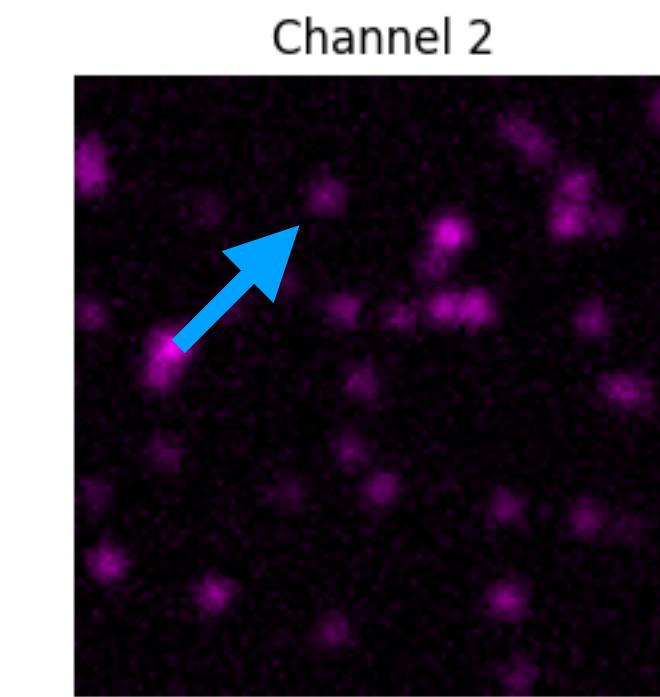
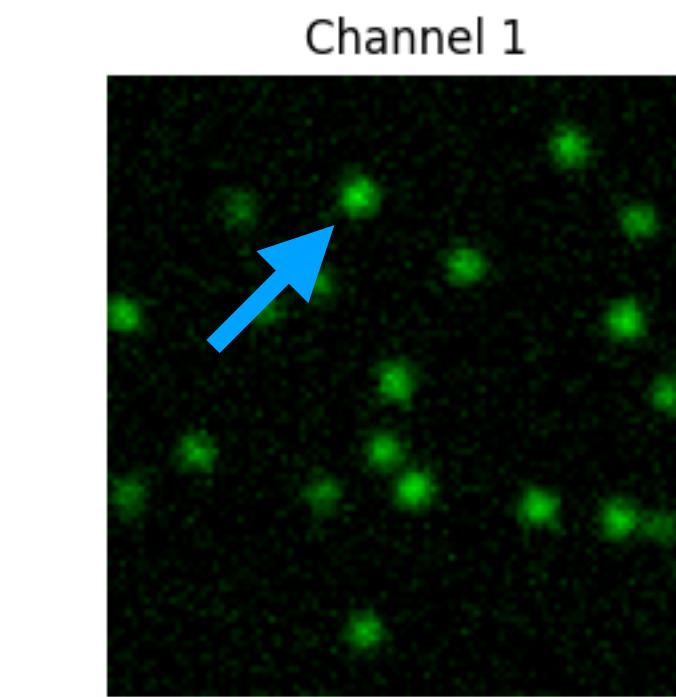
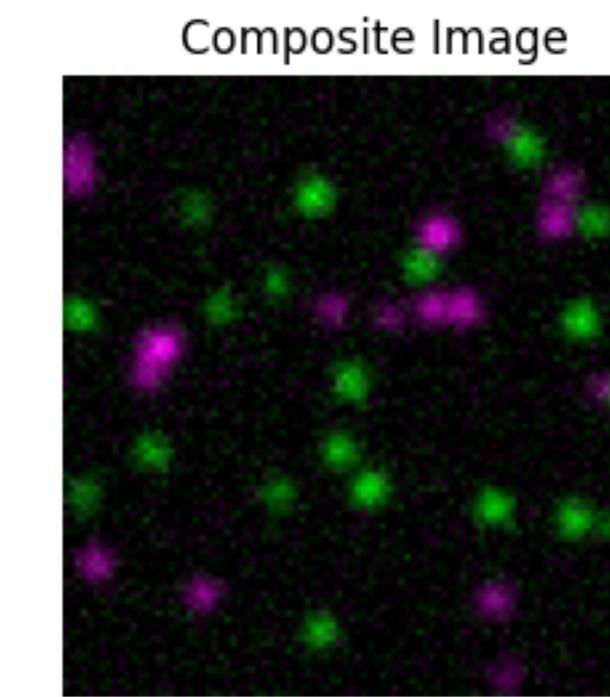
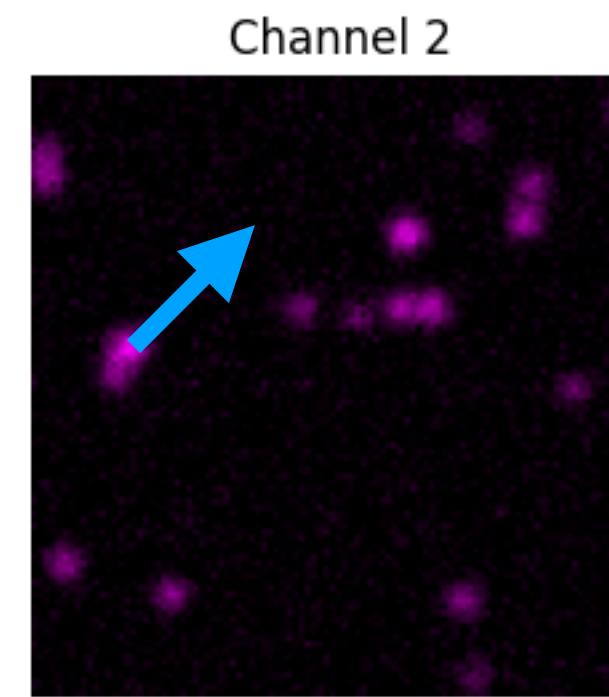
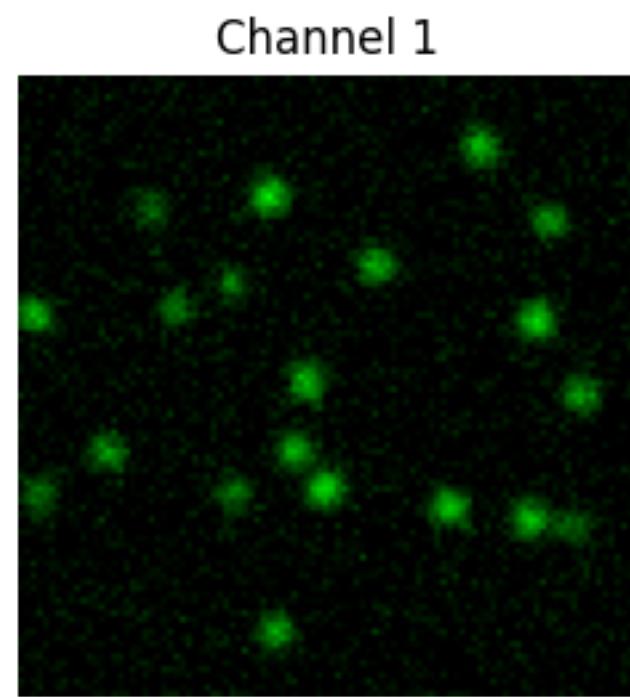
Noise





Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

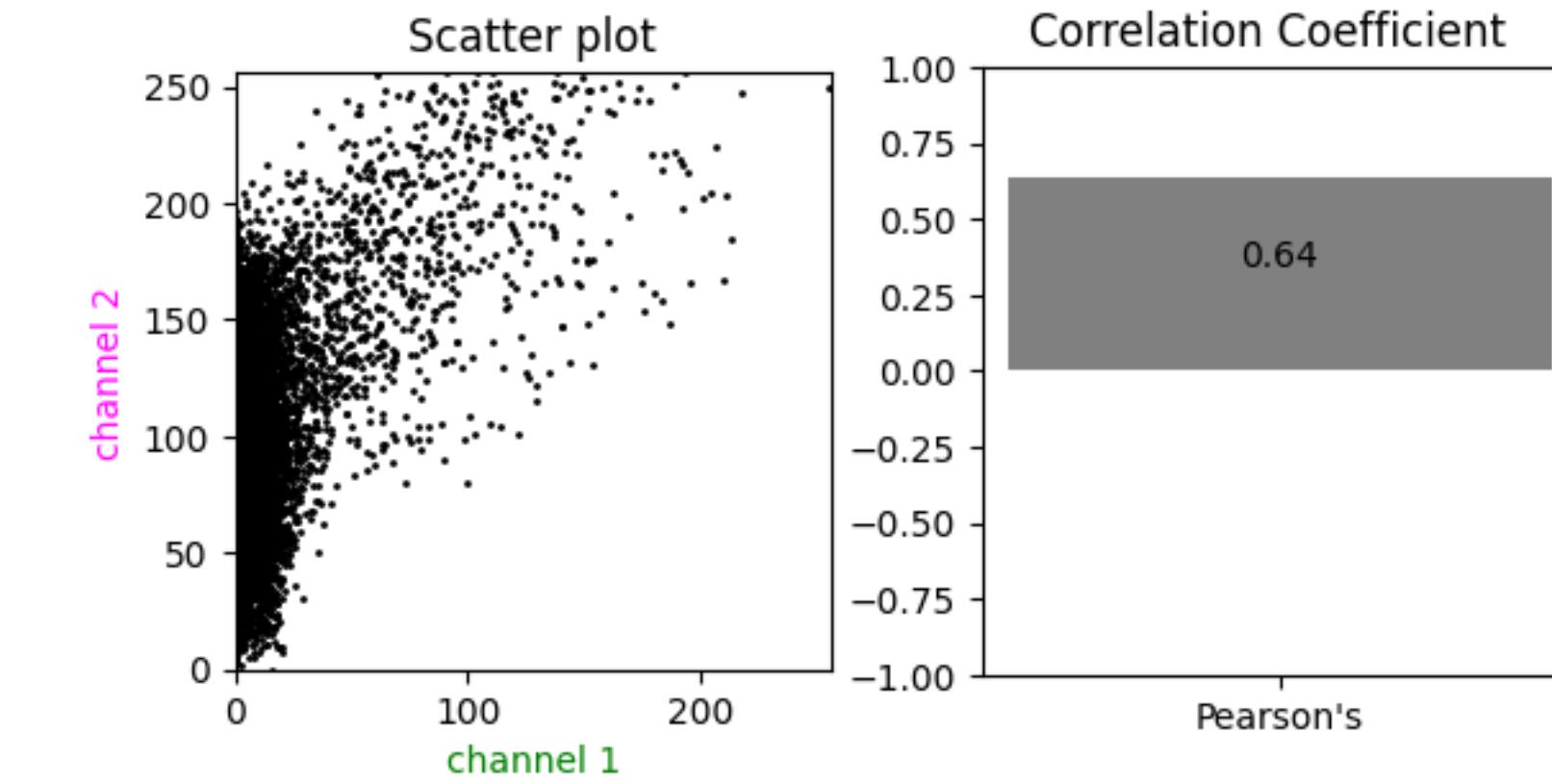
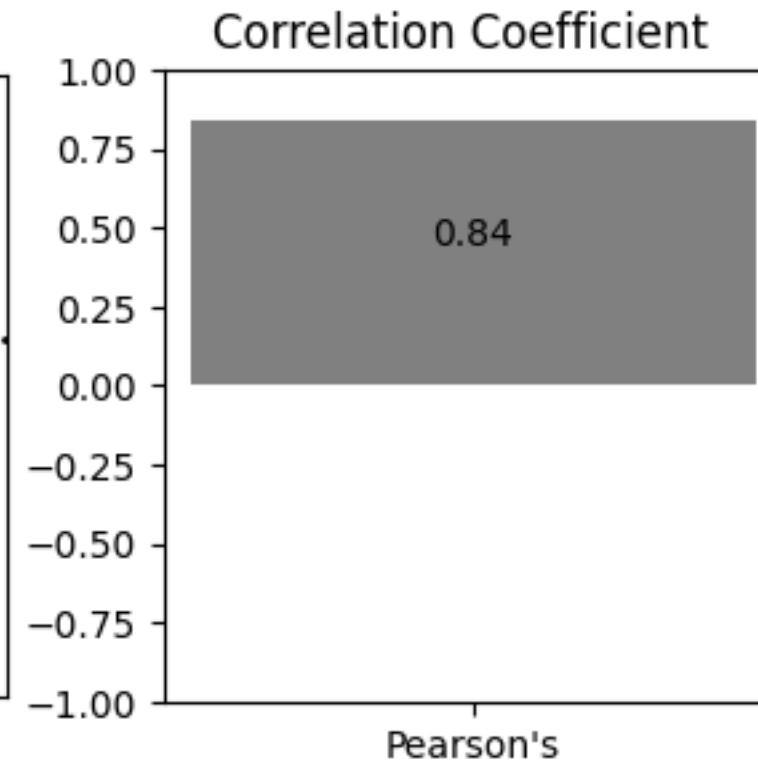
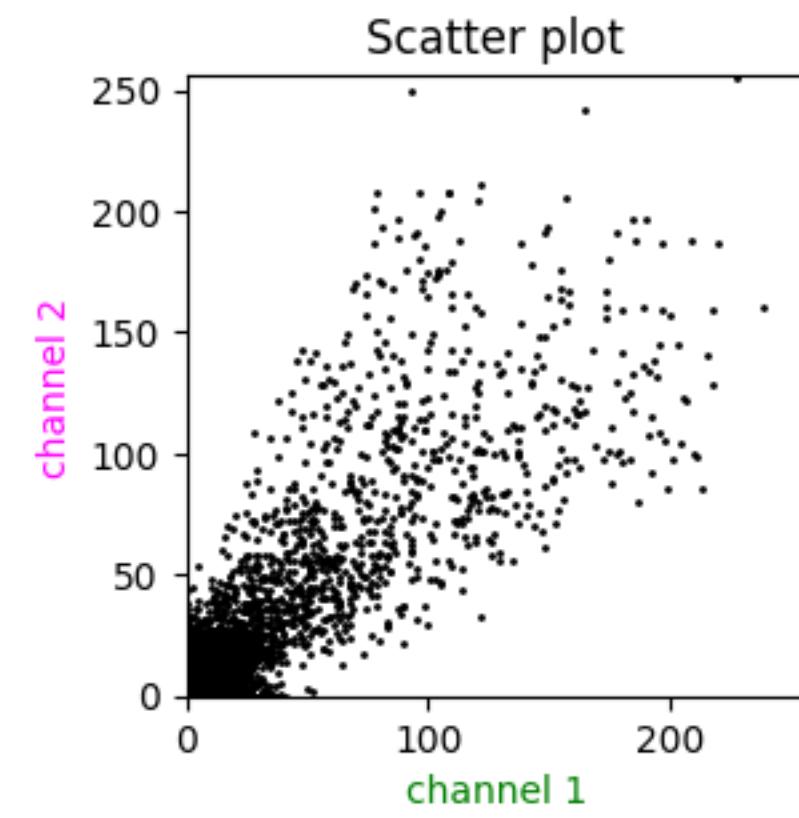
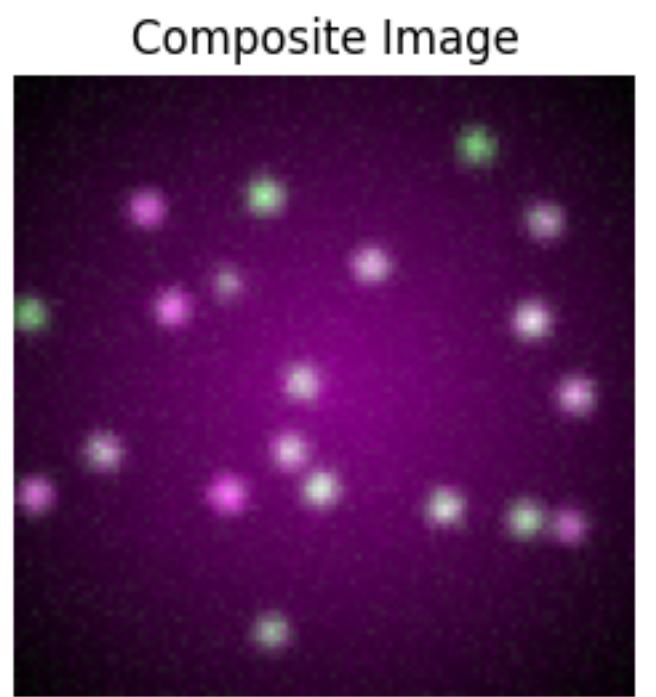
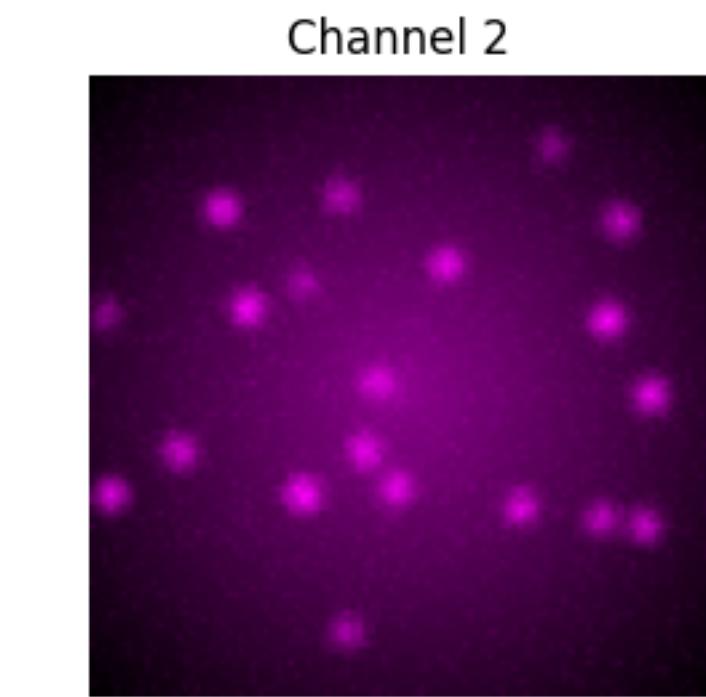
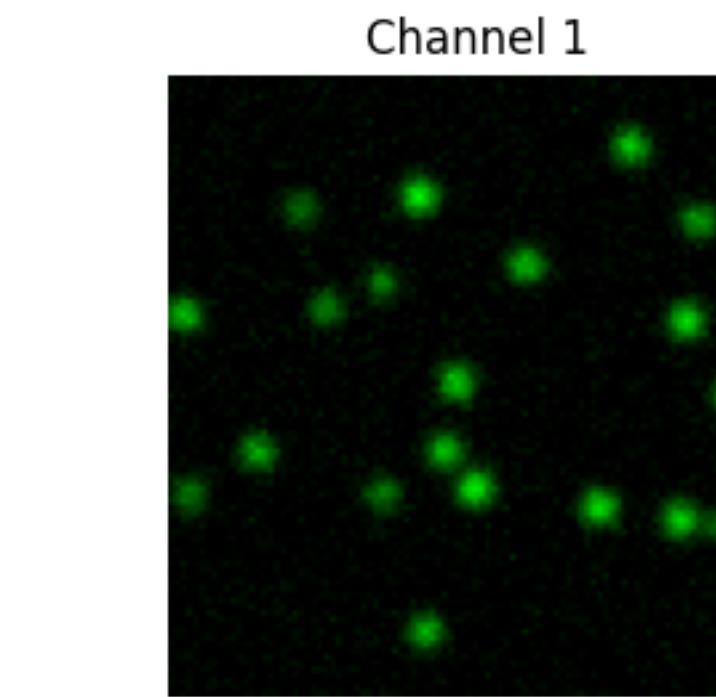
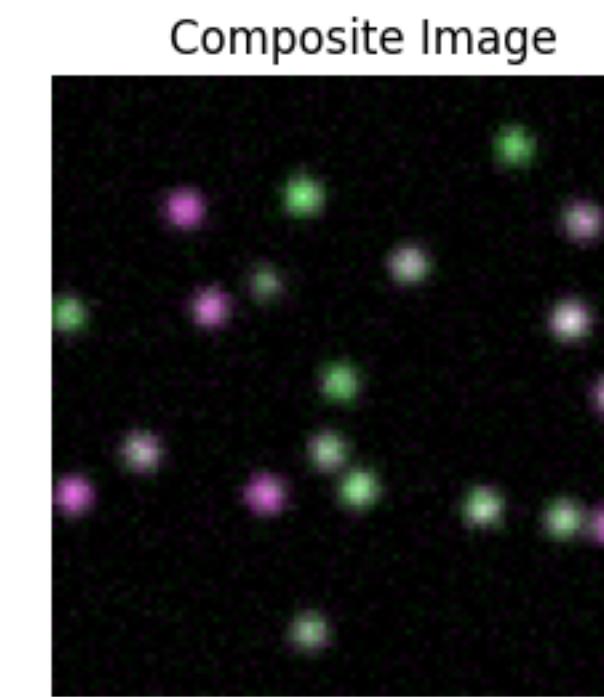
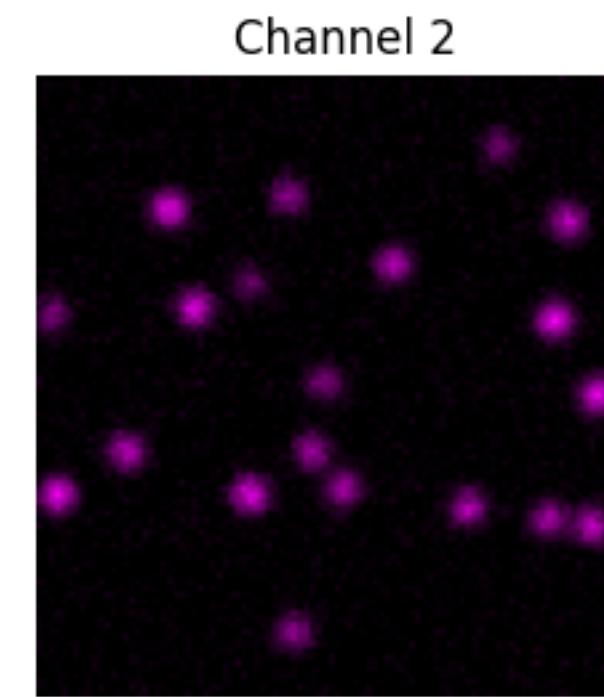
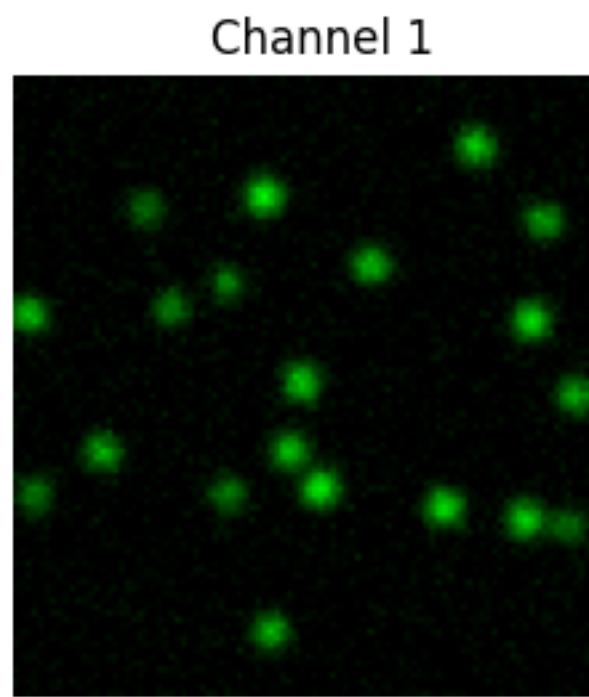
Bleedthrough





Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

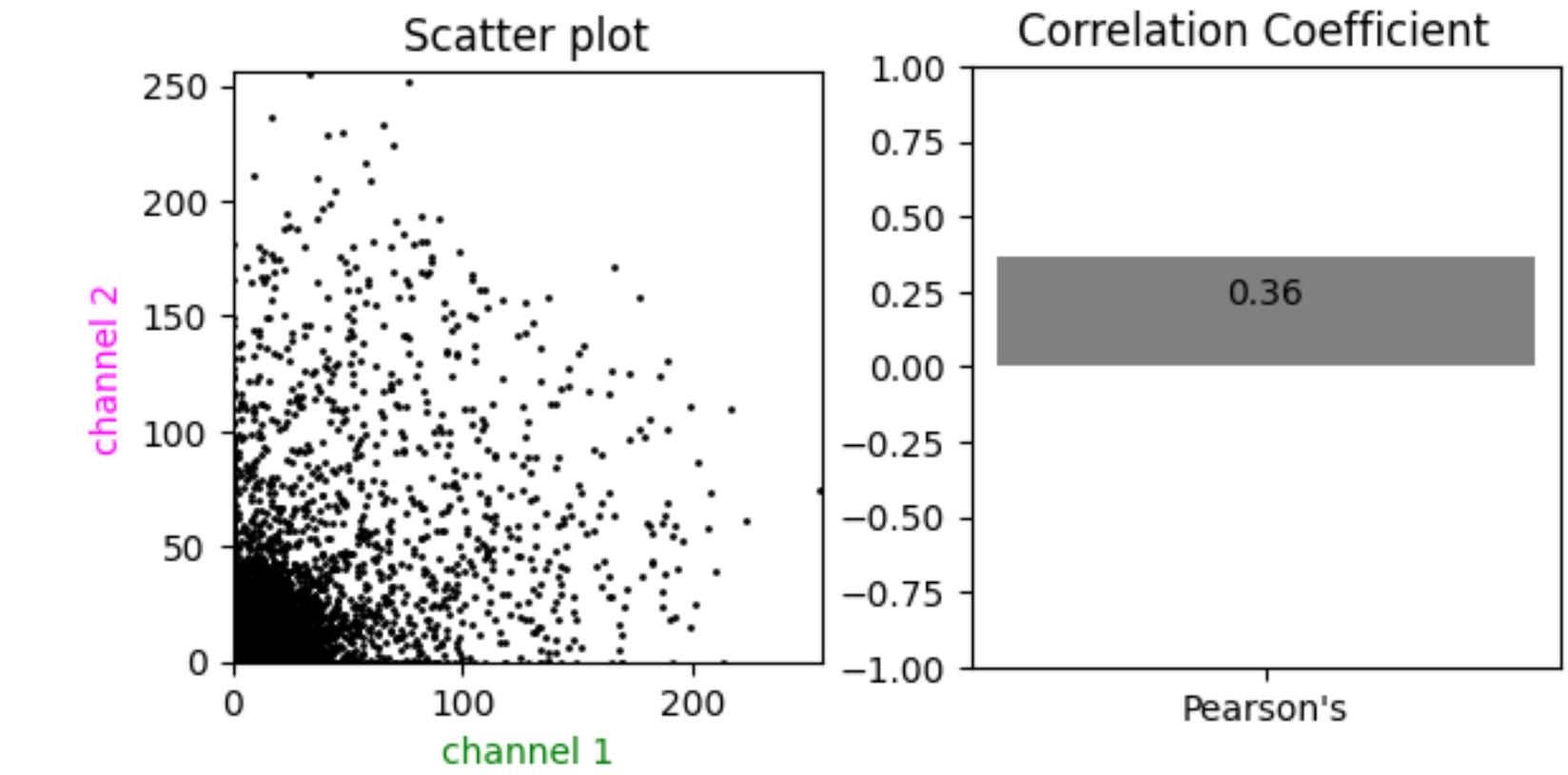
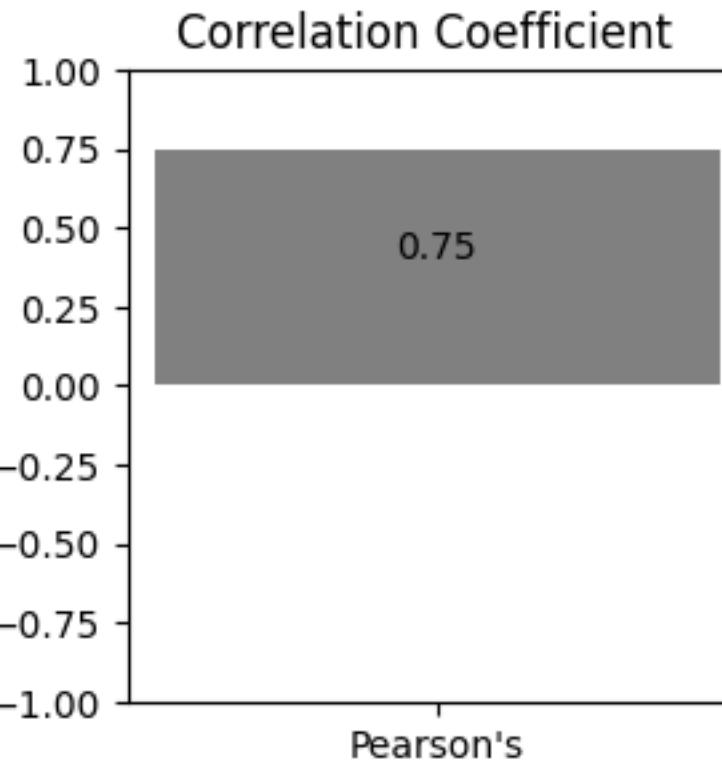
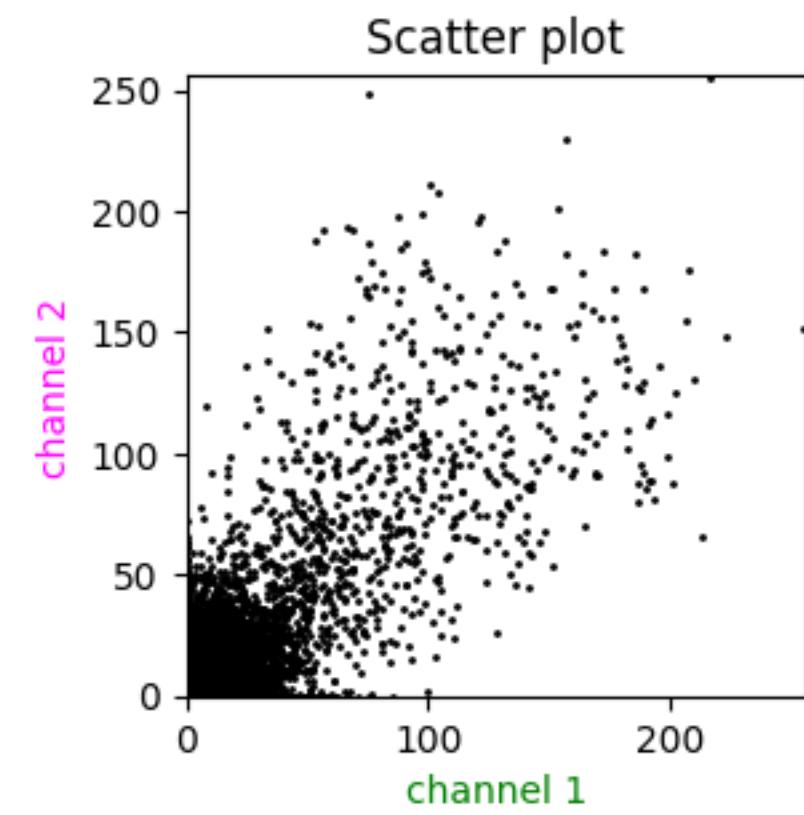
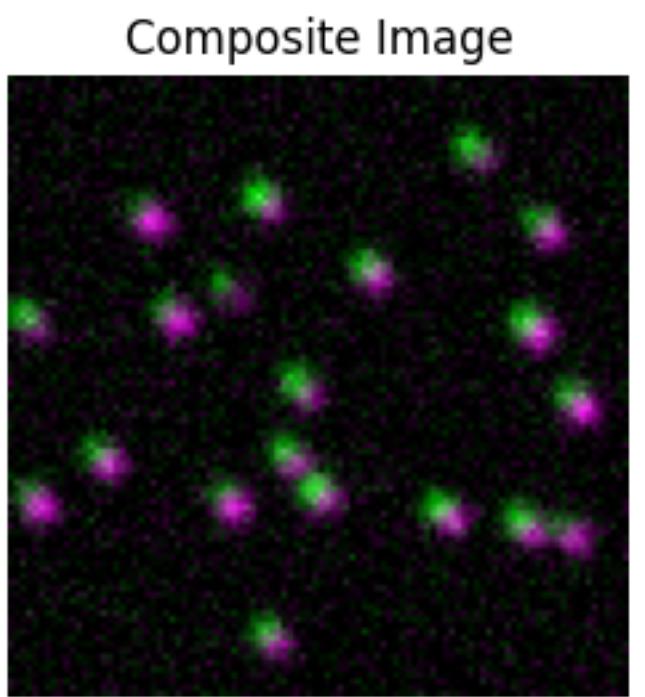
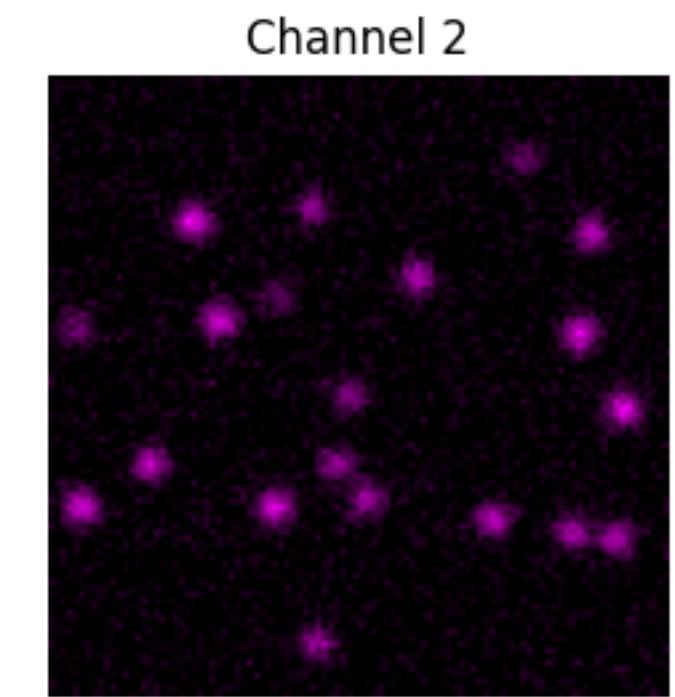
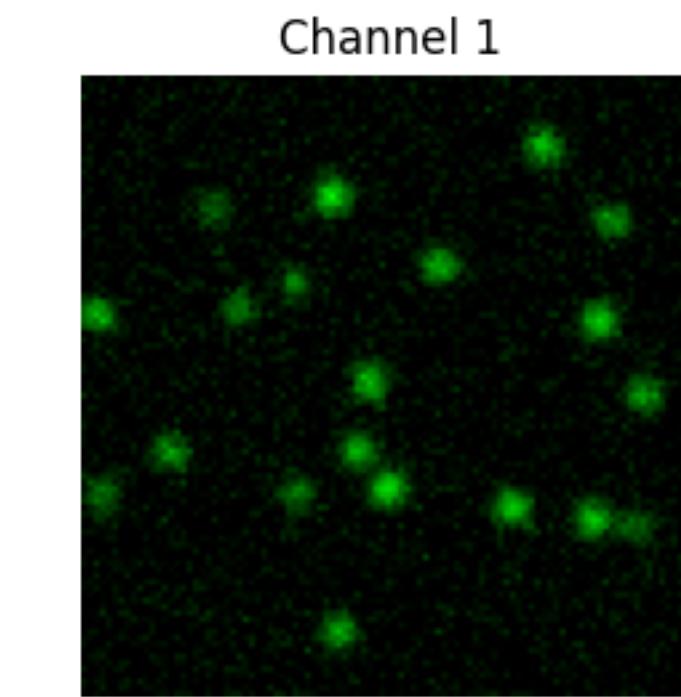
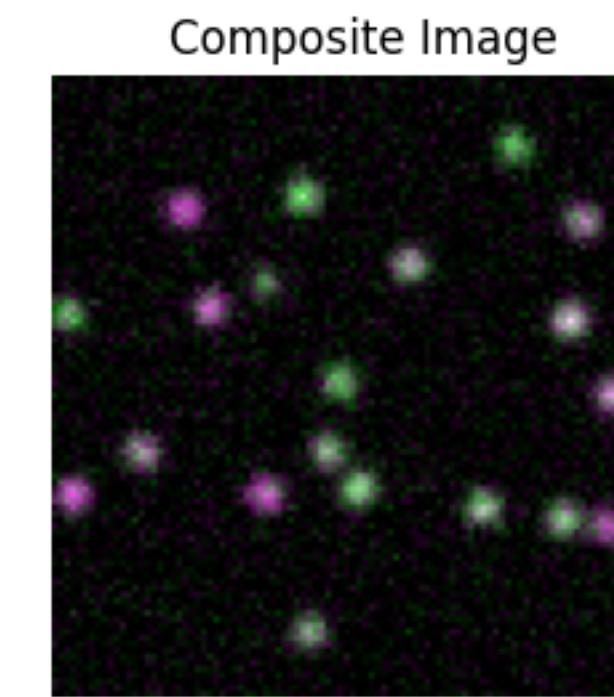
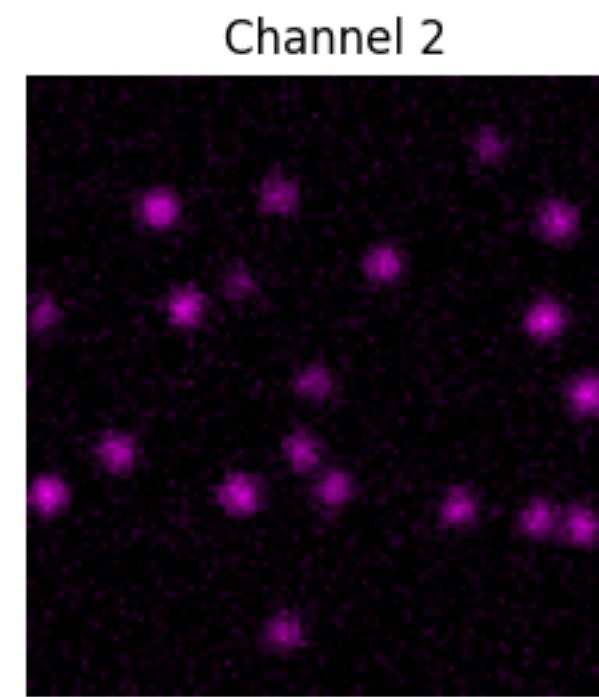
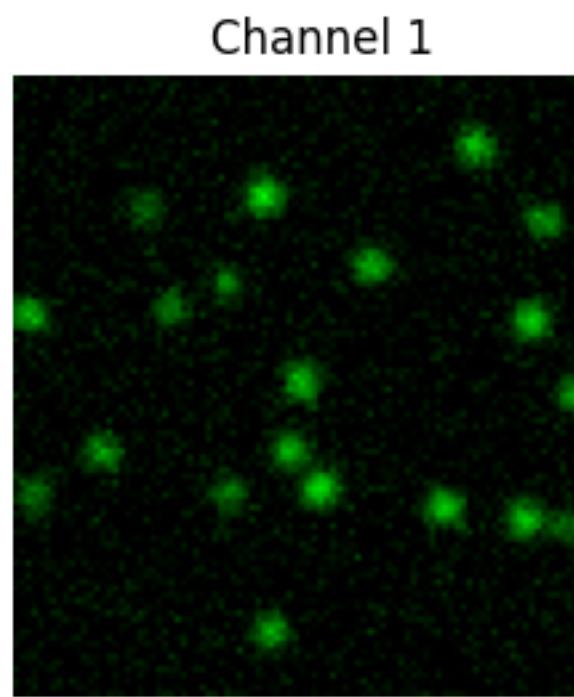
Uneven Illumination





Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

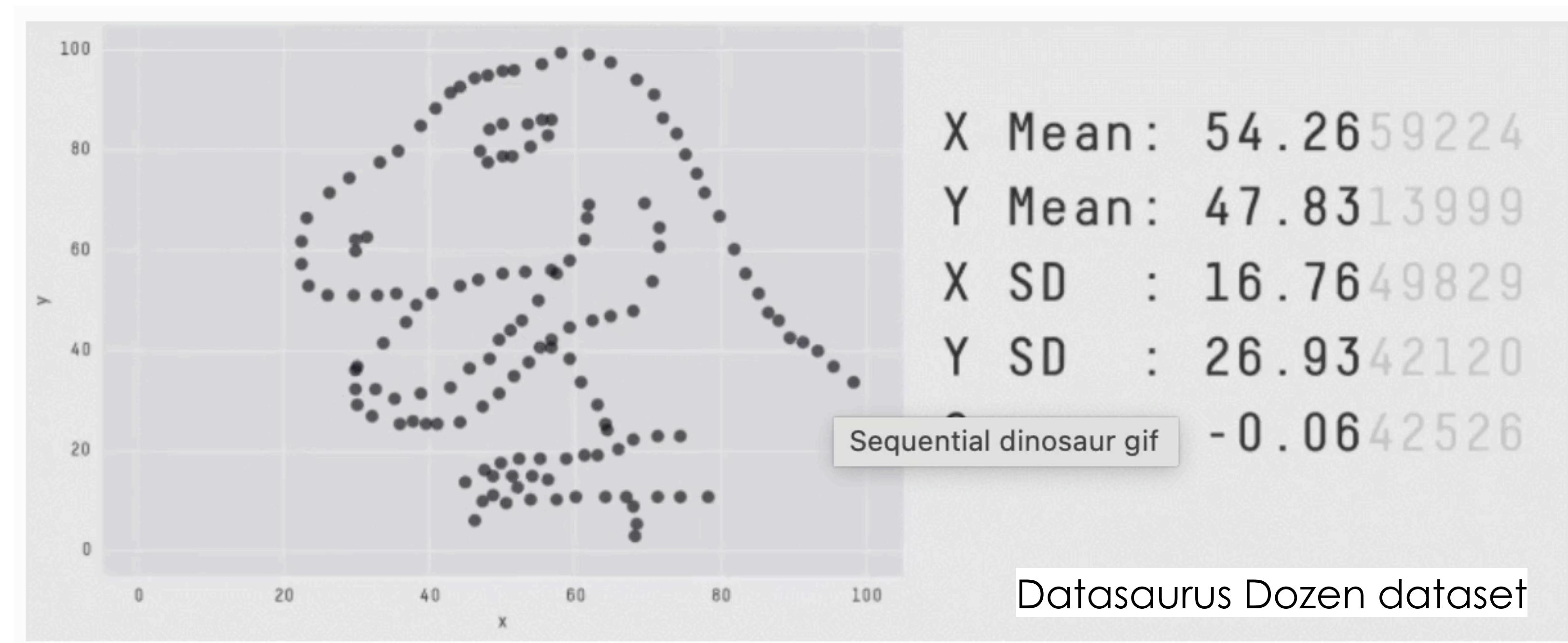
Chromatic Shift





Data Interpretation

plot your data





Intensity/Pixel-based: Manders' correlation coefficients (co-occurrence)

To measure the **degree of spatial overlap** between two signals.

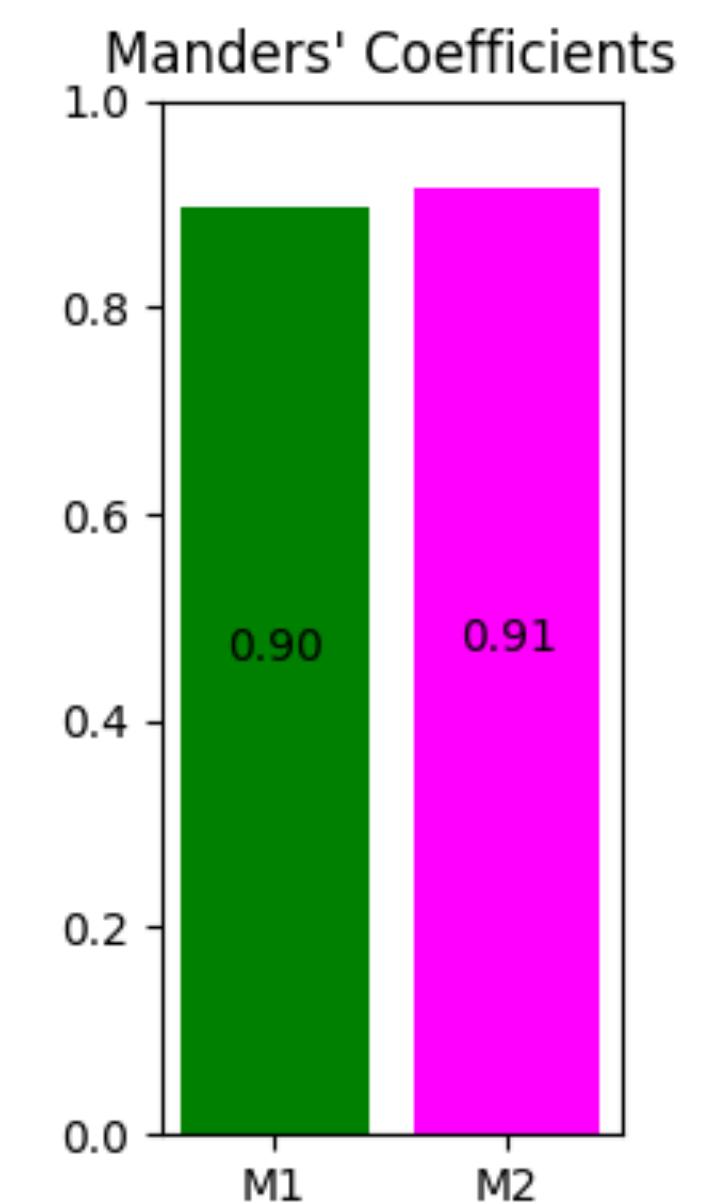
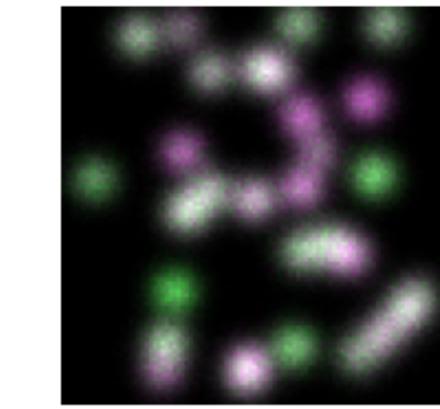
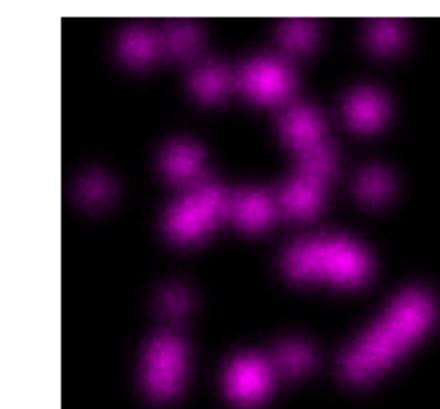
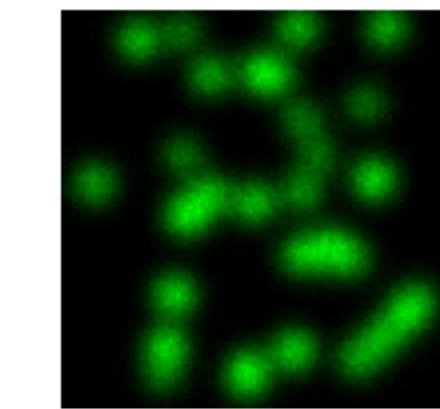
To measure the **proportion of pixel intensity** in one channel **that overlaps with pixel intensity** in the other channel.

$$M_1 = \frac{\sum_i R_i^{coloc}}{\sum_i R_i} \text{ and } M_2 = \frac{\sum_i G_i^{coloc}}{\sum_i G_i}$$

where $R_i^{coloc} = \begin{cases} R_i & \text{if } G_i > G_{thr} \text{ and } R_i > R_{thr} \\ 0 & \text{otherwise} \end{cases}$

where $G_i^{coloc} = \begin{cases} G_i & \text{if } R_i > R_{thr} \text{ and } G_i > G_{thr} \\ 0 & \text{otherwise} \end{cases}$

M1 and **M2** range between 0 and 1



M1 = fraction of channel 1 that co-occurs with channel 2

M2 = fraction of channel 2 that co-occurs with channel 1

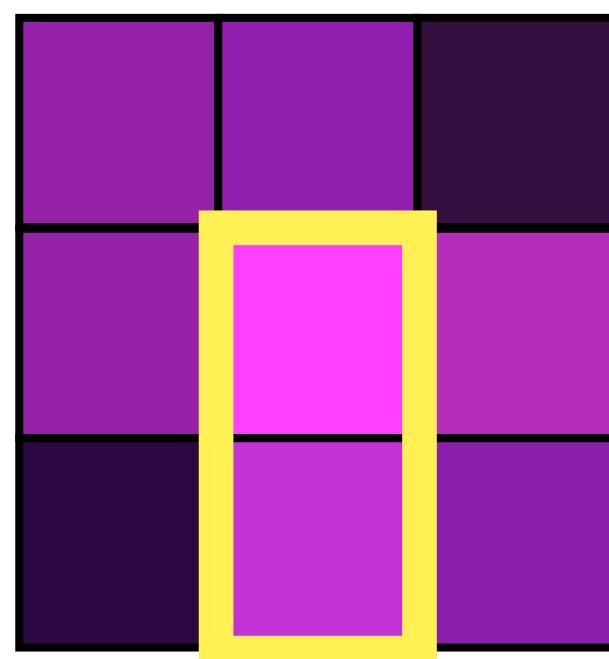


Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)

$$M_1 = \frac{\sum_i R_i^{coloc}}{\sum_i R_i} \text{ and } M_2 = \frac{\sum_i G_i^{coloc}}{\sum_i G_i}$$

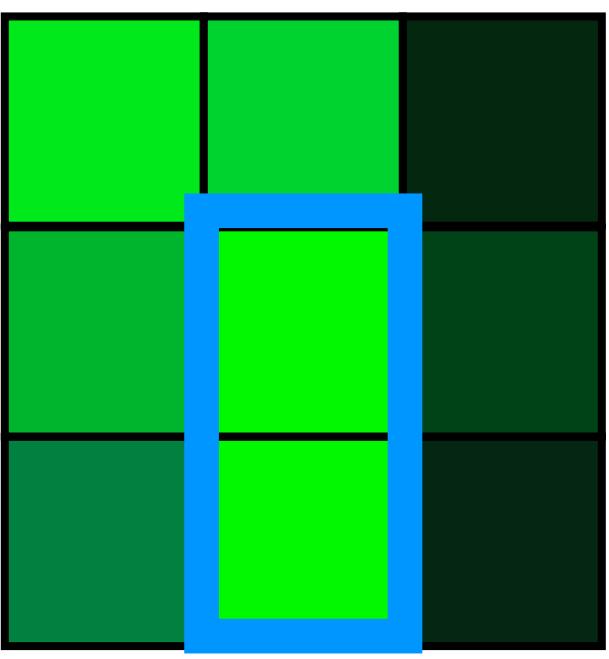
Set a threshold for channel 1:
consider only pixel with a **value > 75**

Set a threshold for channel 2
consider only pixel with a **value > 45**



60	65	10
60	200	100
5	90	76

$$M1 = \frac{200+90=290}{200+90+100+76=466} = 0.62$$



100	90	5
60	150	10
50	150	6

$$M2 = \frac{150+150=300}{100+90+60+150+50+150=600} = 0.5$$

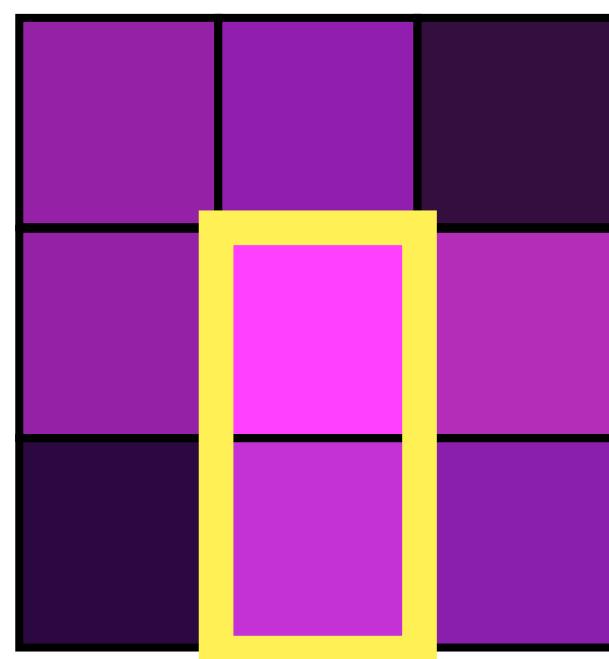


Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)

$$M_1 = \frac{\sum_i R_i^{coloc}}{\sum_i R_i} \quad \text{and} \quad M_2 = \frac{\sum_i G_i^{coloc}}{\sum_i G_i}$$

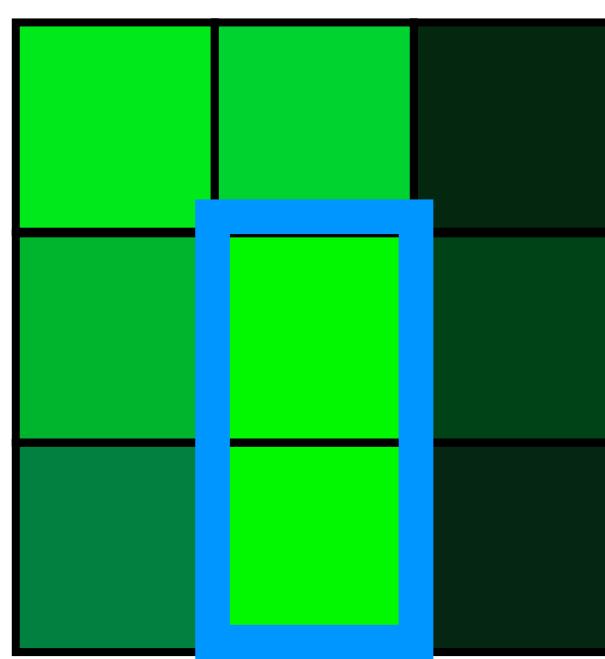
Set a threshold for channel 1:
consider only pixel with a value > 75

Set a threshold for channel 2
consider only pixel with a value > 45



60	65	10
60	200	100
5	90	76

$$\mathbf{M1 = 0.62}$$



100	90	5
60	150	10
50	150	6

$$\mathbf{M2 = 0.5}$$

- Mander's **M1** and **M2** can be different from each other

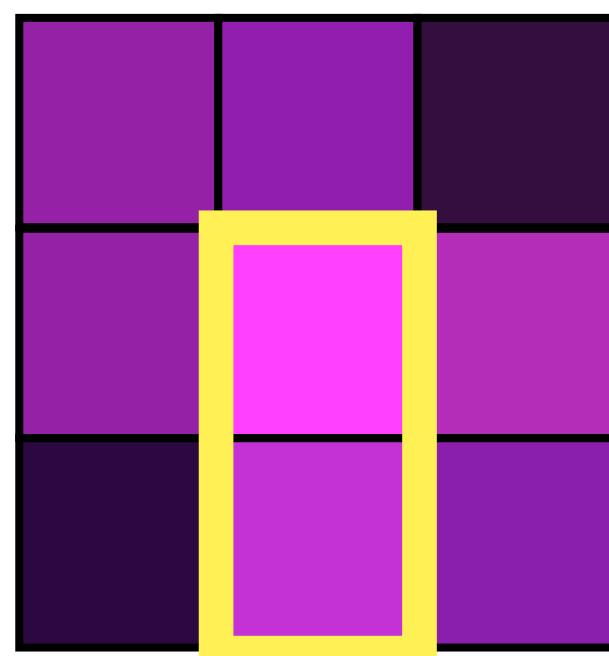


Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)

$$M_1 = \frac{\sum_i R_i^{coloc}}{\sum_i R_i} \quad \text{and} \quad M_2 = \frac{\sum_i G_i^{coloc}}{\sum_i G_i}$$

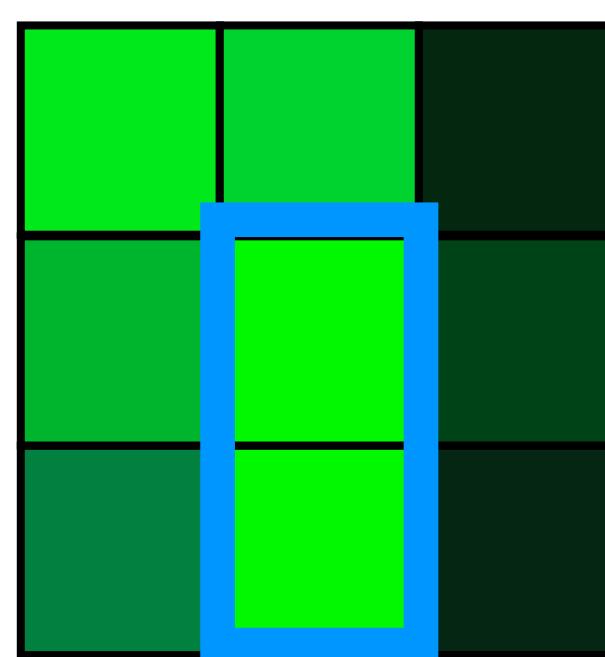
Set a threshold for channel 1:
consider only pixel with a value > 75

Set a threshold for channel 2
consider only pixel with a value > 45



60	65	10
60	200	100
5	90	76

$$\mathbf{M1 = 0.62}$$



100	90	5
60	150	10
50	150	6

$$\mathbf{M2 = 0.5}$$

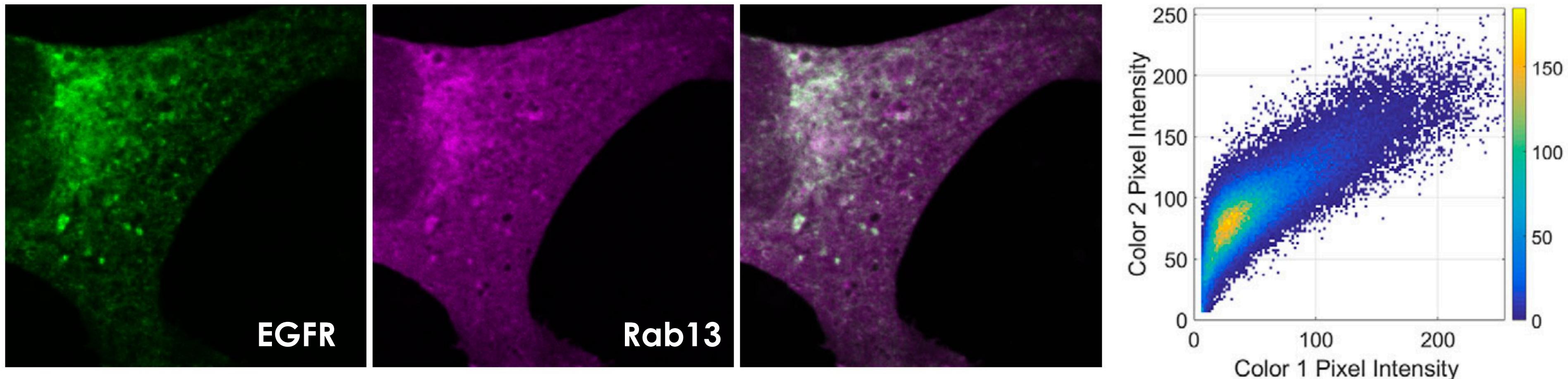
- Mander's **M1** and **M2** can be different from each other

- Mander's **M1** and **M2** ≠ ratio of areas
in the **magenta** channel we have **2 pixel** in the overlap region (yellow) out of **4 total**, thus the **50%**, but **M1 is 62%** since we take into consideration the intensity values.

- in the **green** channel we have **2 pixel** in the overlap region (cyan) out of **6 total**, thus the **~33%**, but **M2 is 50%** since we take into consideration the intensity values.



Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

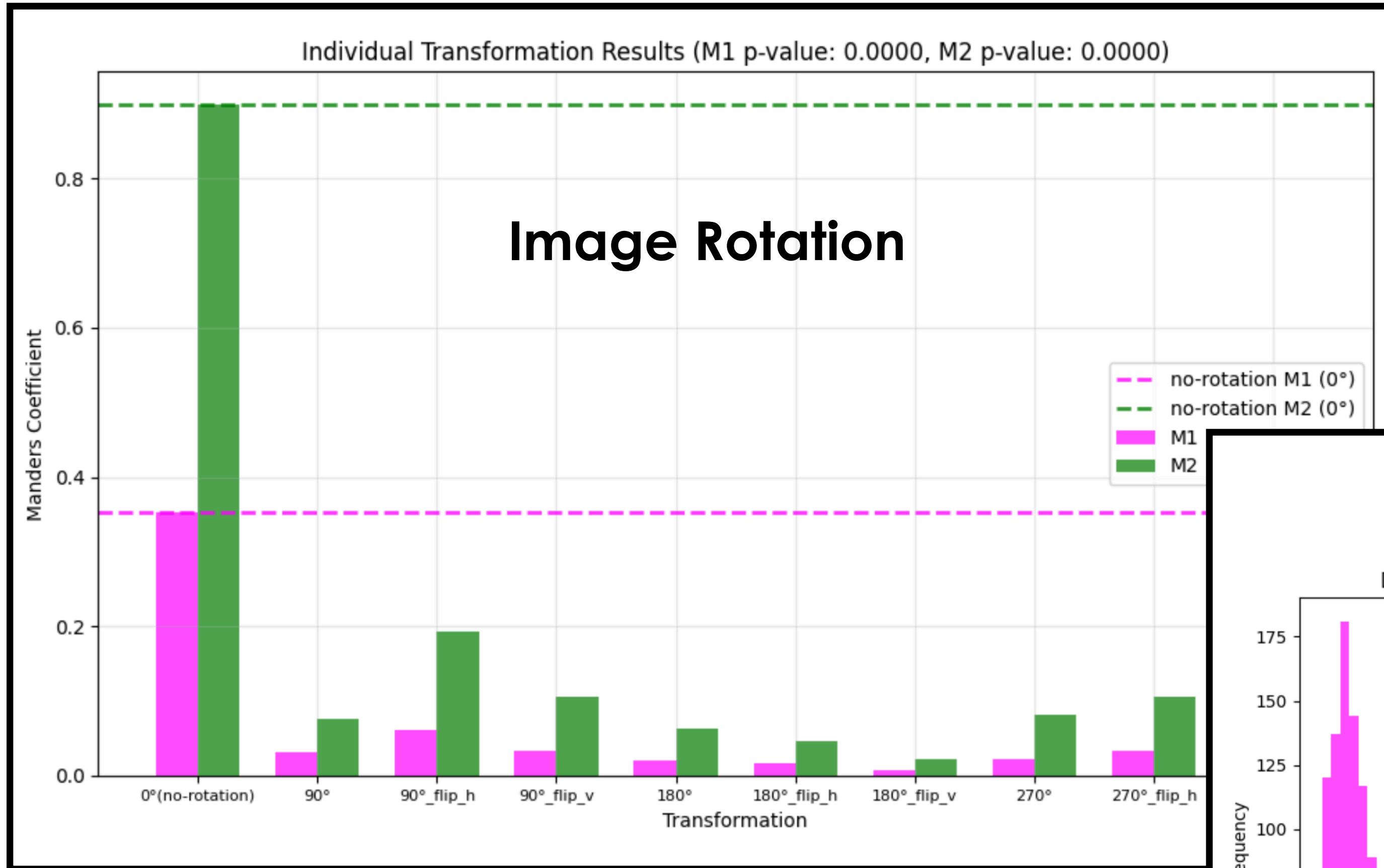


$r_P = 0.76$ EGFR and Rab13 concentrations predict each other relatively well, indicating a concentration-dependent relationship between these molecules.

$M1 = 0.99$ all of the EGFR signal overlaps with that of Rab13, not all Rab13 co-occurs with EGFR.
 $M2 = 0.44$ This suggests that, although Rab13 may associate with EGFR, it may also be associated with other molecules at different cellular locations.

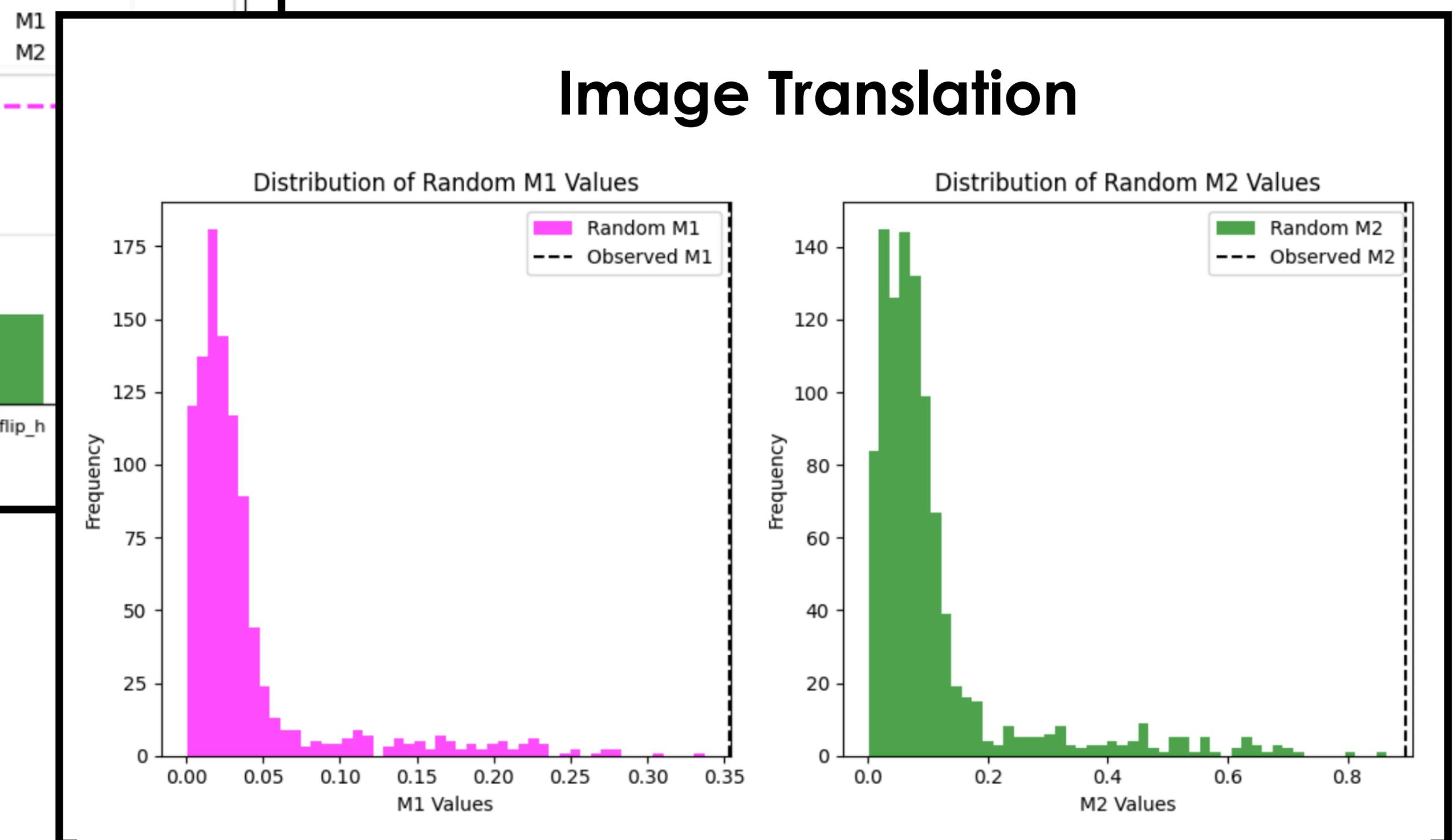


Intensity/Pixel-based: Pearson's correlation coefficient (correlation)



Block Scrambling

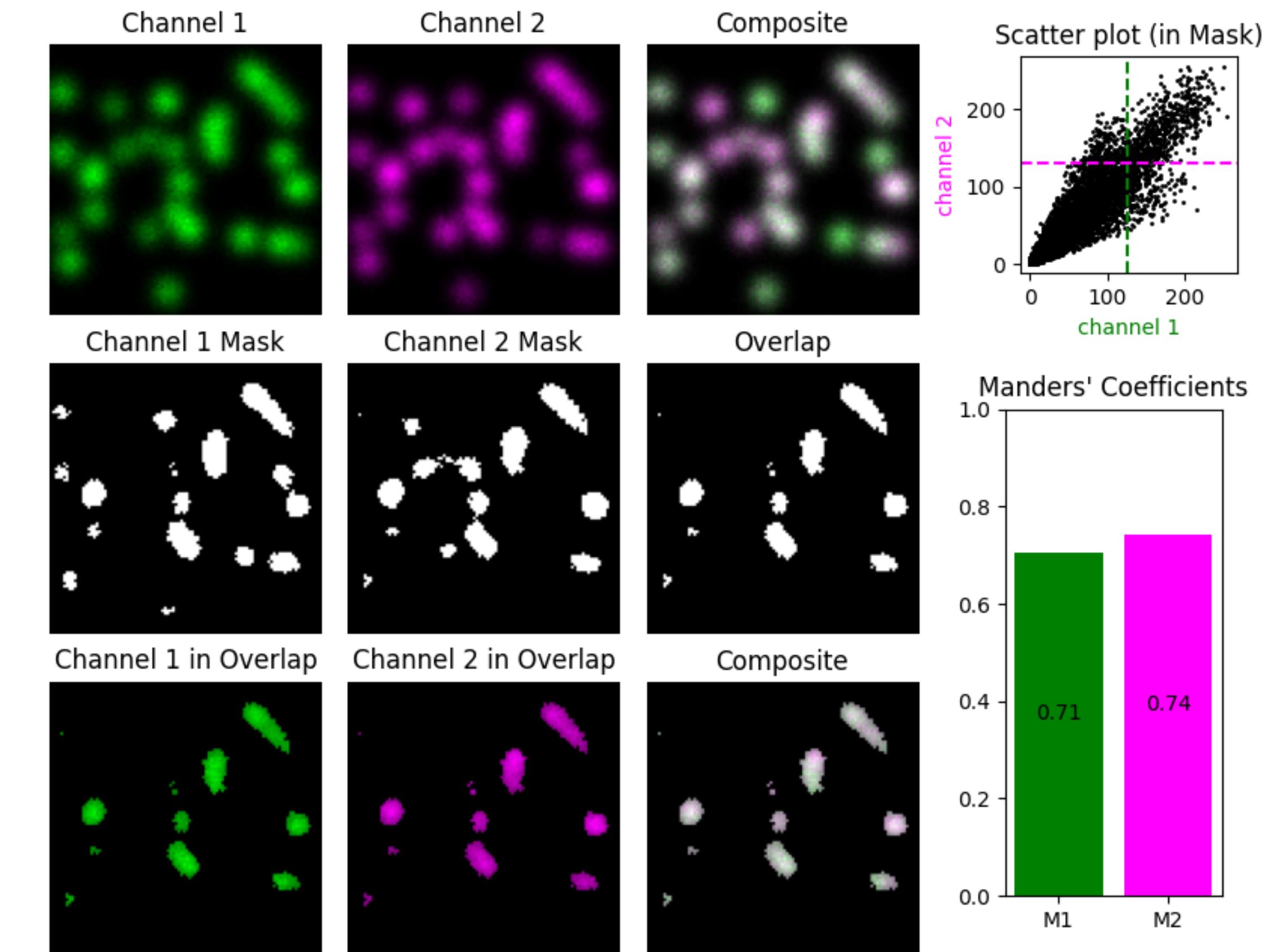
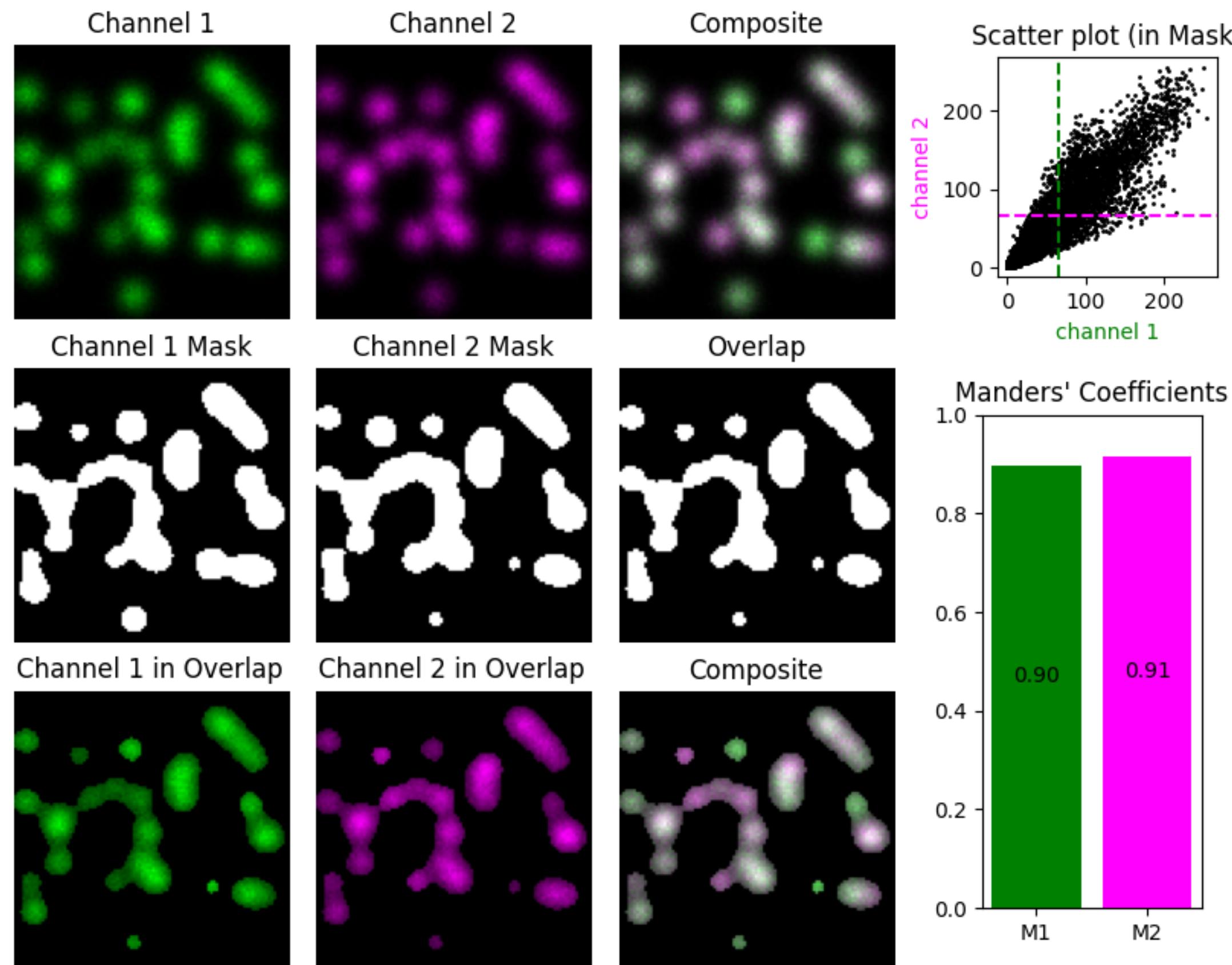
...





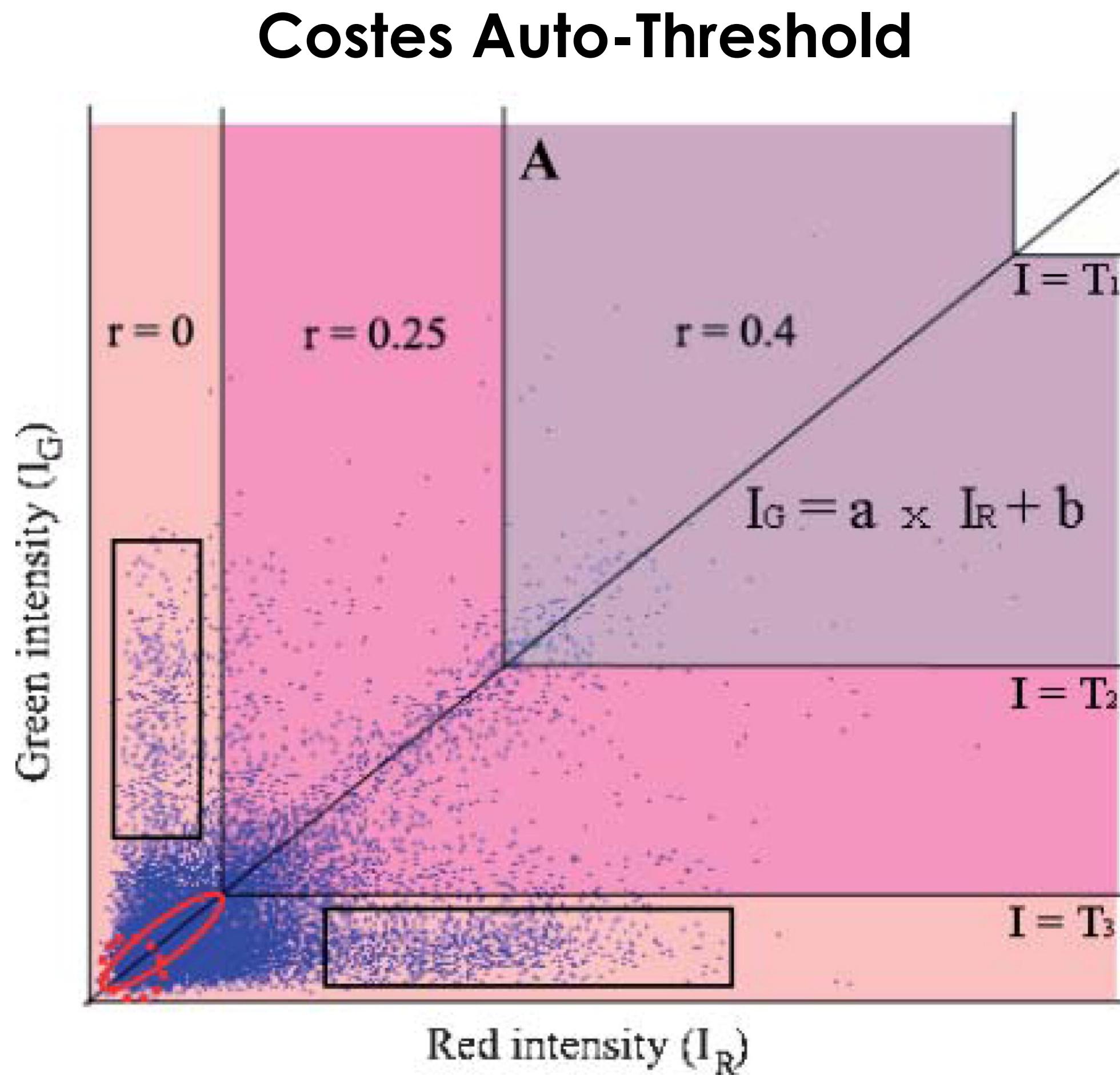
Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)

Highly depends on threshold





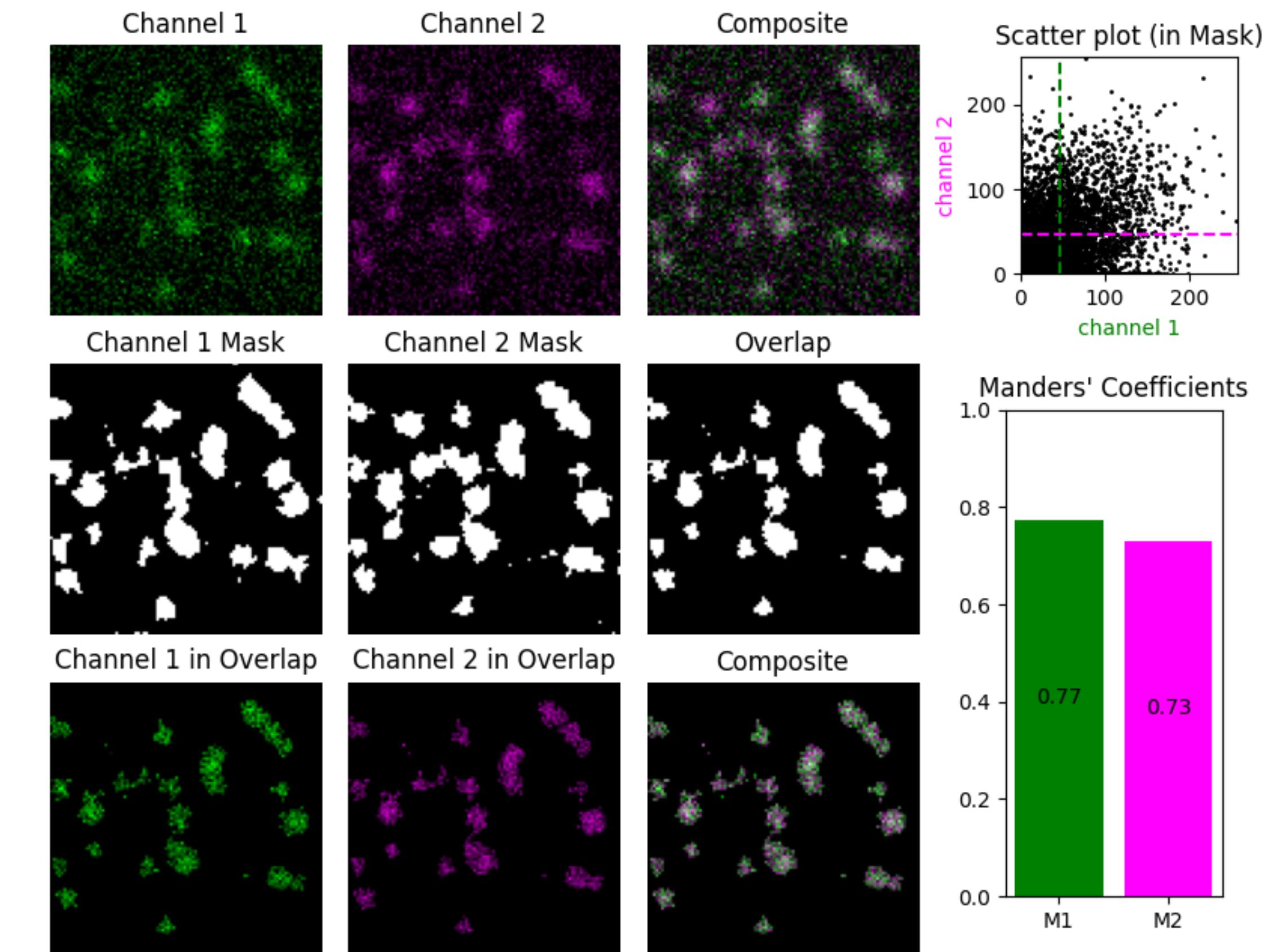
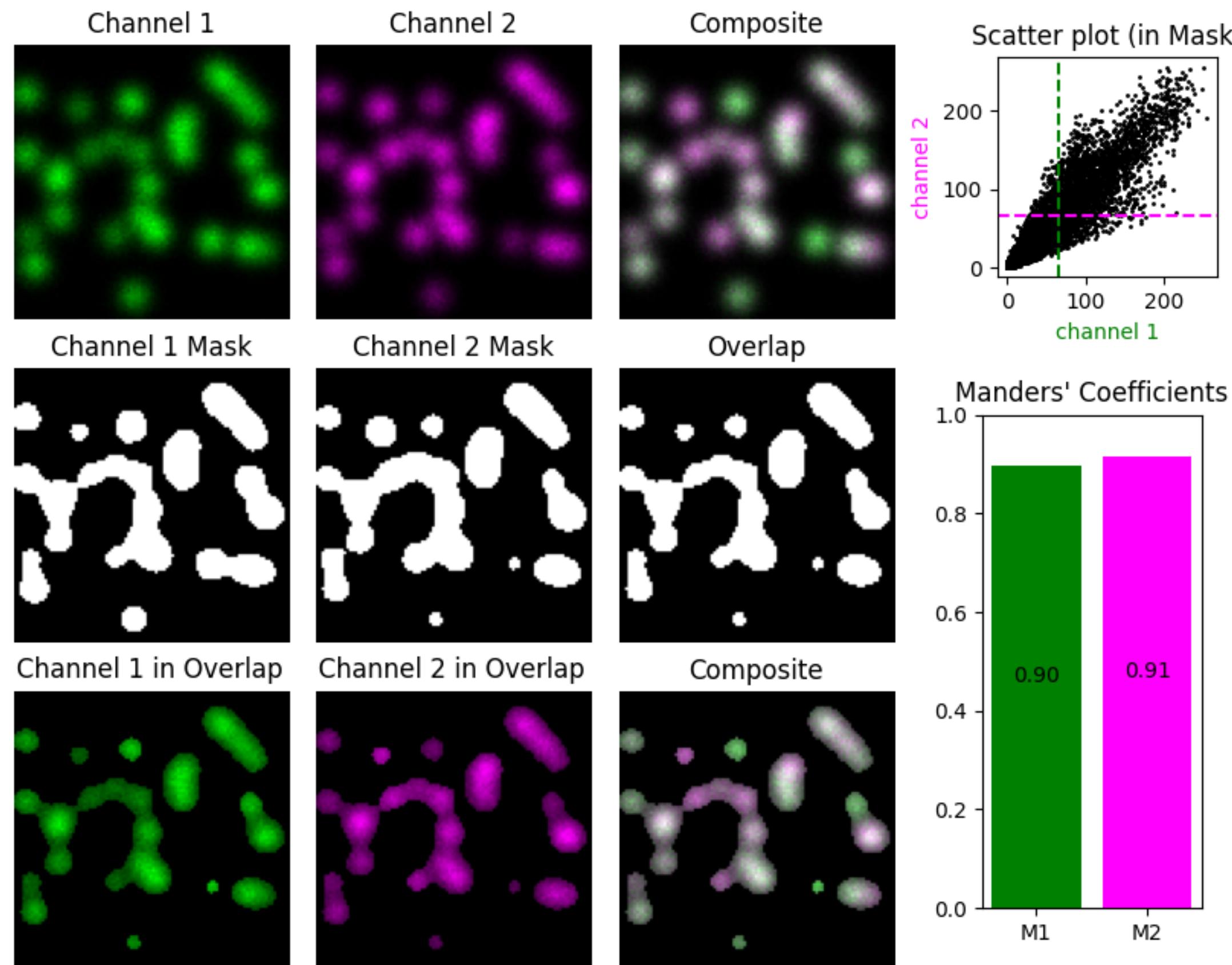
Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)





Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)

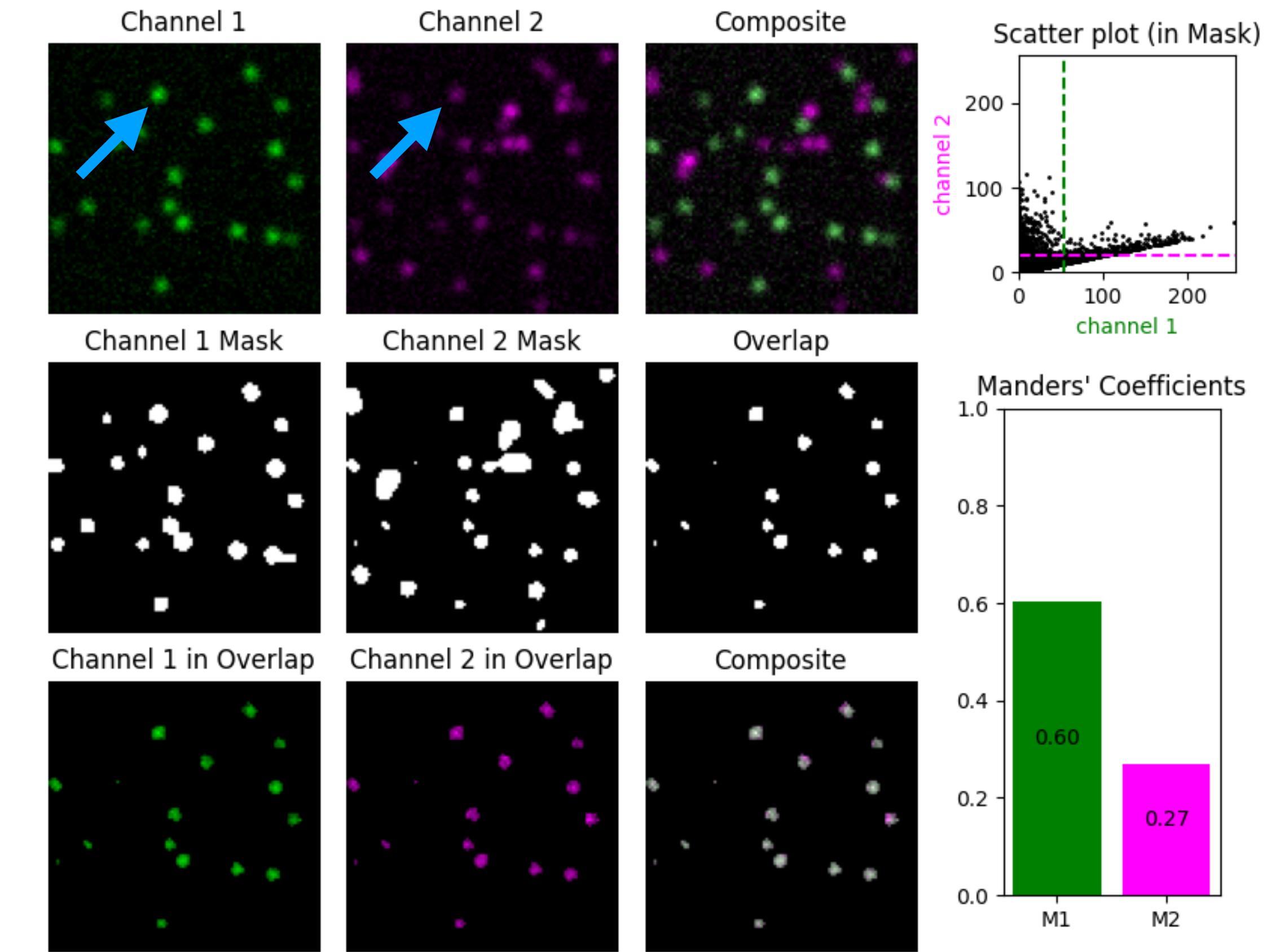
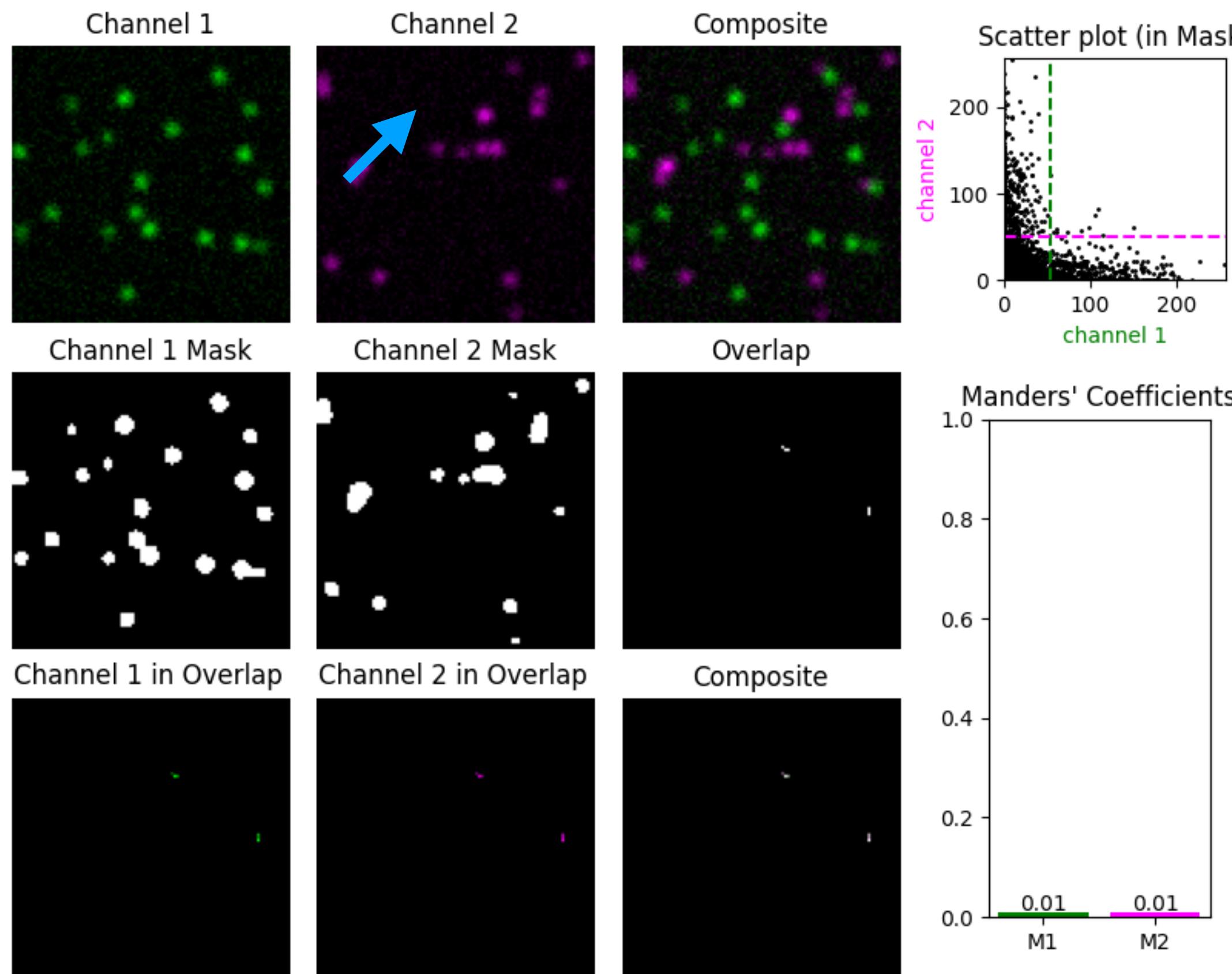
Noise





Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)

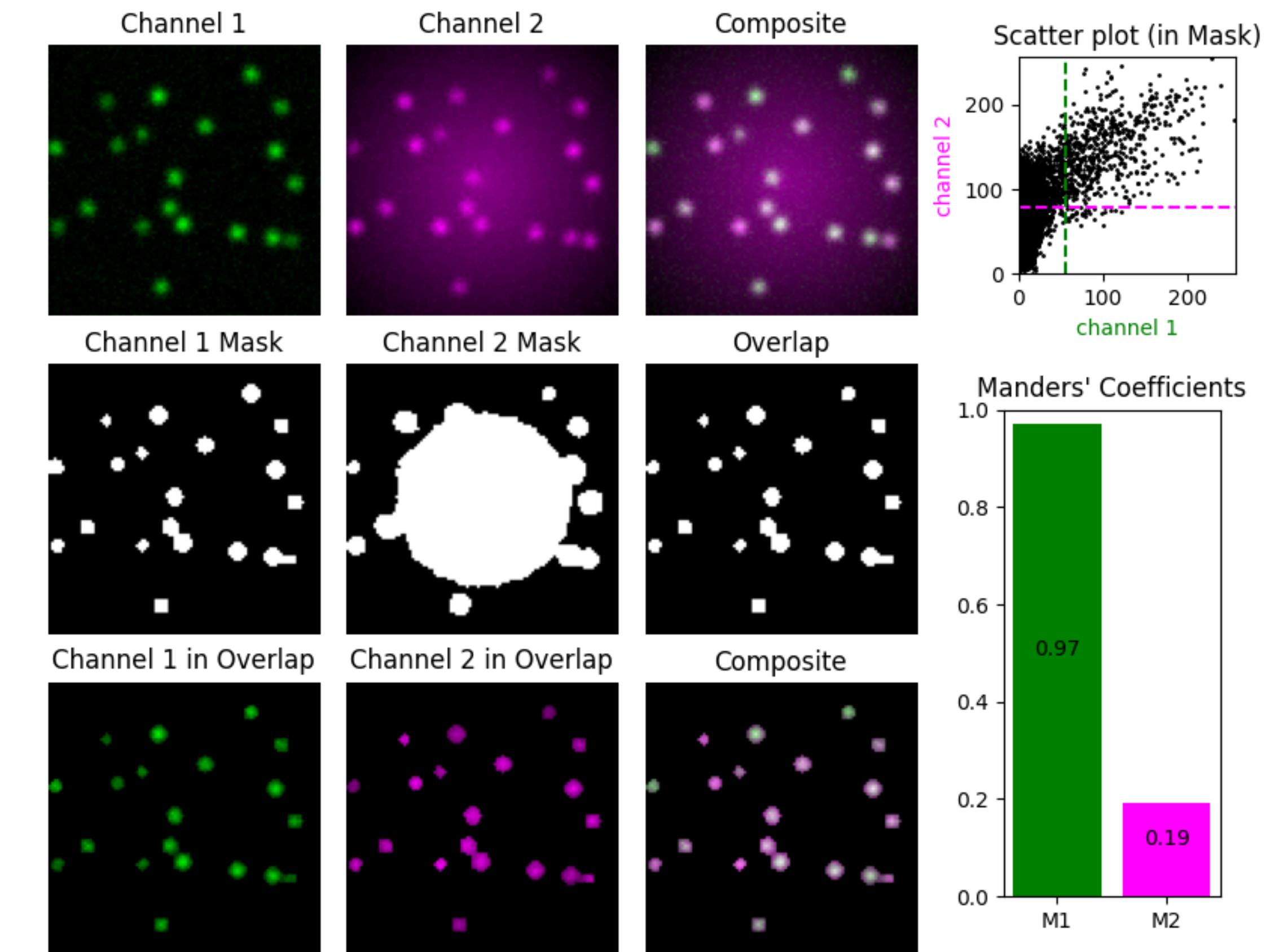
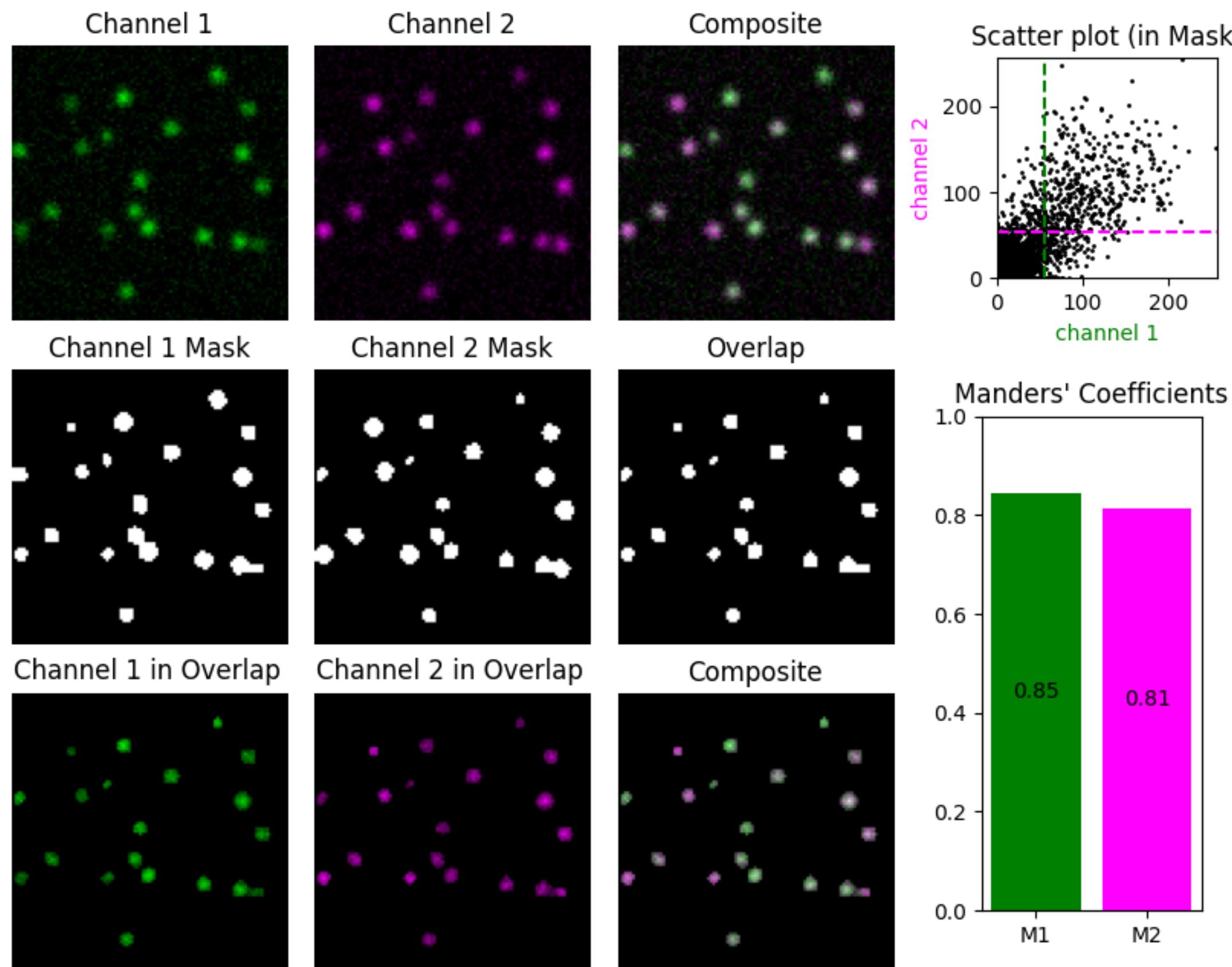
Bleedthrough





Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)

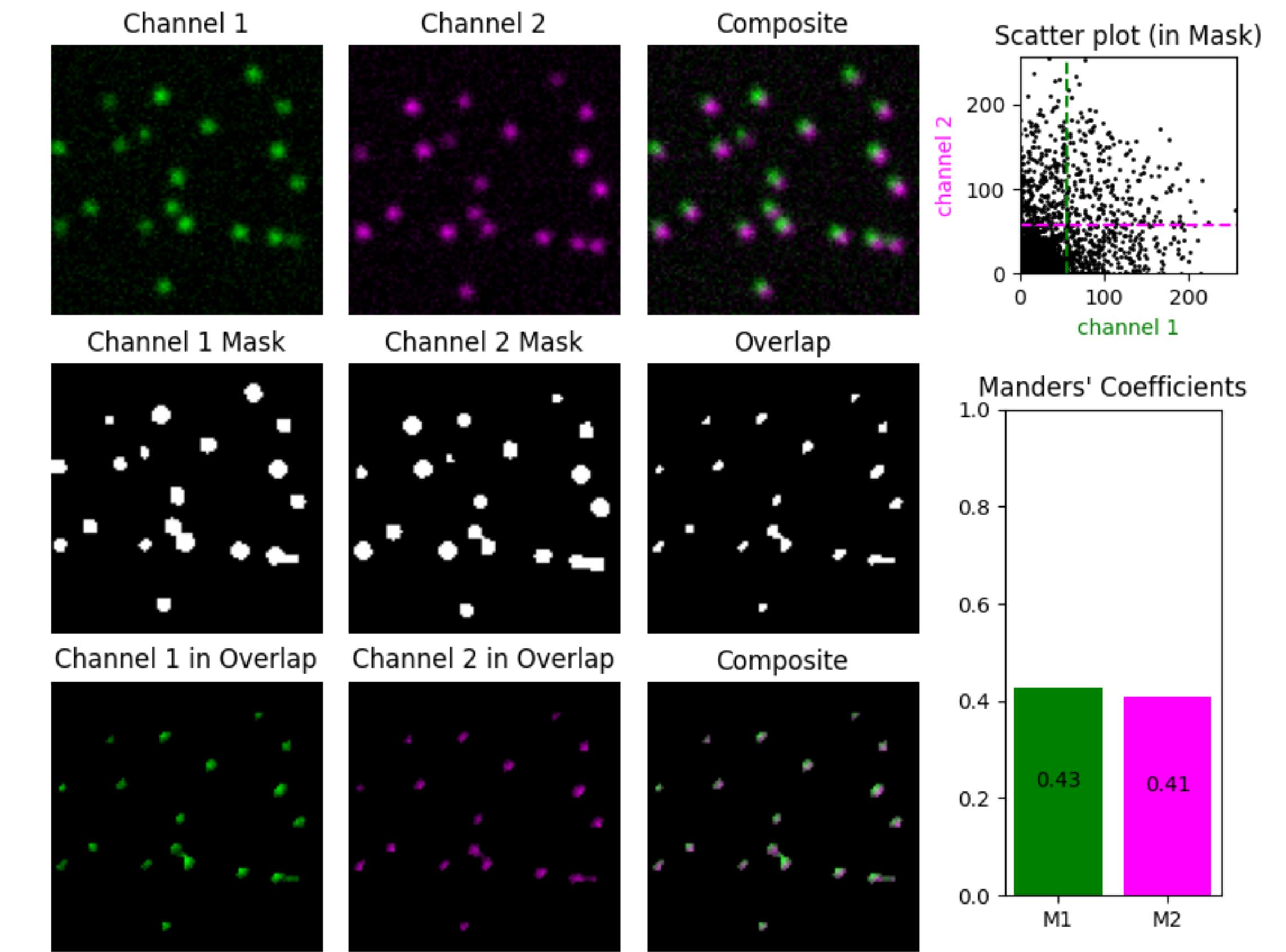
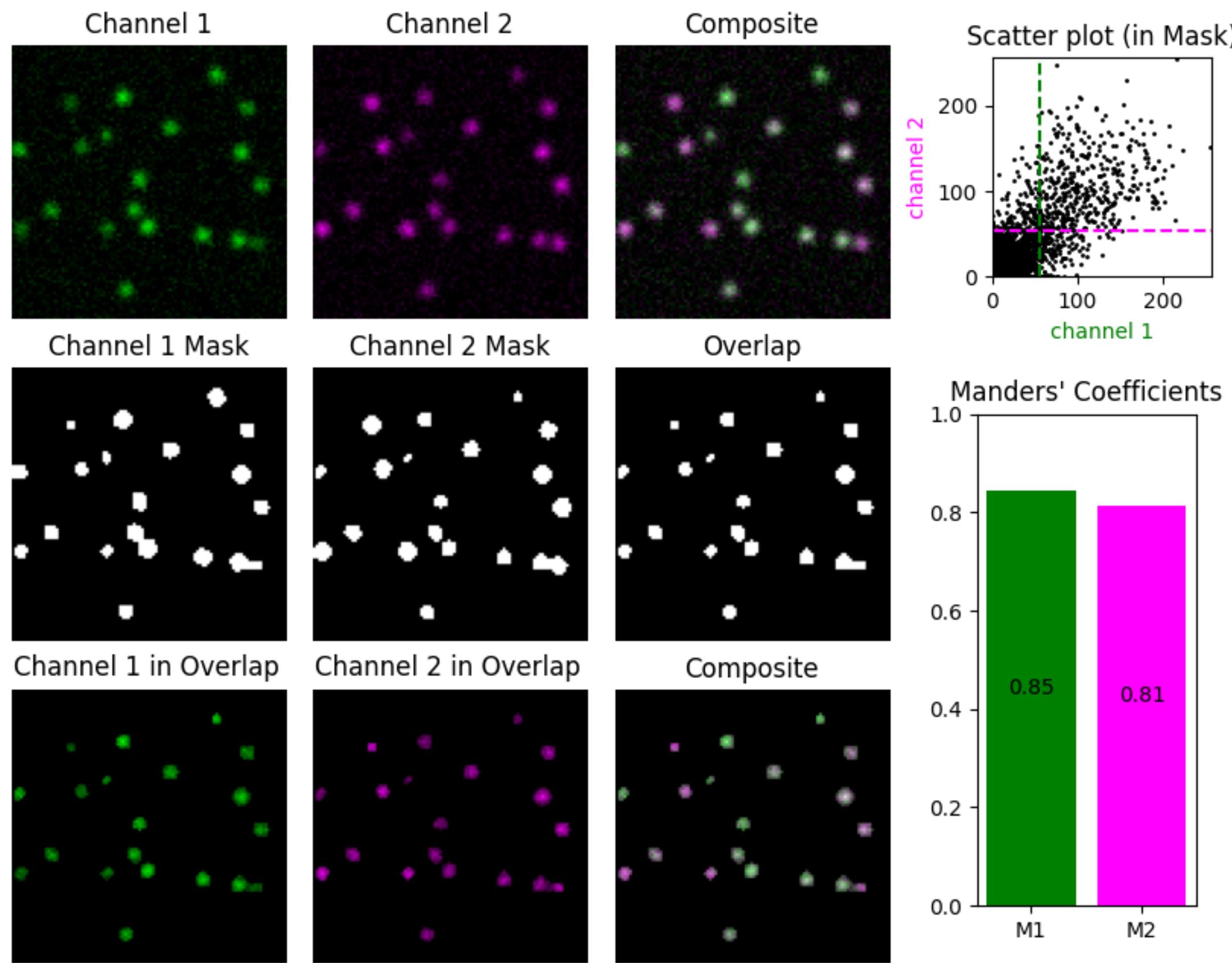
Uneven Illumination





Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)

Chromatic Shift





Summary

Colocalization in fluorescence microscopy cannot prove molecular interaction

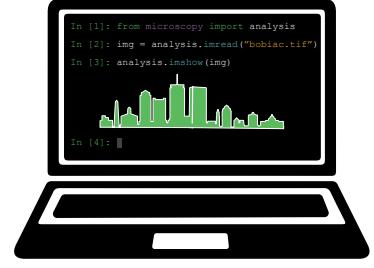
As with any other fluorescence microscopy experiments, it is important to...

- use a suitable fluorescence microscopy technique to study colocalization (resolution, optical sectioning, ...)
- perform controls (e.g bleedthrough, chromatic shift, ...)
- have an idea on how to approach the image analysis before acquiring the data

Image pre-processing is likely needed before analyzing your data (noise, uneven illumination, background...)

The colocalization analysis method depends on the data and on the question we are trying to answer. Interpreting the results can be hard. Perform statistical analysis.

Report how you did the analysis ("Analysis was performed with ImageJ." is not a good way to report what you did)



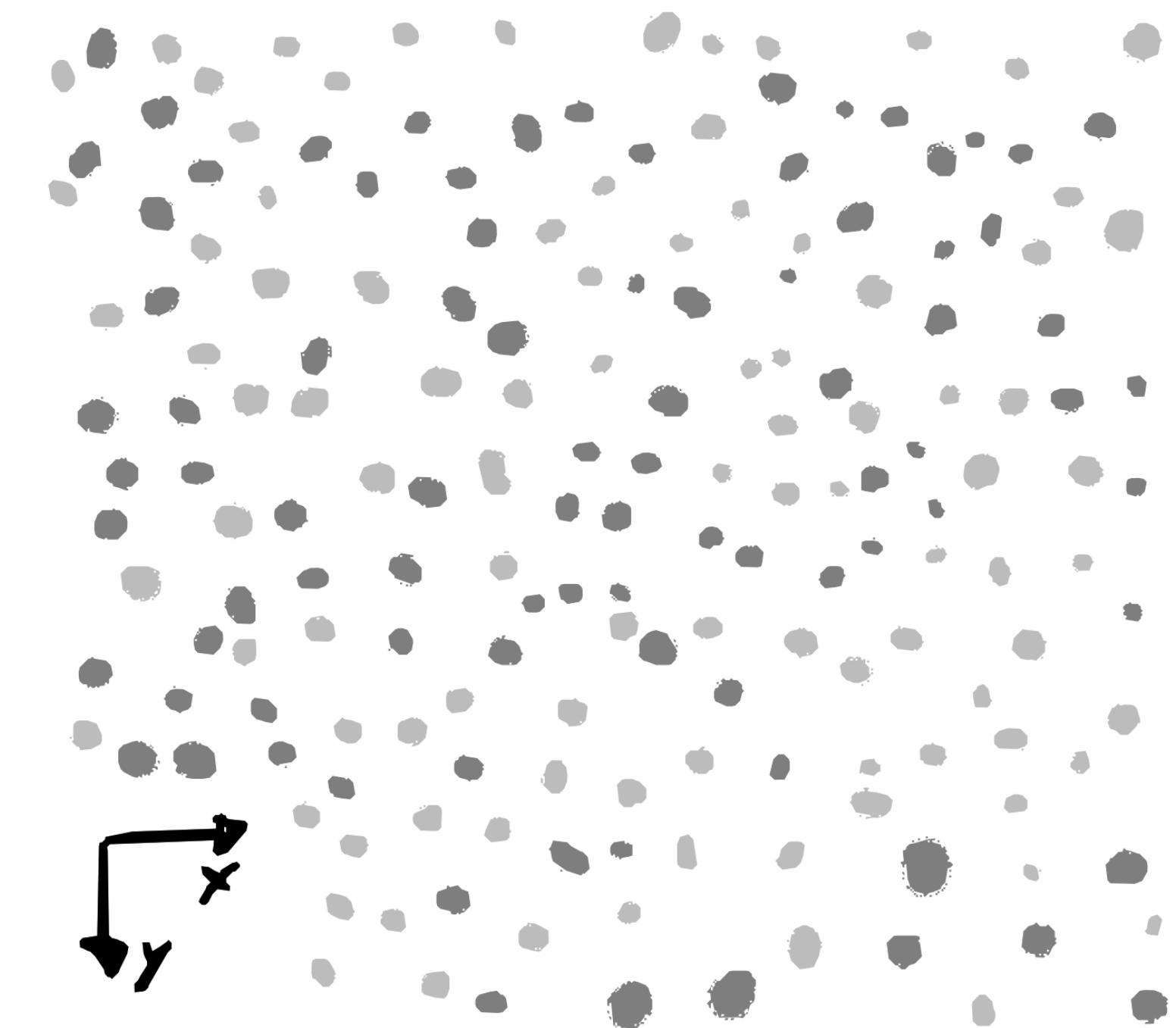
Spatial statistics: Object-based colocalization



What are (2D) objects?

Points

- Represented as coordinates
- Obtained by extracting coordinates*
- Example: Membrane proteins



Extracting coordinates — examples:

Spotiflow: Dominguez Mantes, A., Herrera, A., Khven, I. *et al.* Spotiflow: accurate and efficient spot detection for fluorescence microscopy with deep stereographic flow regression. *Nat Methods* **22**, 1495–1504 (2025). <https://doi.org/10.1038/s41592-025-02662-x>

SMLM fitting in Python: E.g. <https://github.com/ZhuangLab/storm-analysis>

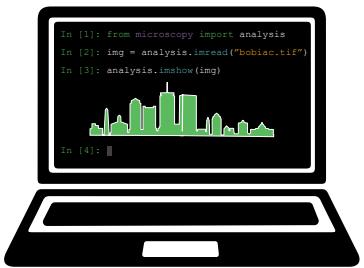


What are (2D) objects?

Lines

- Geometrically: length, but no width
- Obtained by segmentation
- Example: A tissue boundary

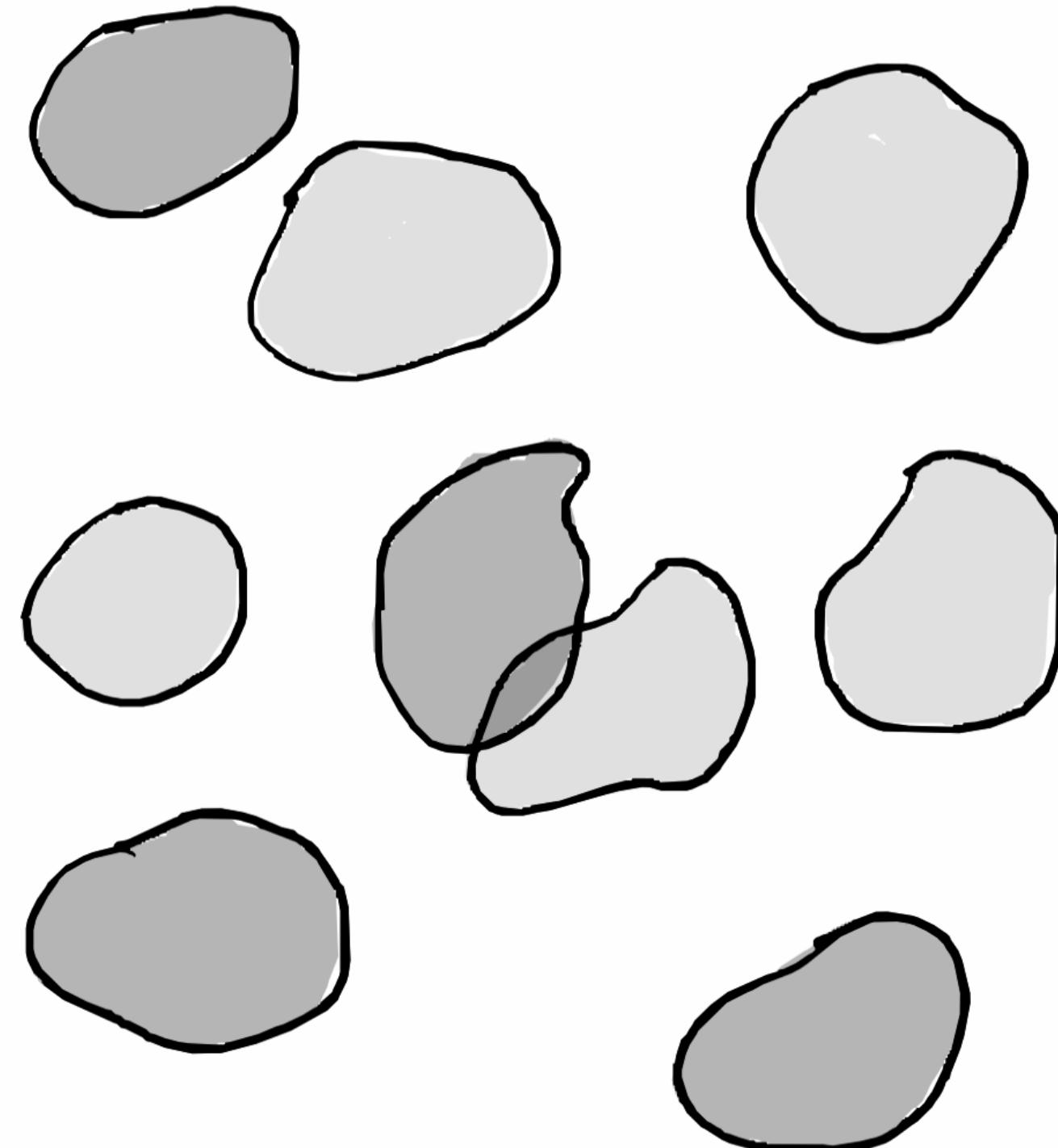




What are (2D) objects?

Bounded regions

- Have an area
- Obtained by segmentation
(Cellpose, StarDist, ...)
- Example: Nuclei, vesicles...



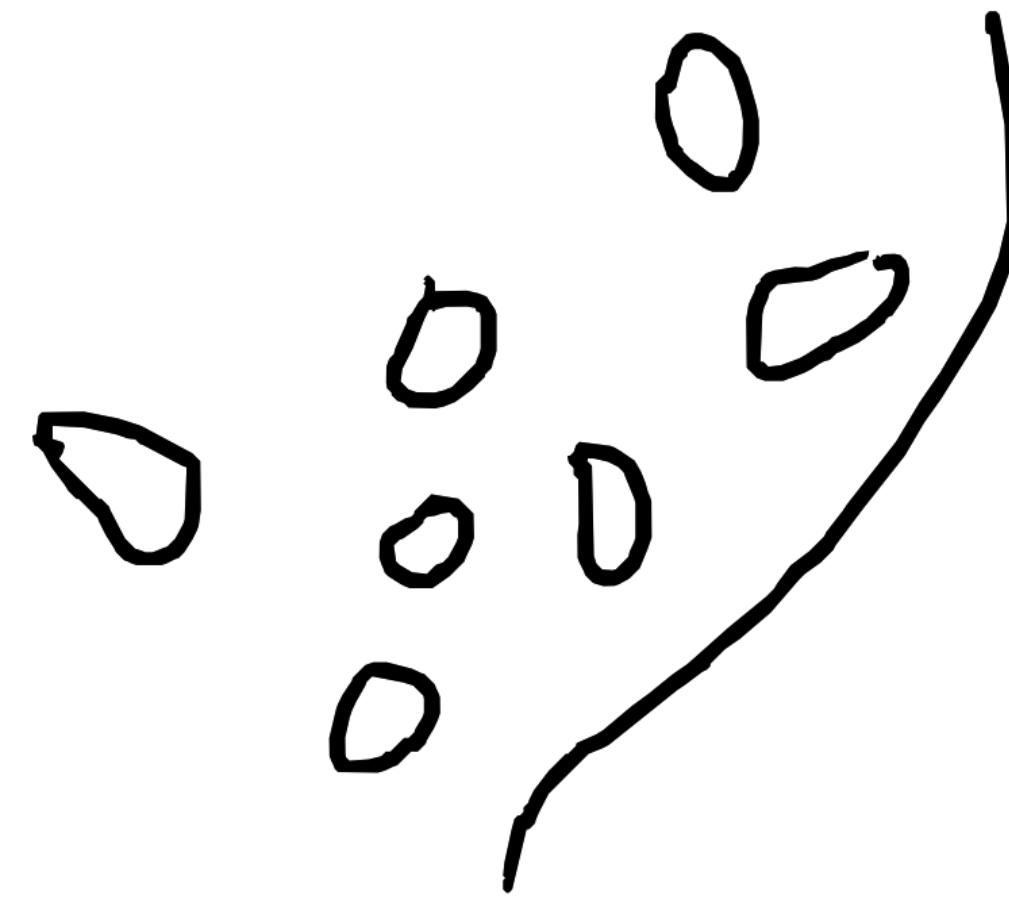
Cellpose-SAM: superhuman generalization for cellular segmentation
Marius Pachitariu, Michael Rariden, Carsen Stringer
bioRxiv 2025.04.28.651001; doi: <https://doi.org/10.1101/2025.04.28.651001>

M. Weigert and U. Schmidt, "Nuclei Instance Segmentation and Classification in Histopathology Images with Stardist," *2022 IEEE International Symposium on Biomedical Imaging Challenges (ISBIC)*, Kolkata, India, 2022, pp. 1-4, doi: 10.1109/ISBIC56247.2022.9854534.
keywords: {Deep learning;Image segmentation;Histopathology;Microscopy;Fluorescence;Colon;Task analysis;image segmentation;challenge;deep learning;histopathology},



What are (2D) objects?

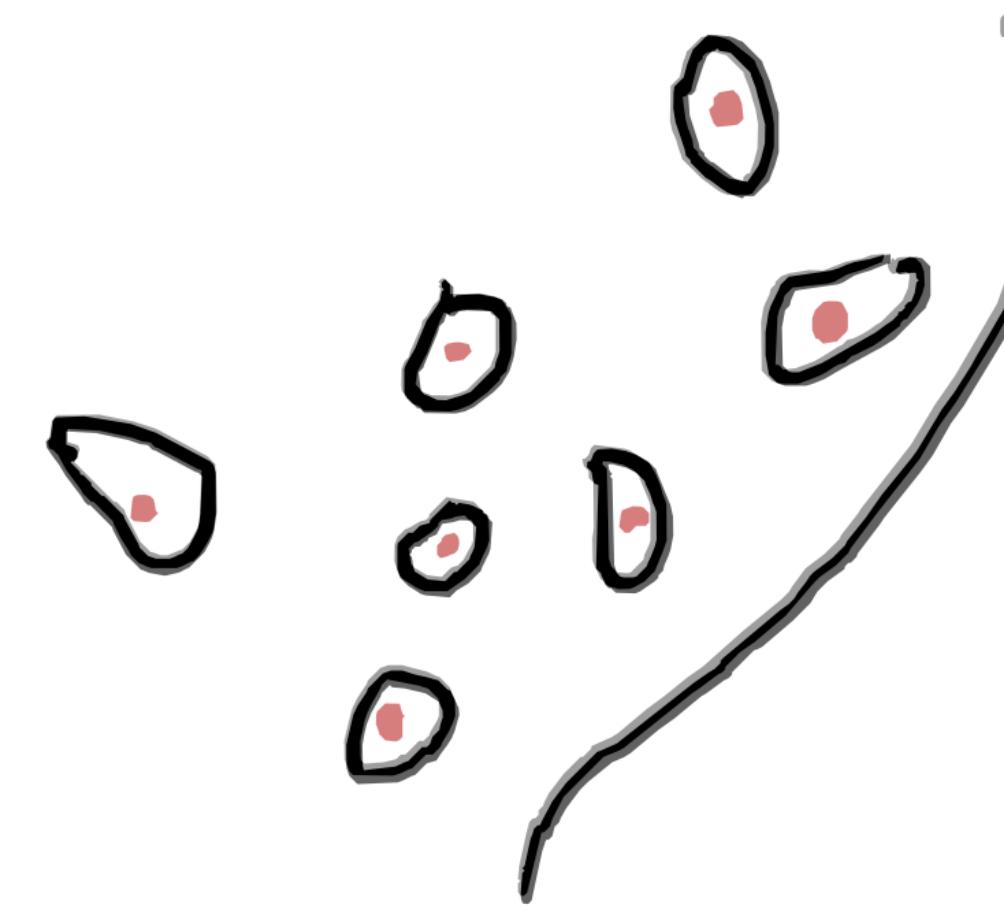
- Points
- Lines
- Bounded regions
- Objects can appear together





What are (2D) objects?

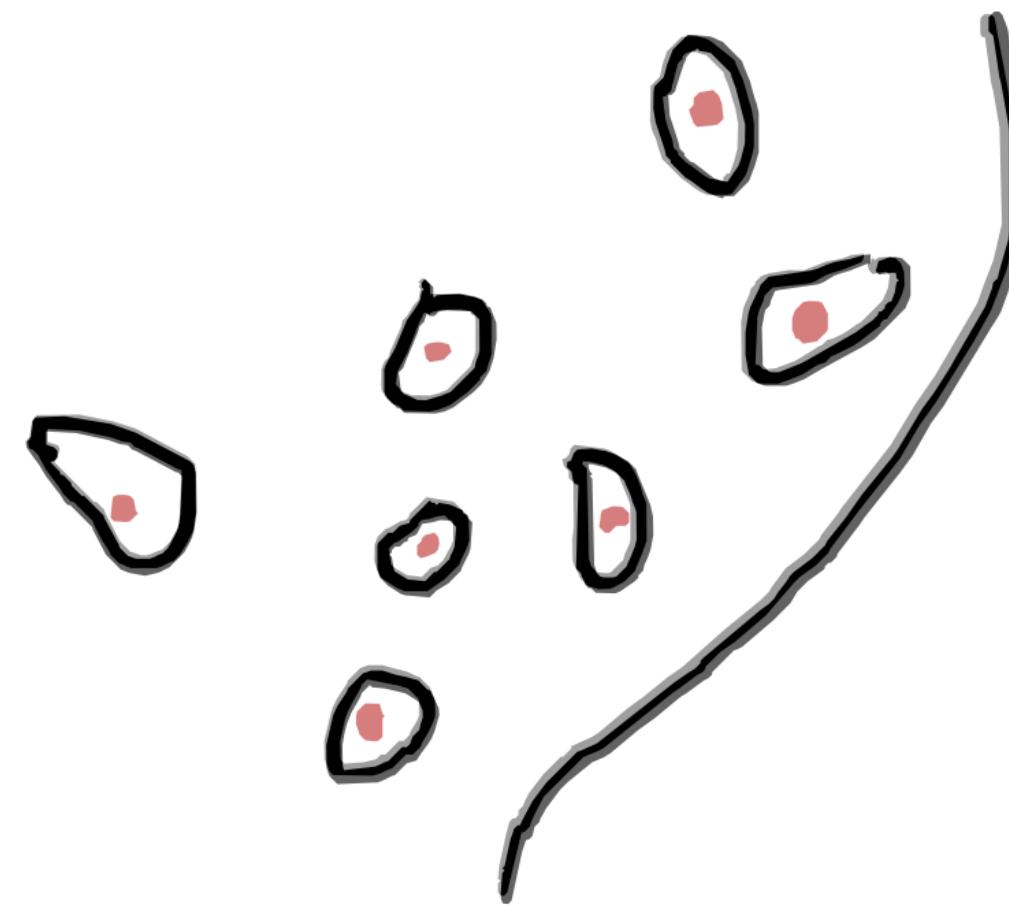
- Points
- Lines
- Bounded regions
- Objects can appear together
- Objects can often be derived from one another

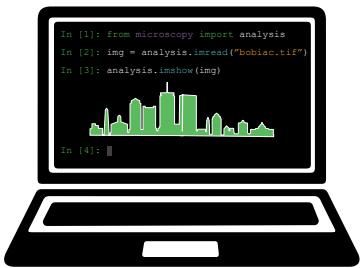




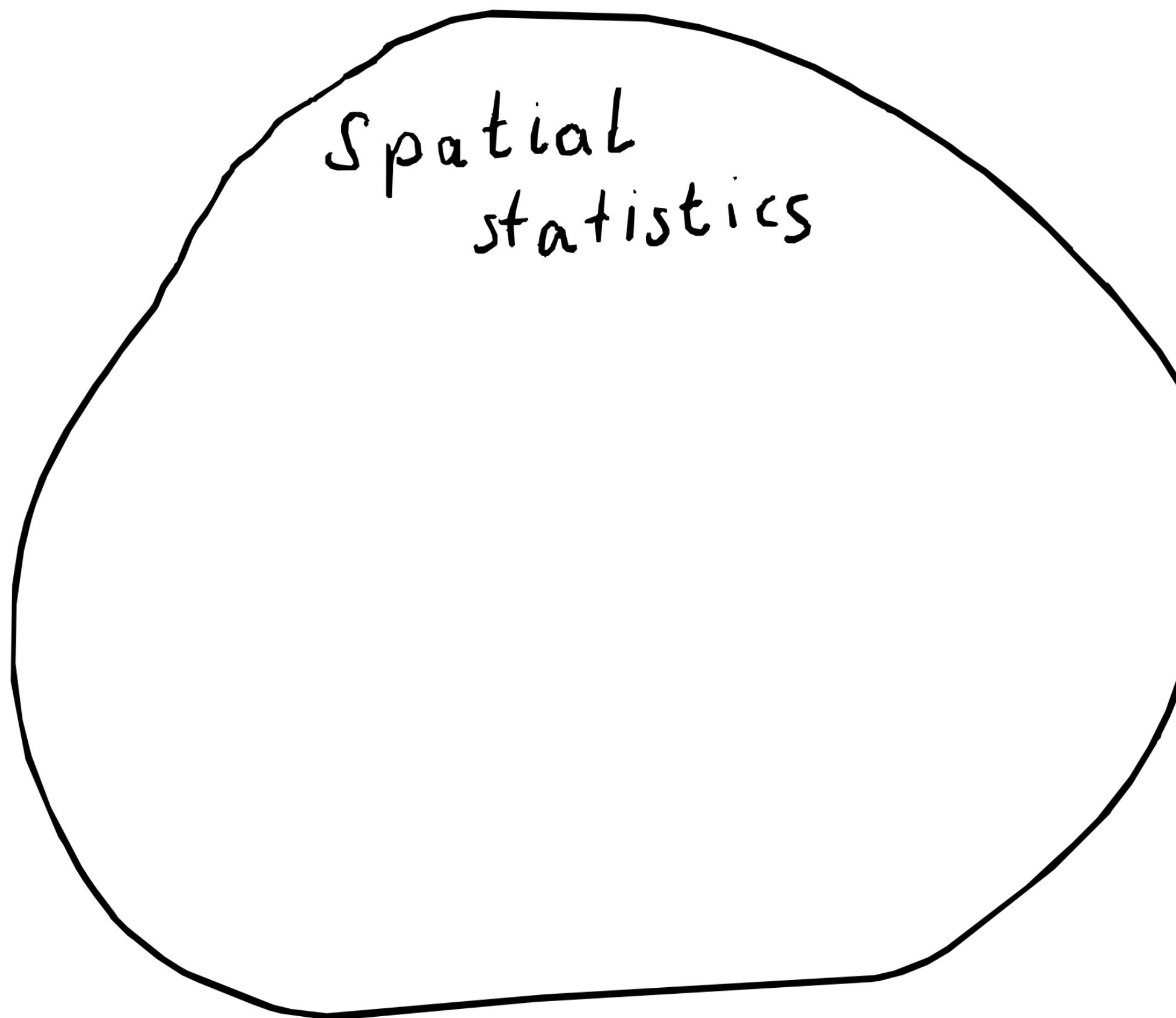
What are (2D) objects?

Or more simply: An object is anything you can count!





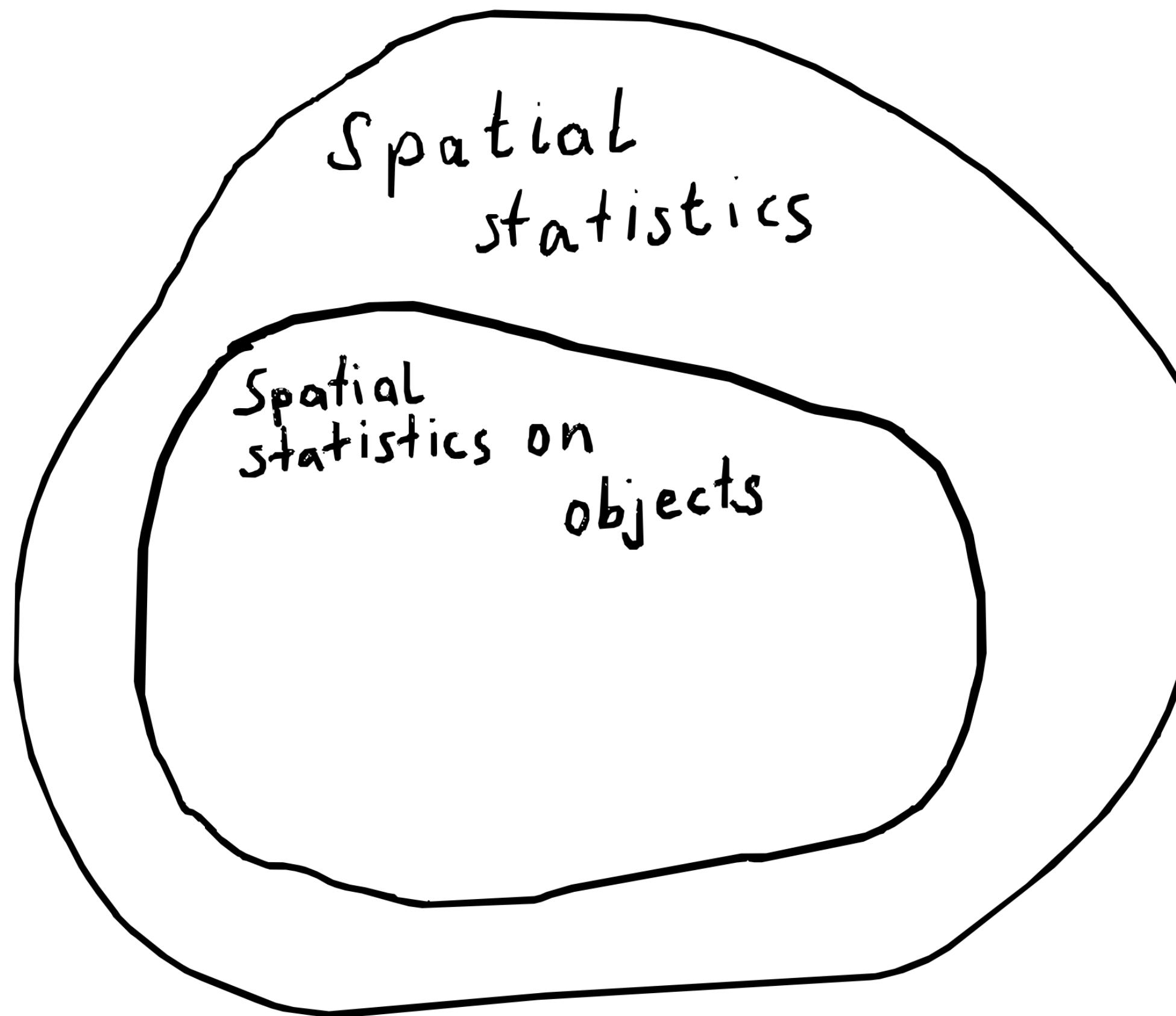
What is “object-based” colocalization



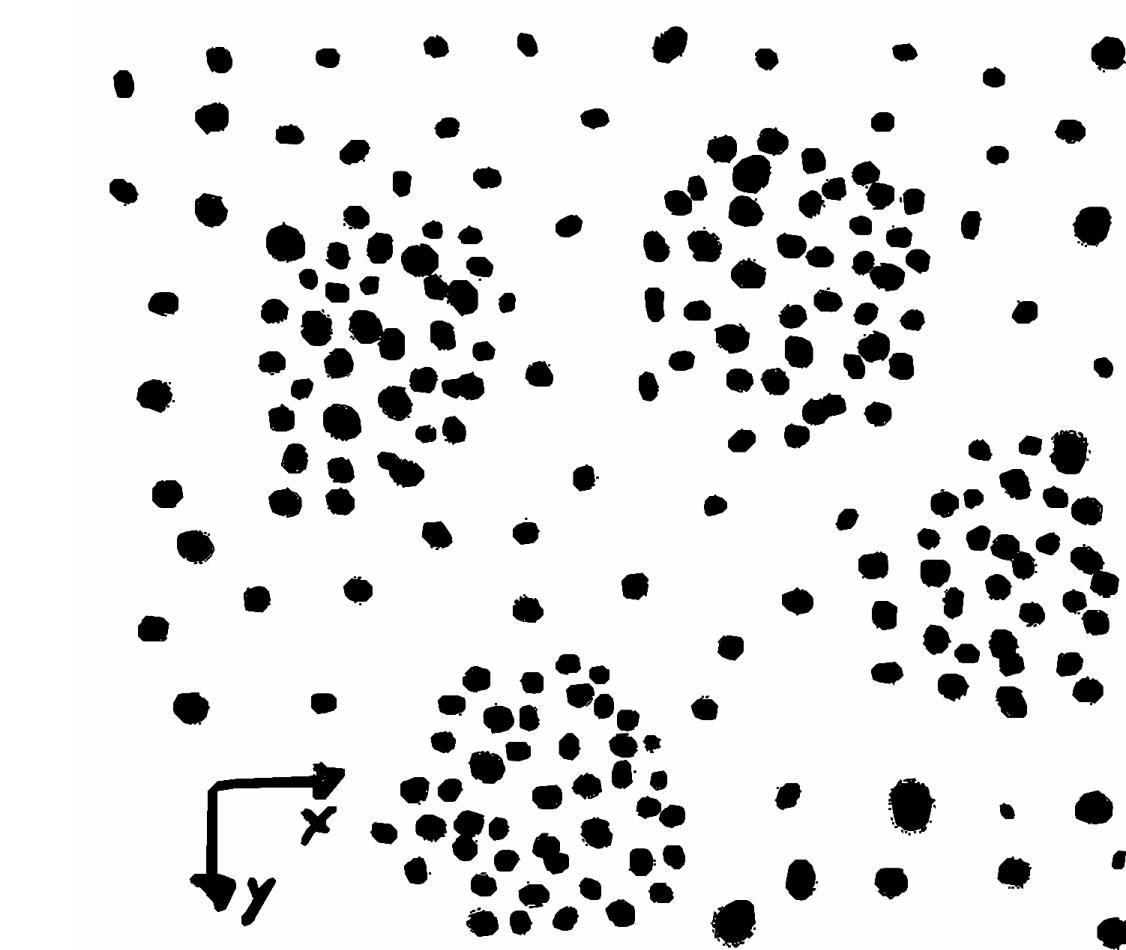
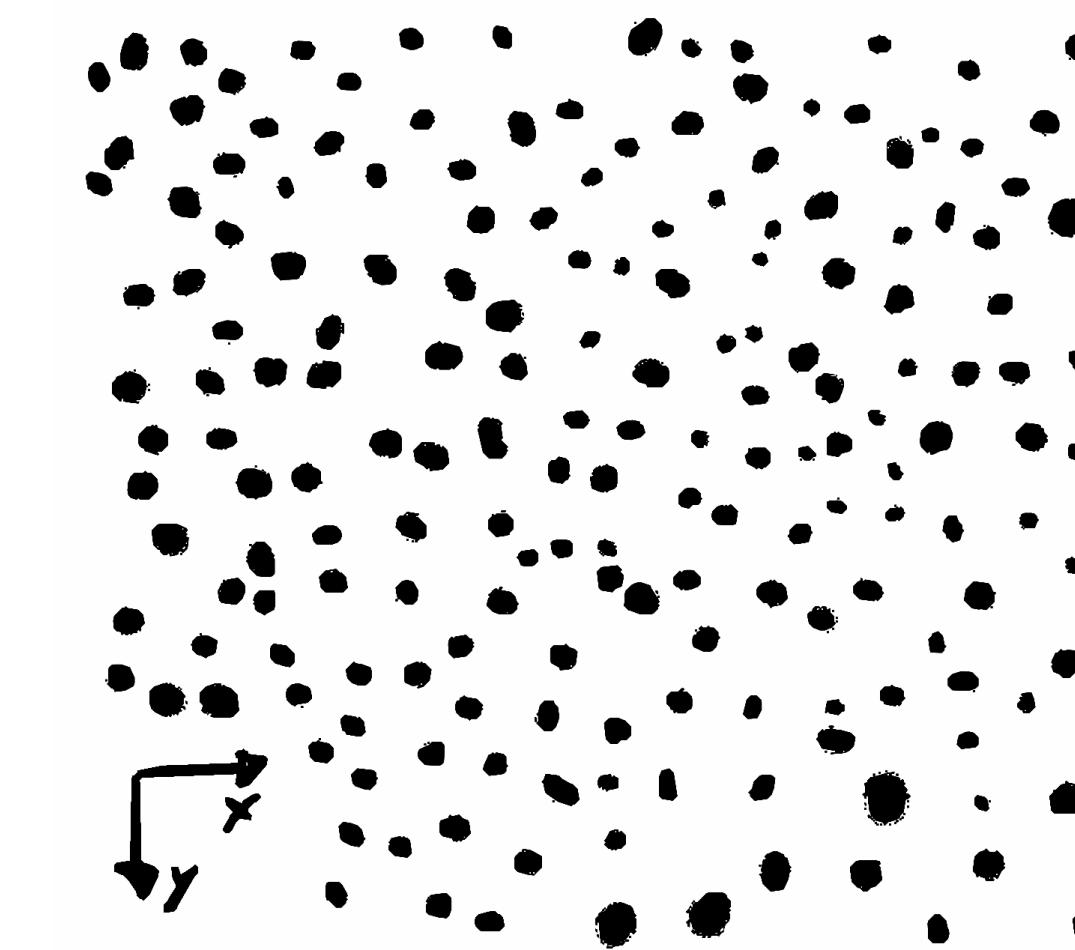
- Spatial statistics deals with spatial data



What is “object-based” colocalization

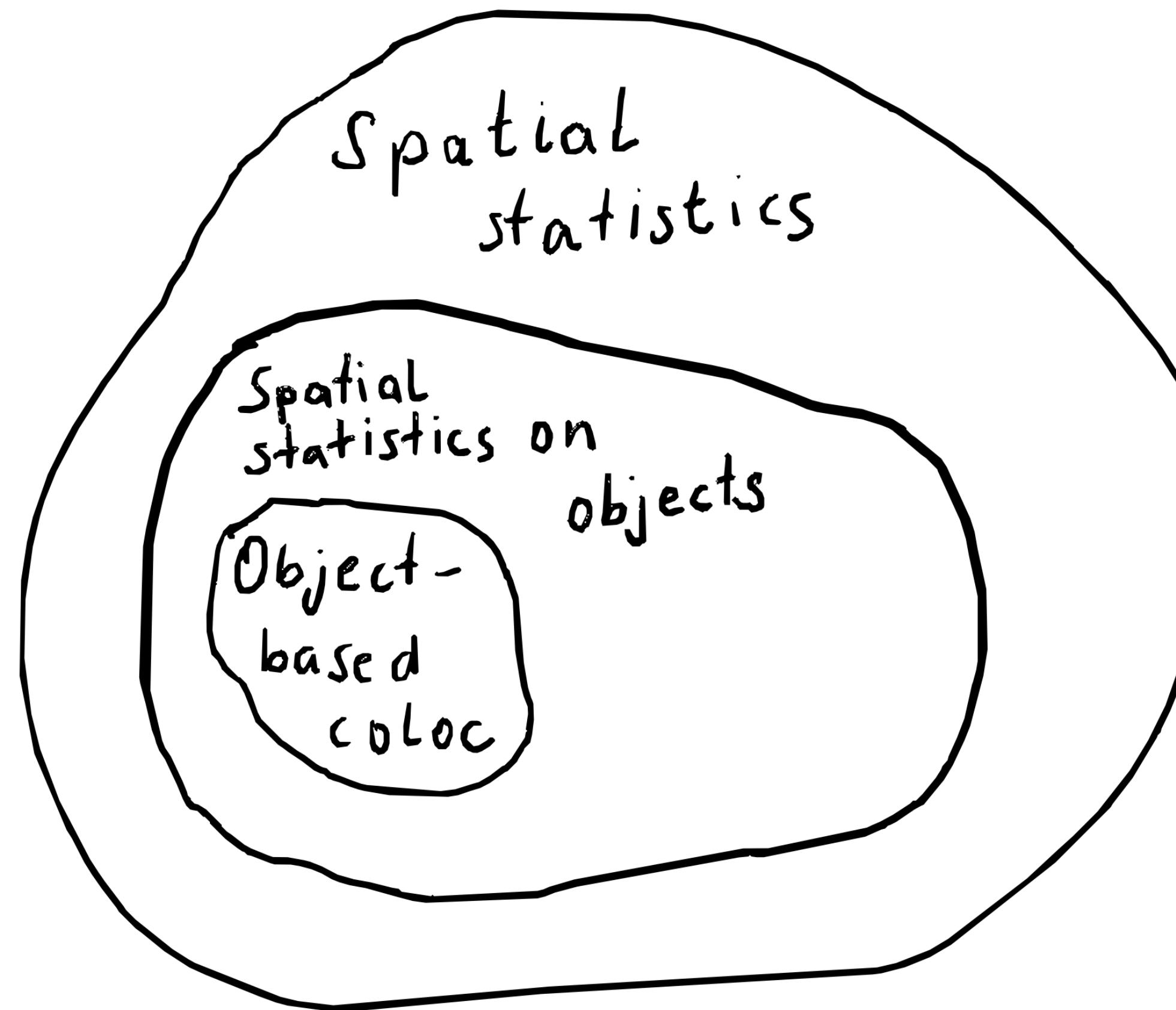


- Spatial statistics deals with spatial data
- These data can be objects

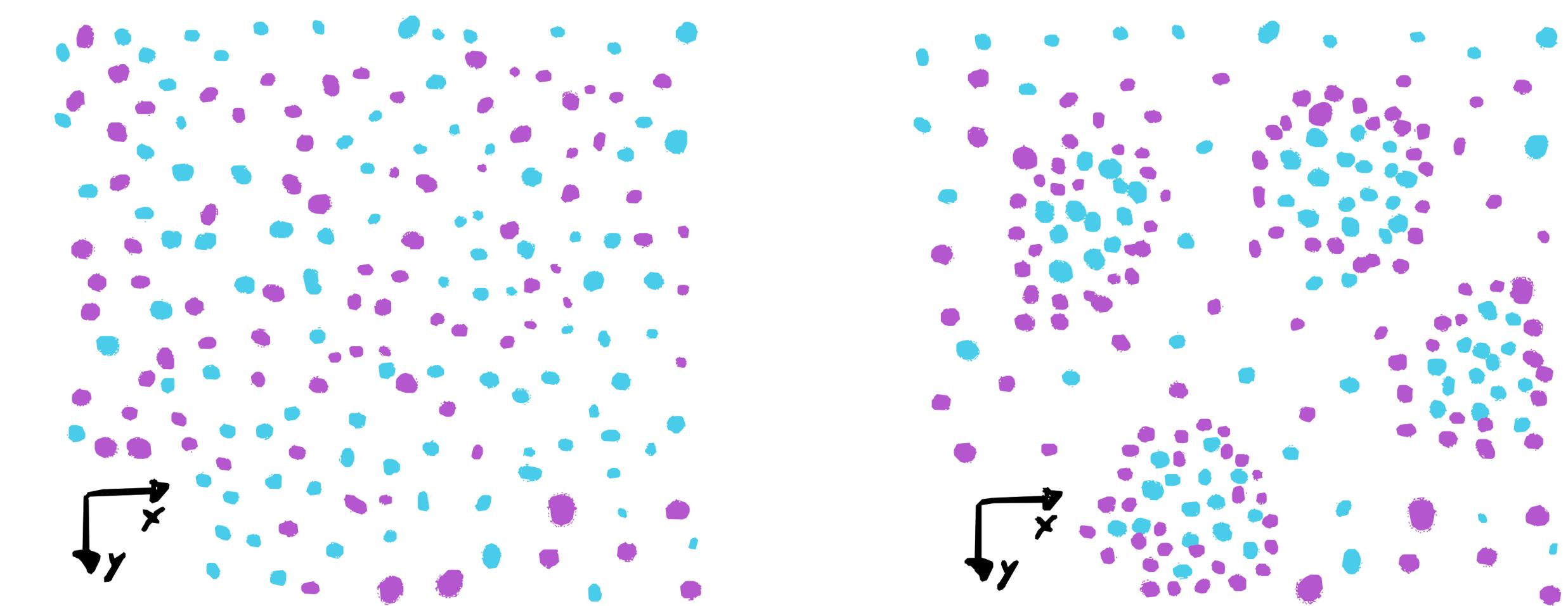




What is “object-based” colocalization

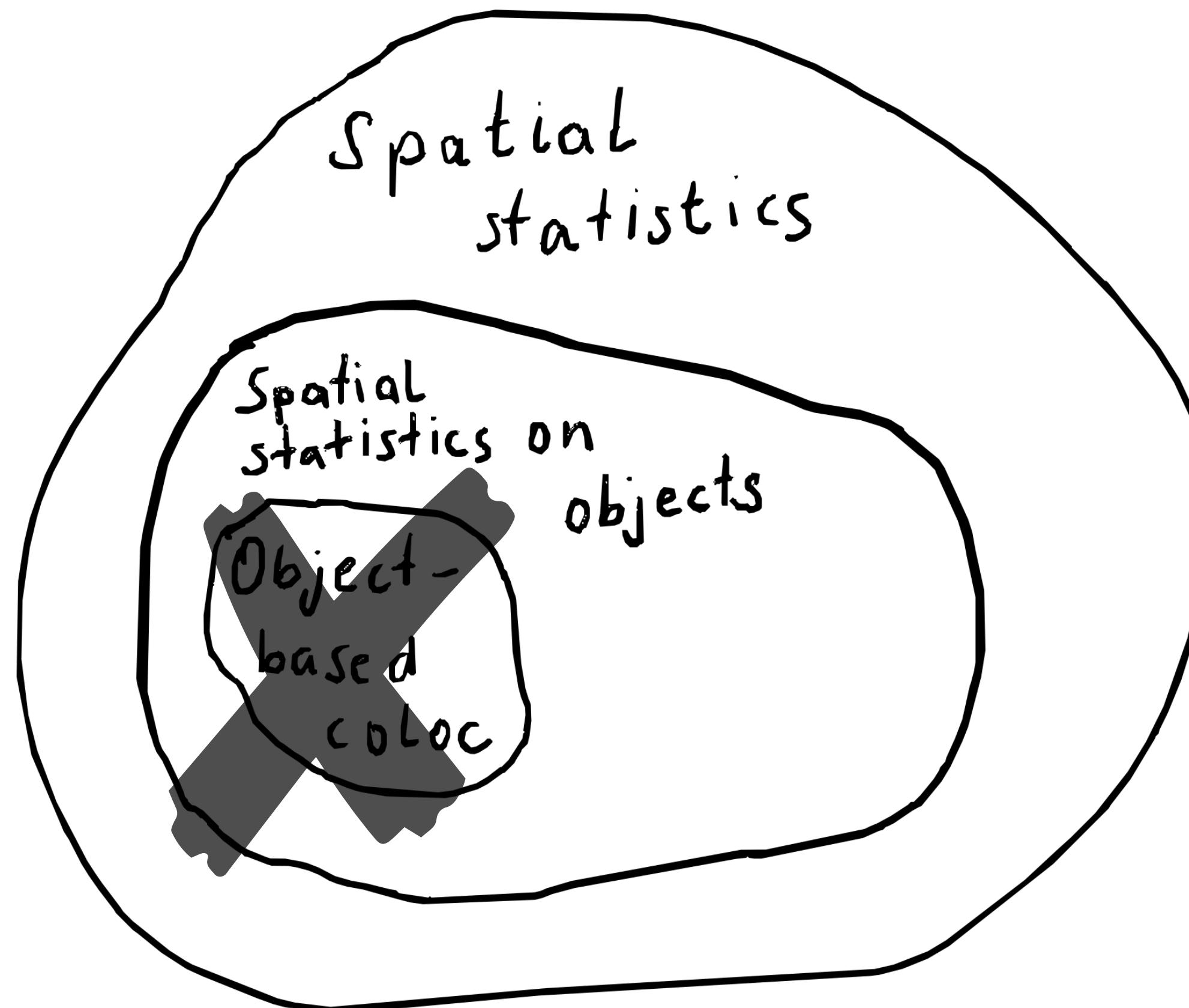


- Spatial statistics deals with spatial data
- These data can be objects
- Objects can be of more than one class

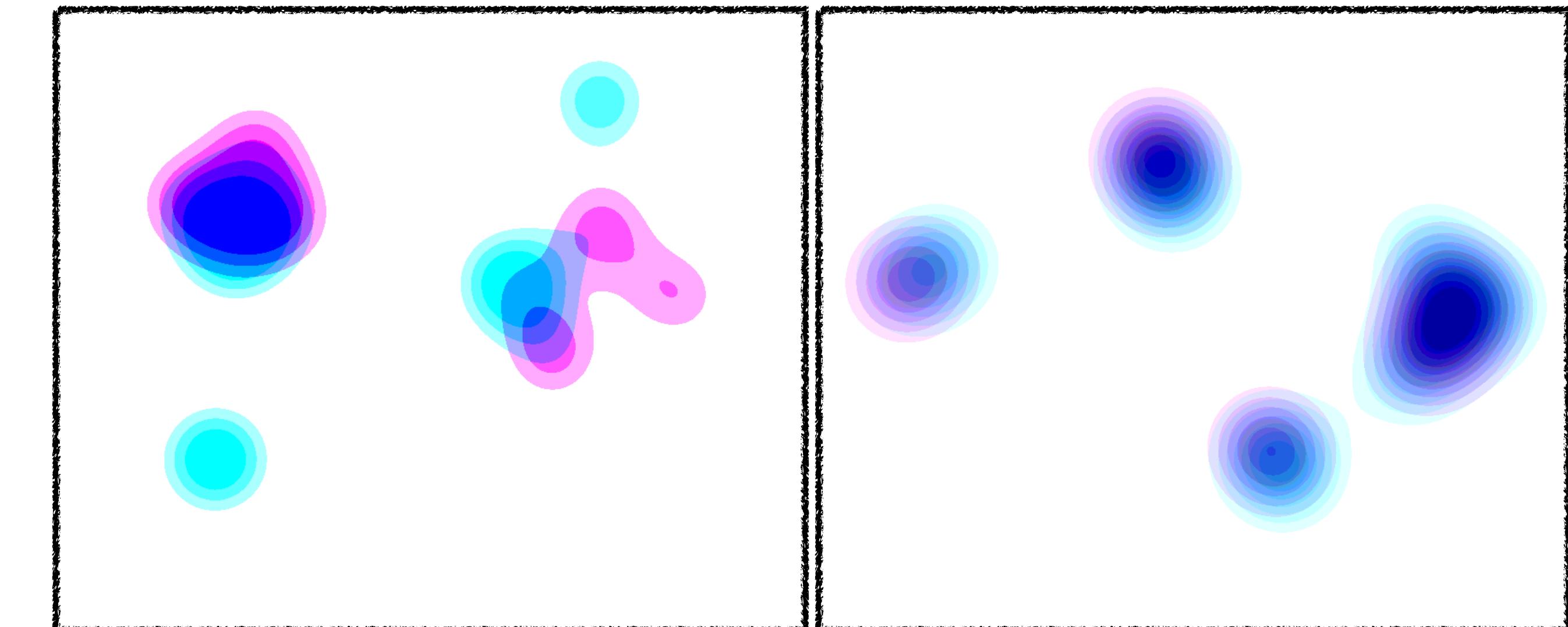




What is “object-based” colocalization



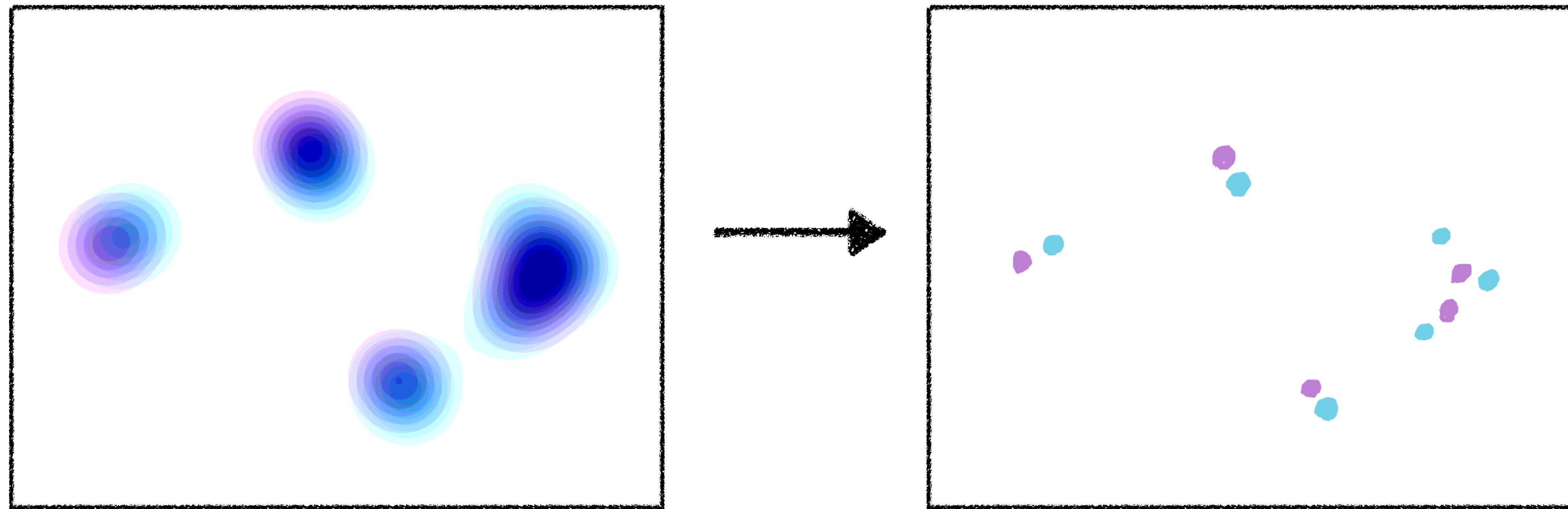
- Colocalization is an illusion of the diffraction limit of microscopy
- Colocalization does not exist!





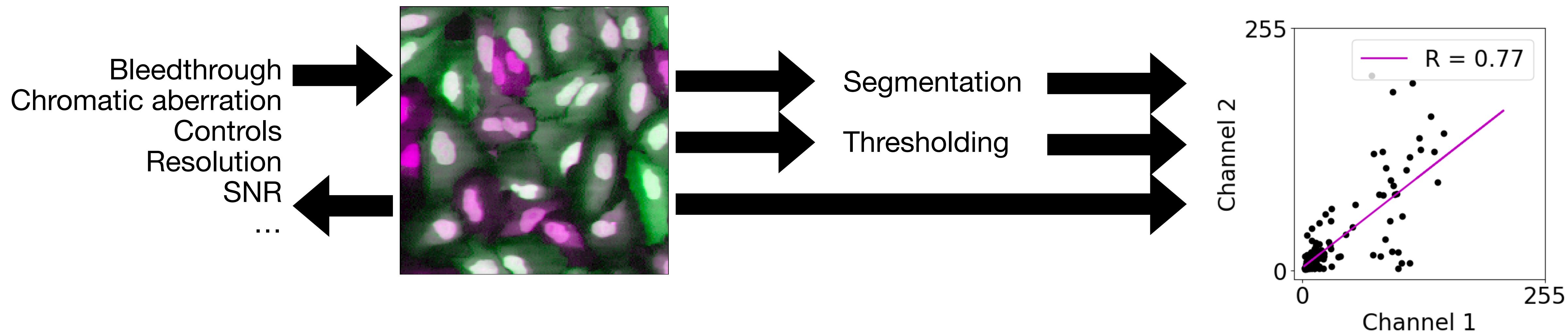
What is “object-based” colocalization

- Extracting objects often allows us to rephrase colocalization questions as spatial statistics questions





Recap: Intensity-based coloc

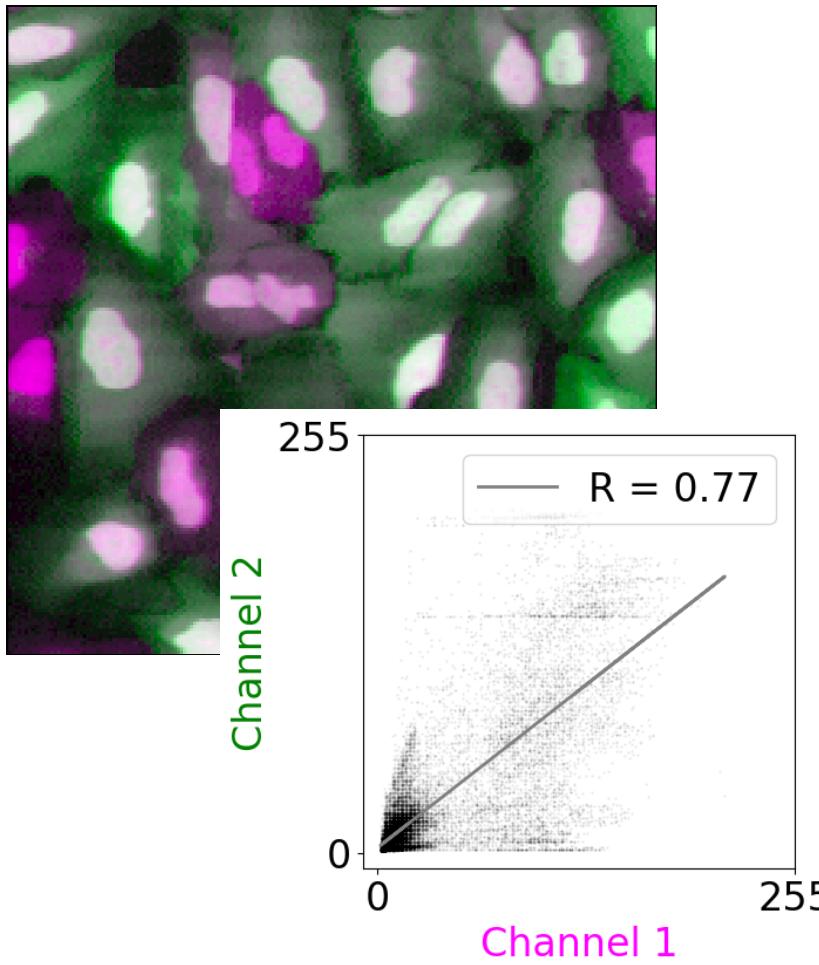


Data: Image set [BBBC014v1](#) provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [[dx.doi.org/10.1101/2014.09.03.100001](https://doi.org/10.1101/2014.09.03.100001)].

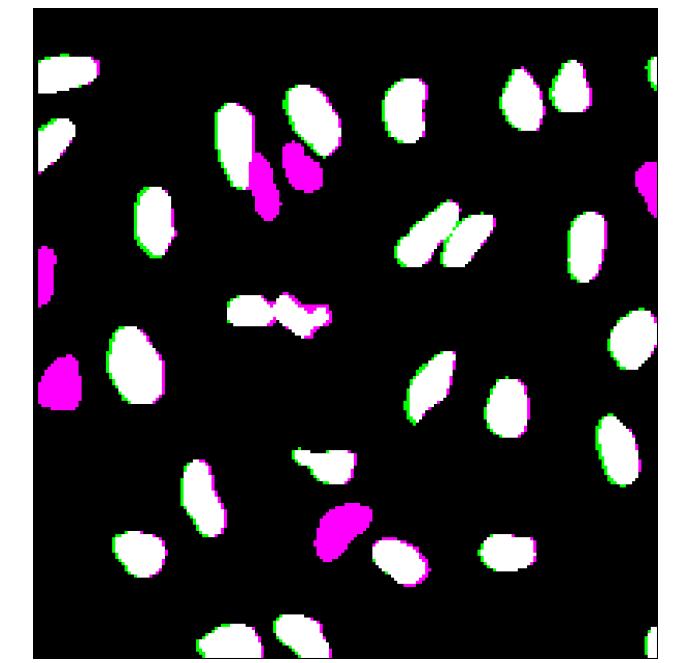


Intensity-based vs. Object-based coloc

Purely intensity-based

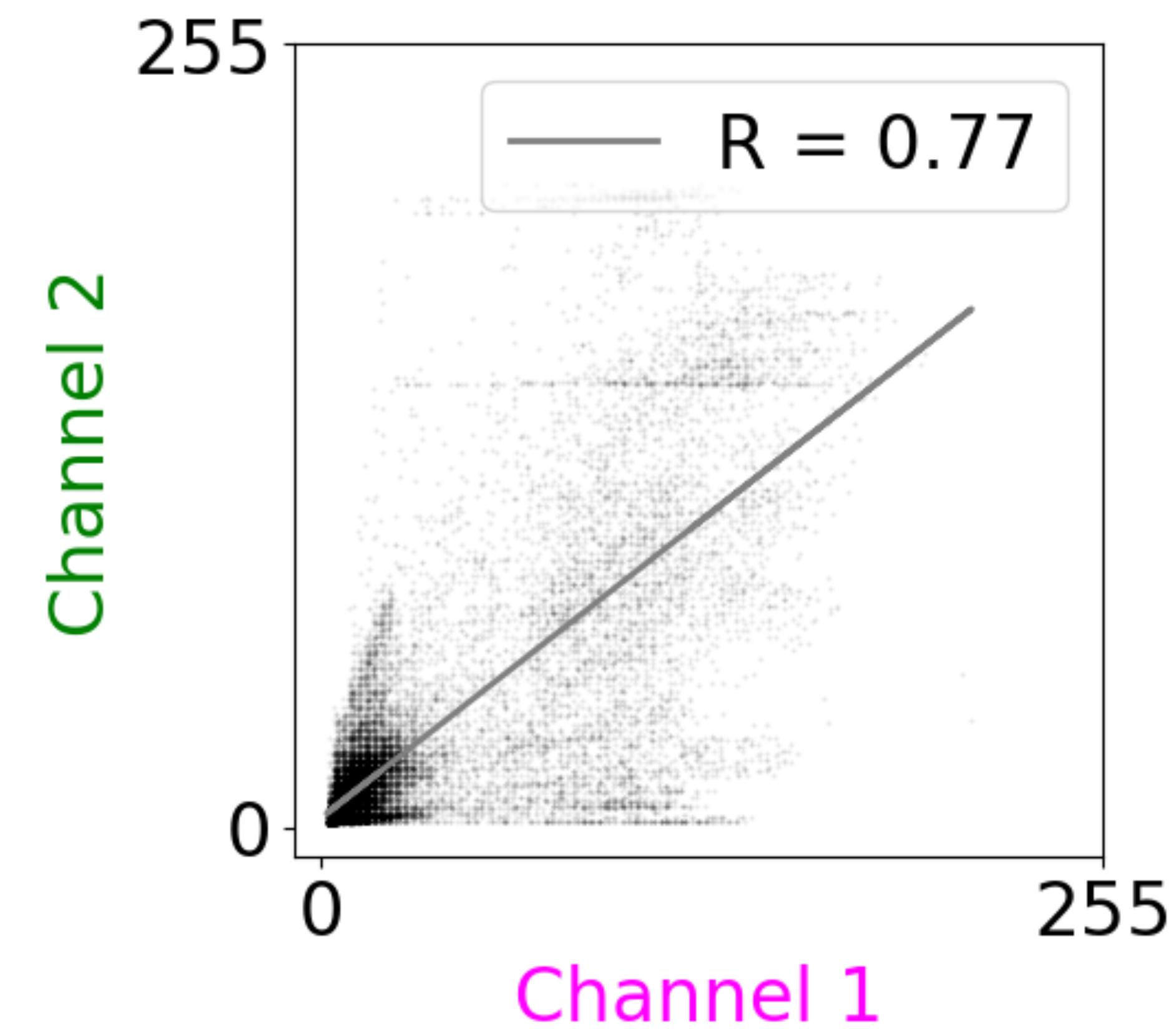
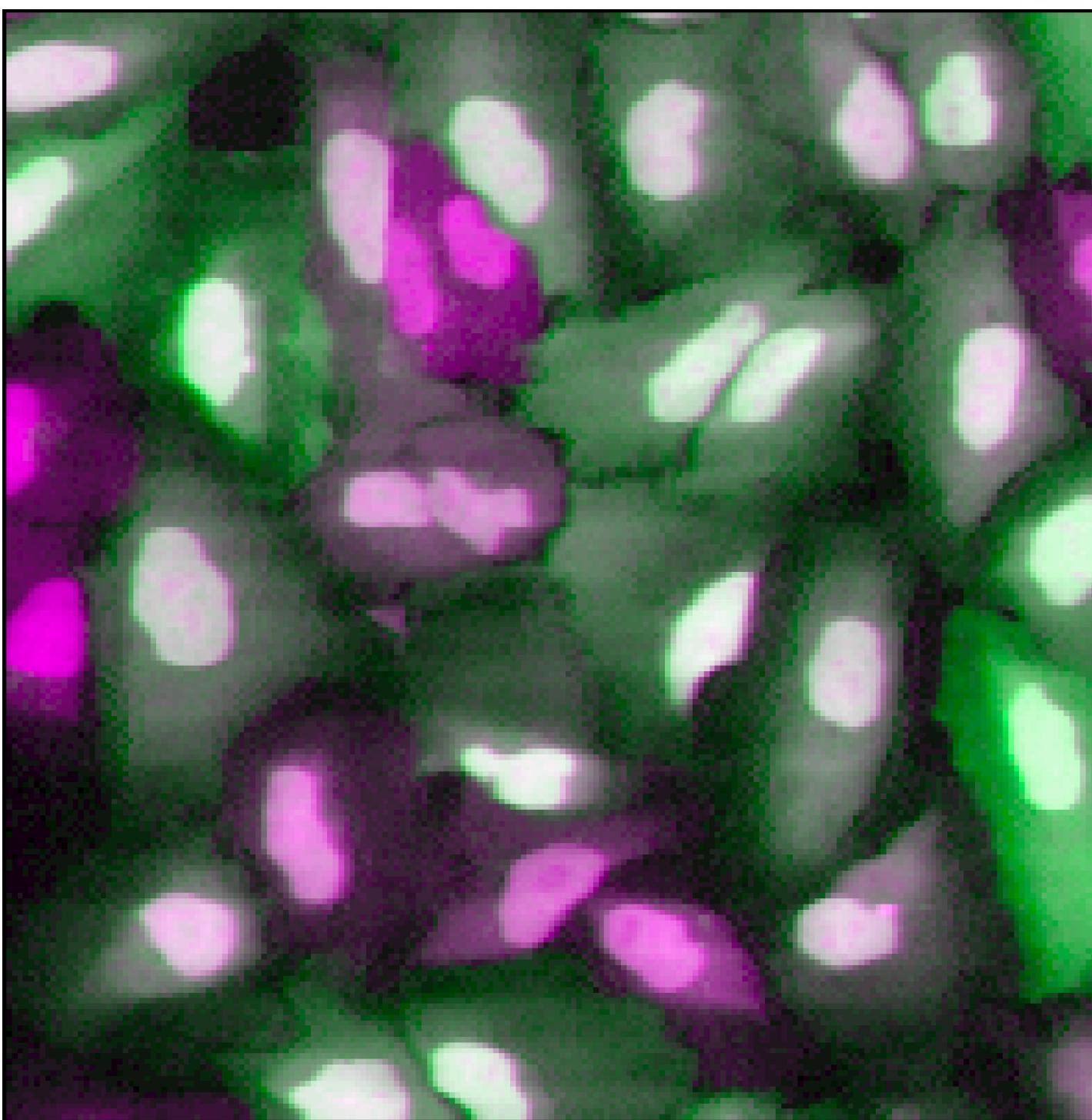


Object-based



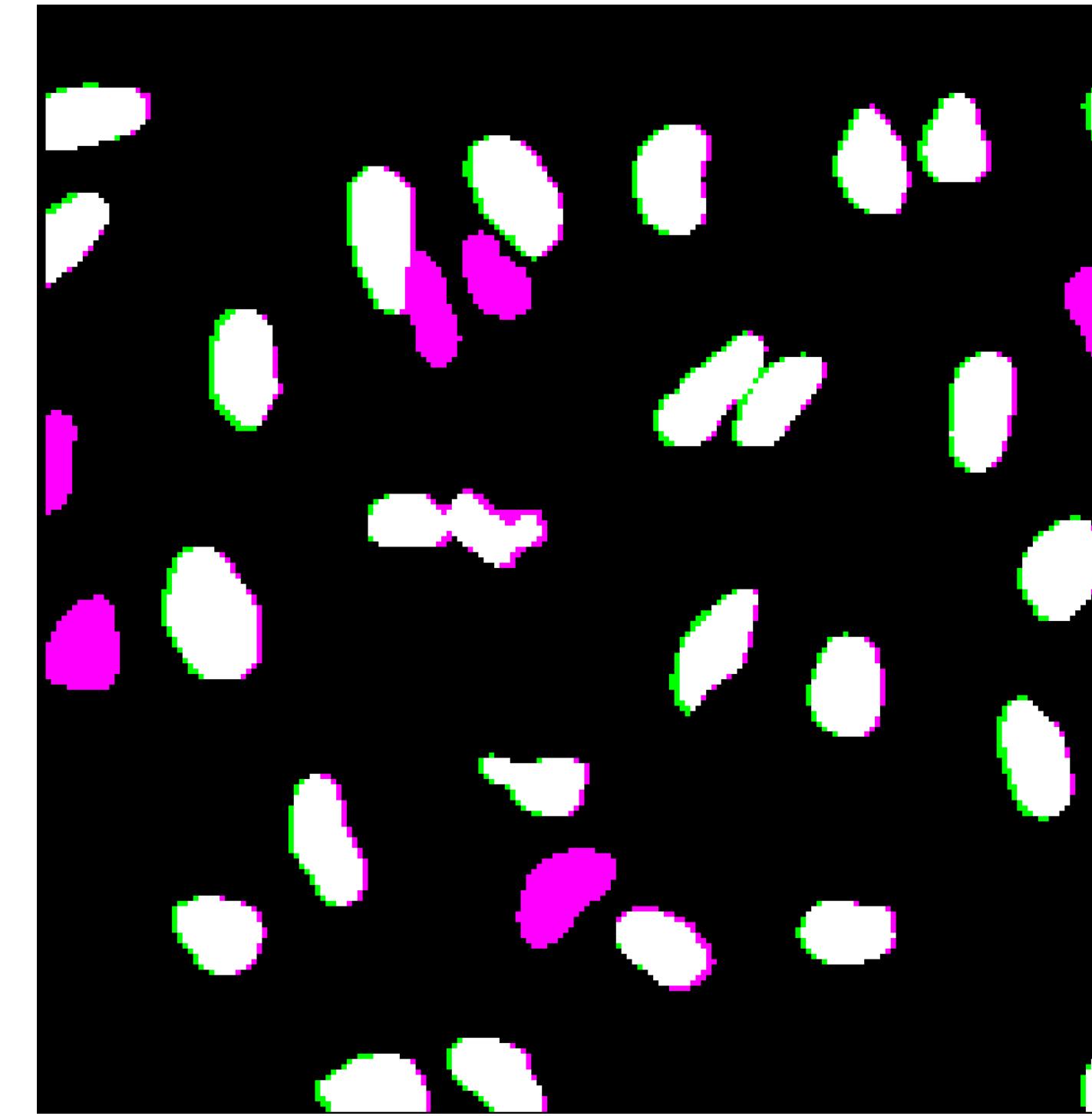
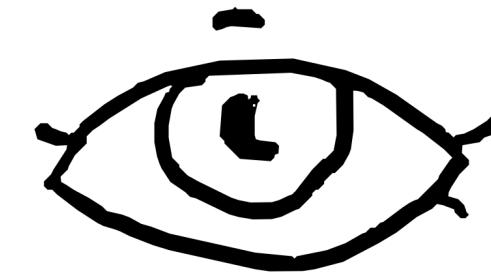
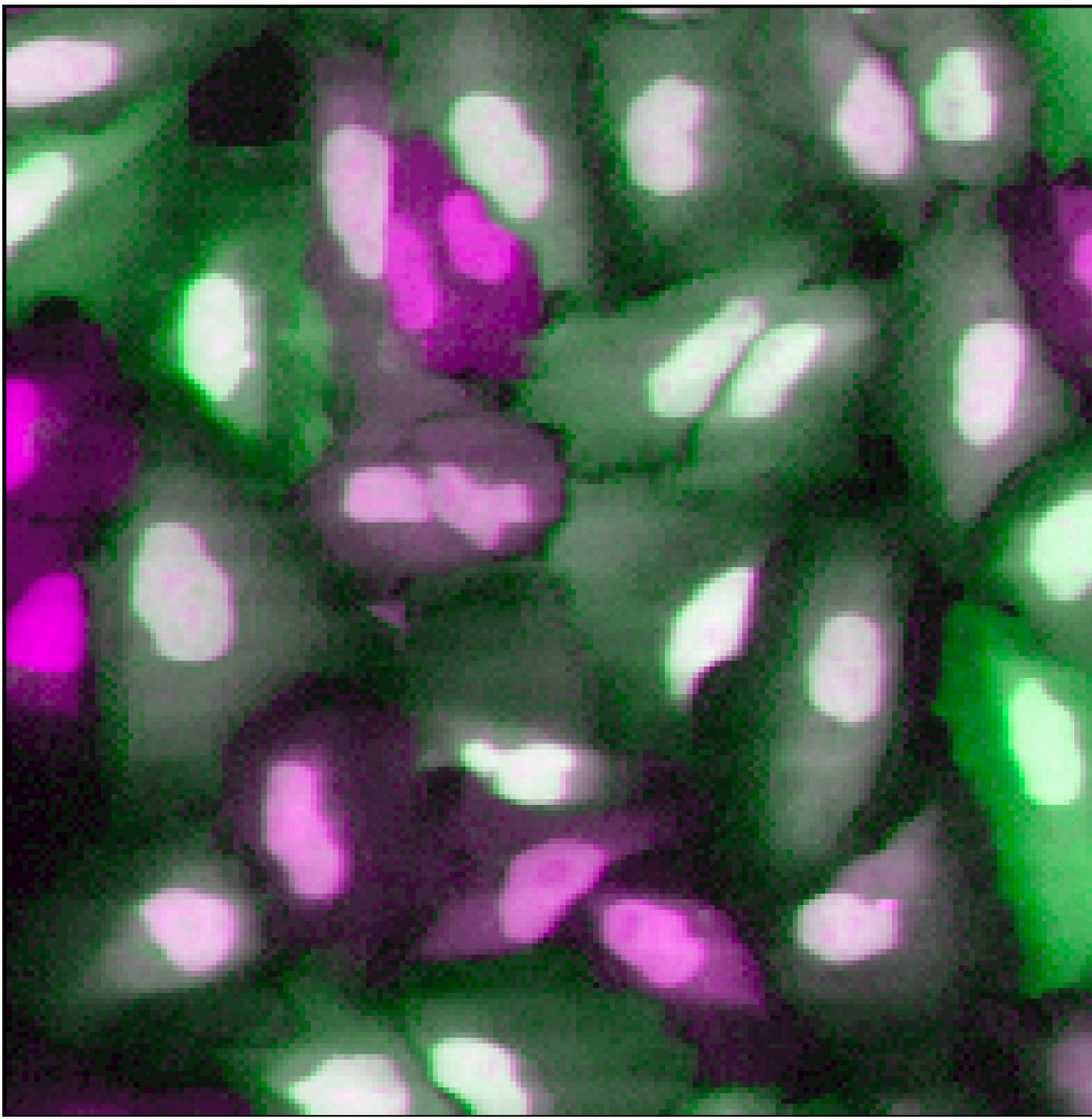
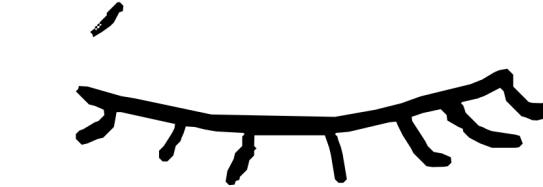


Intensity-based *could* discard spatial information





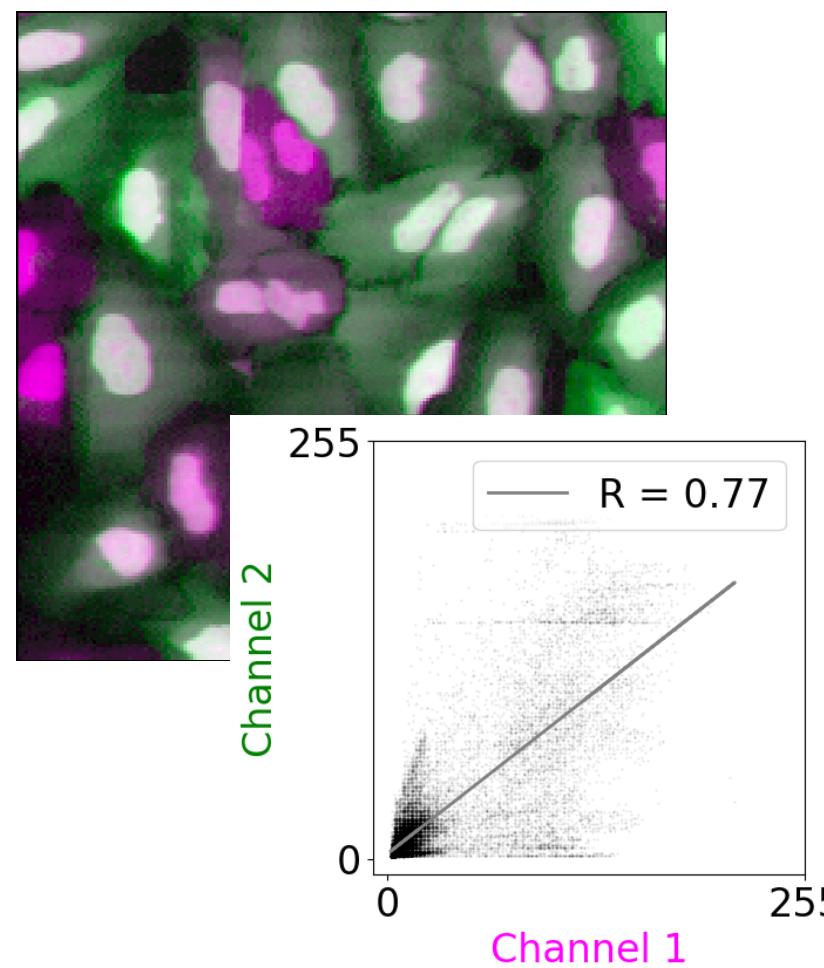
Object based analysis discards intensity-values





Intensity-based vs. Object-based coloc

Purely intensity-based



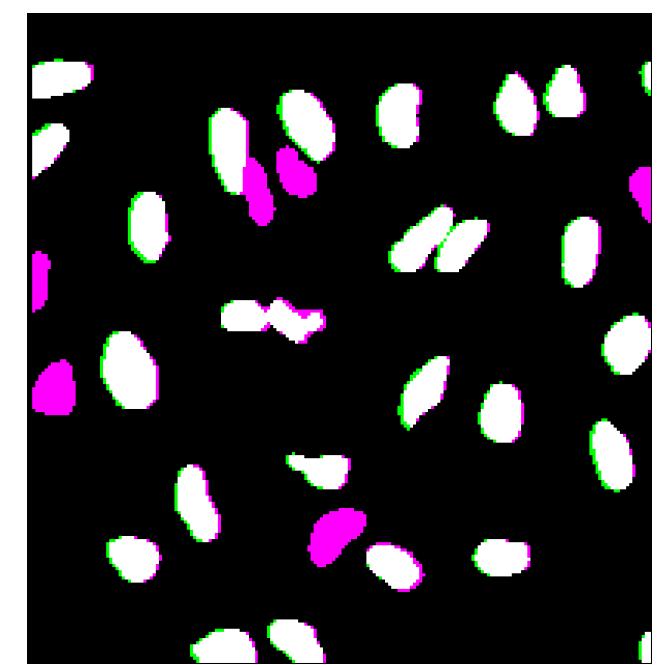
▲
Only pixel
intensity values

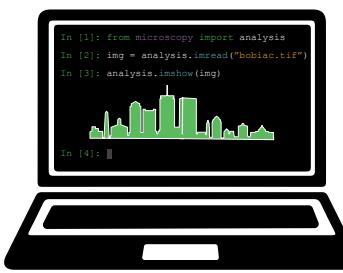
▲
Thresholding

▲
Segmentation

▲
Discards pixel
intensity values

Object-based

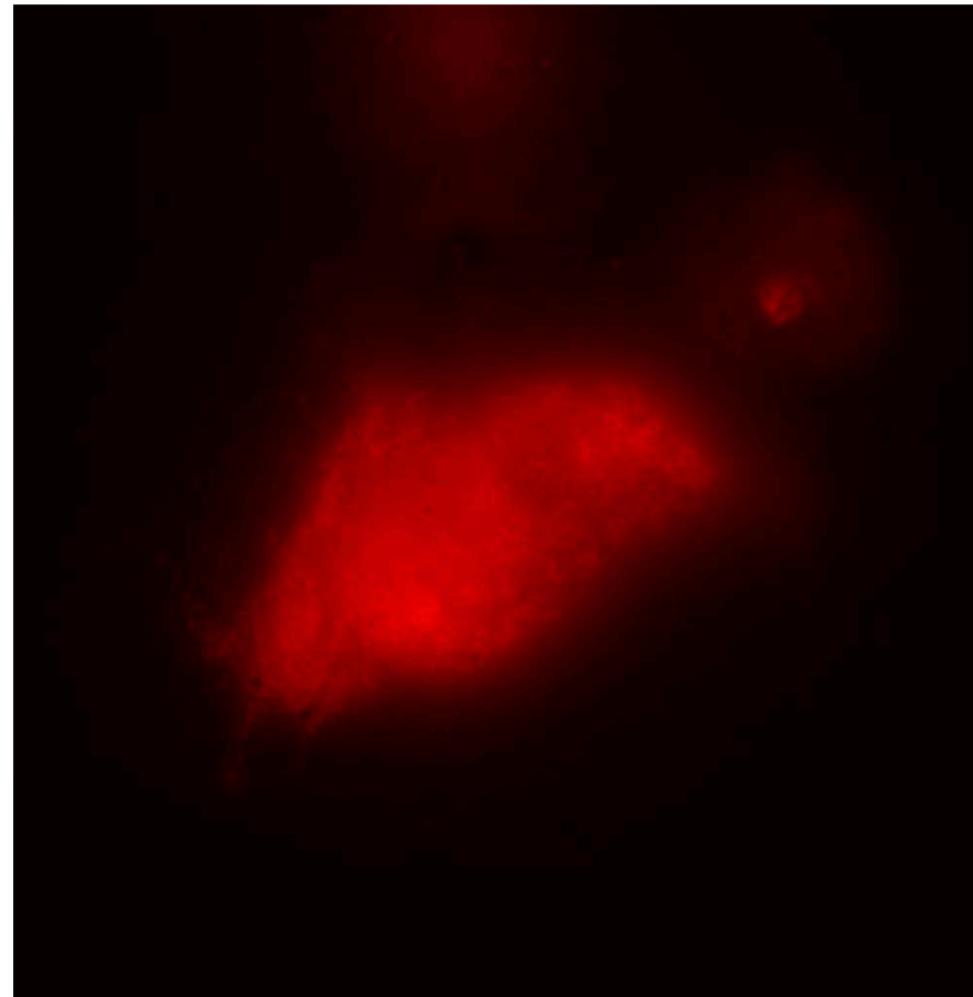




When to use object-based methods

Intensity-based

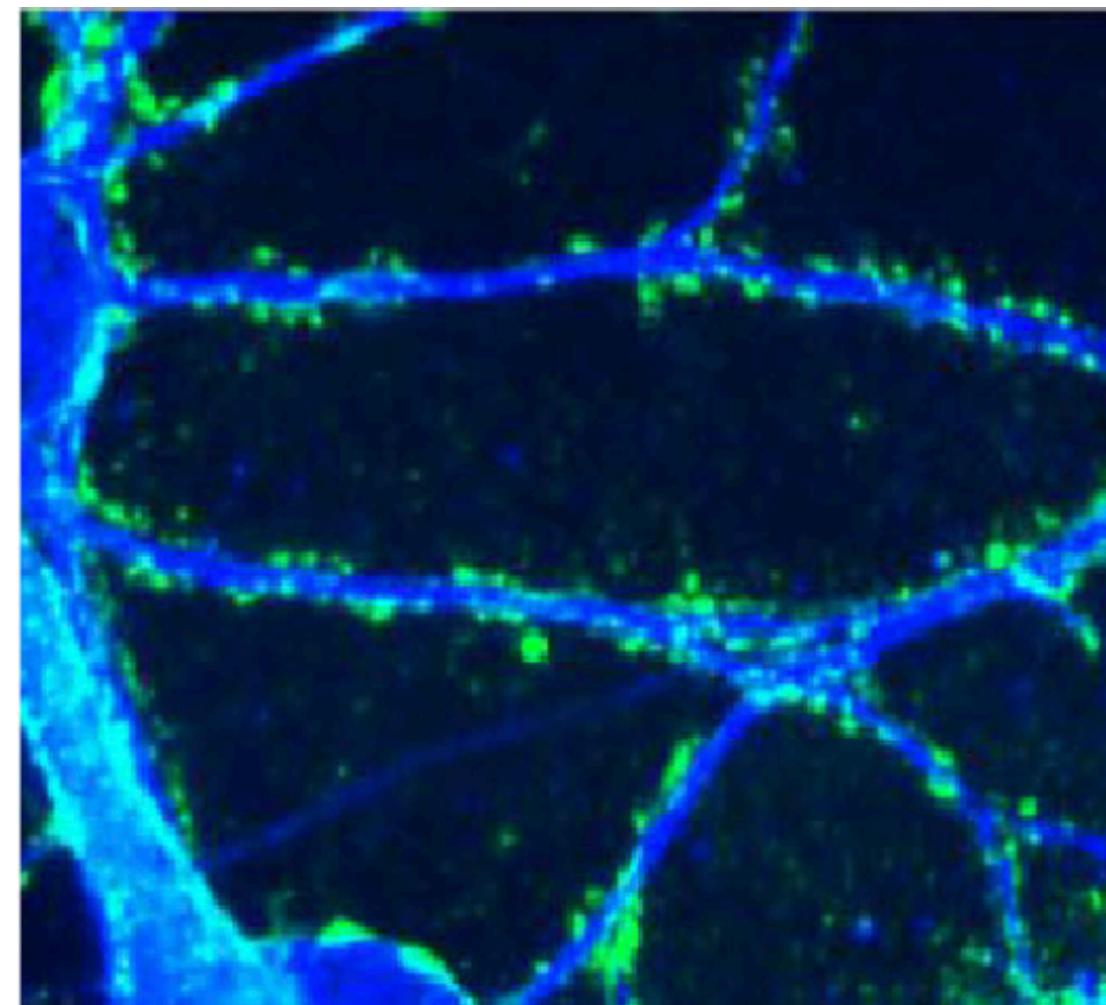
- Segmentation difficult



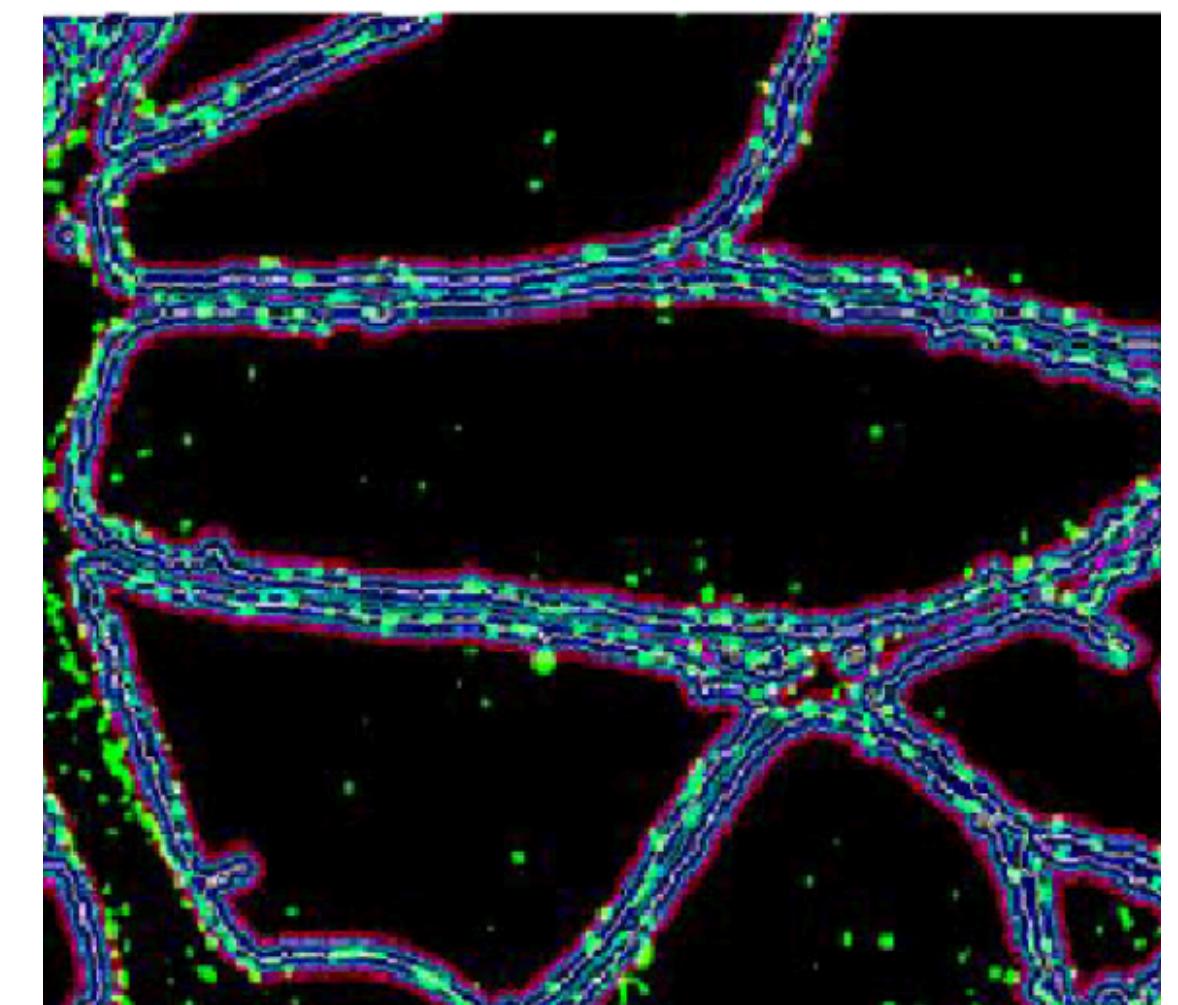
Two HIV components in HeLa cells

Object-based

- Objects are discrete & segmentable



Neuronal dendrites (blue)
and synaptic spots (green)

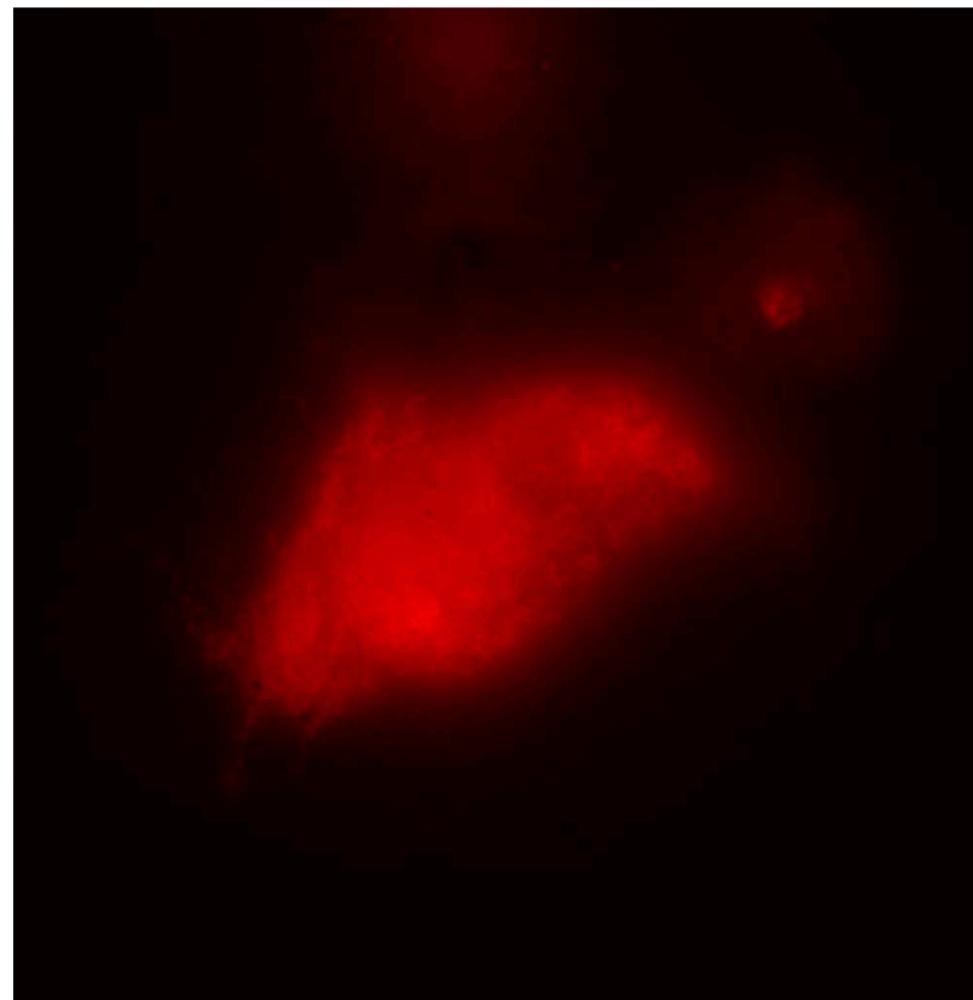




When to use object-based methods

Intensity-based

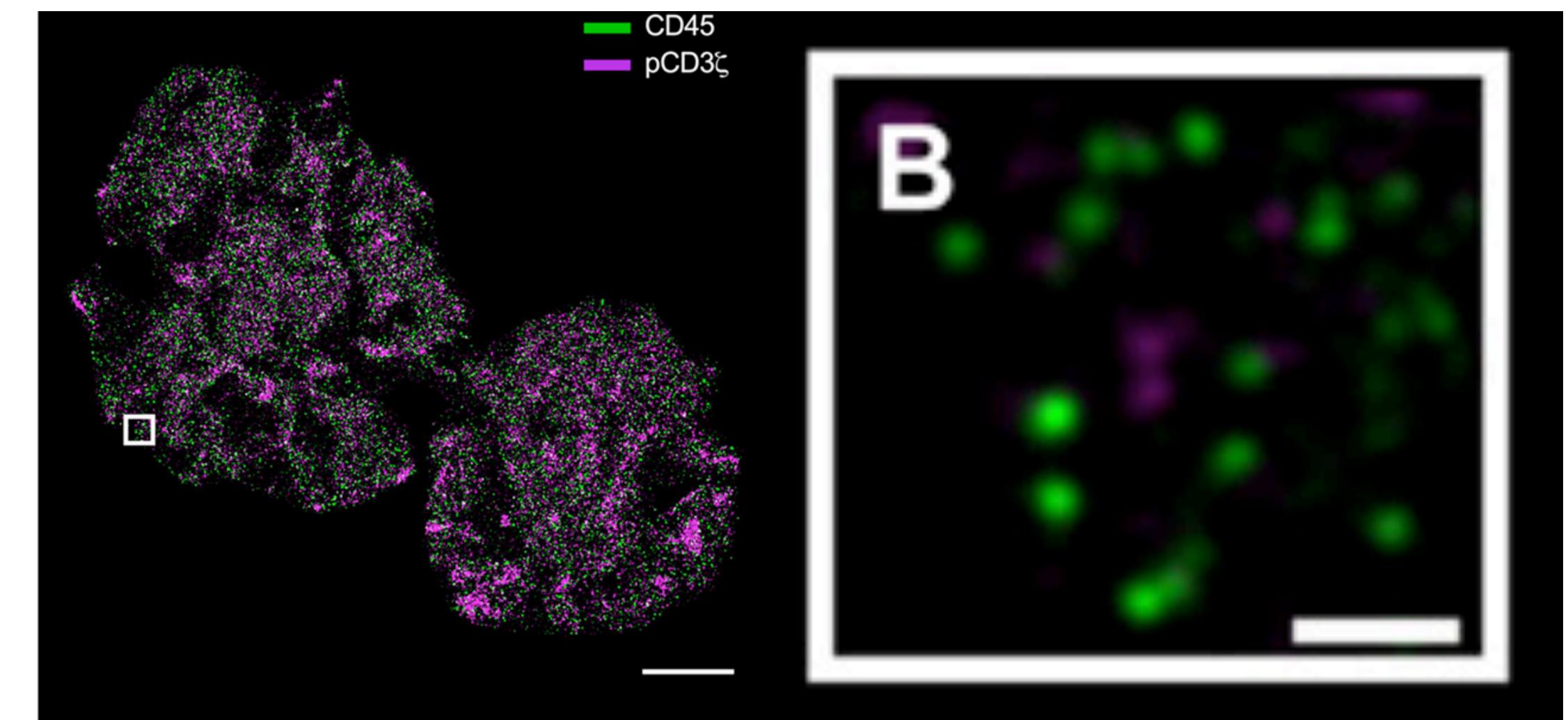
- Often in diffraction limited settings



Two HIV components in HeLa cells

Object-based

- Often in super-resolution settings



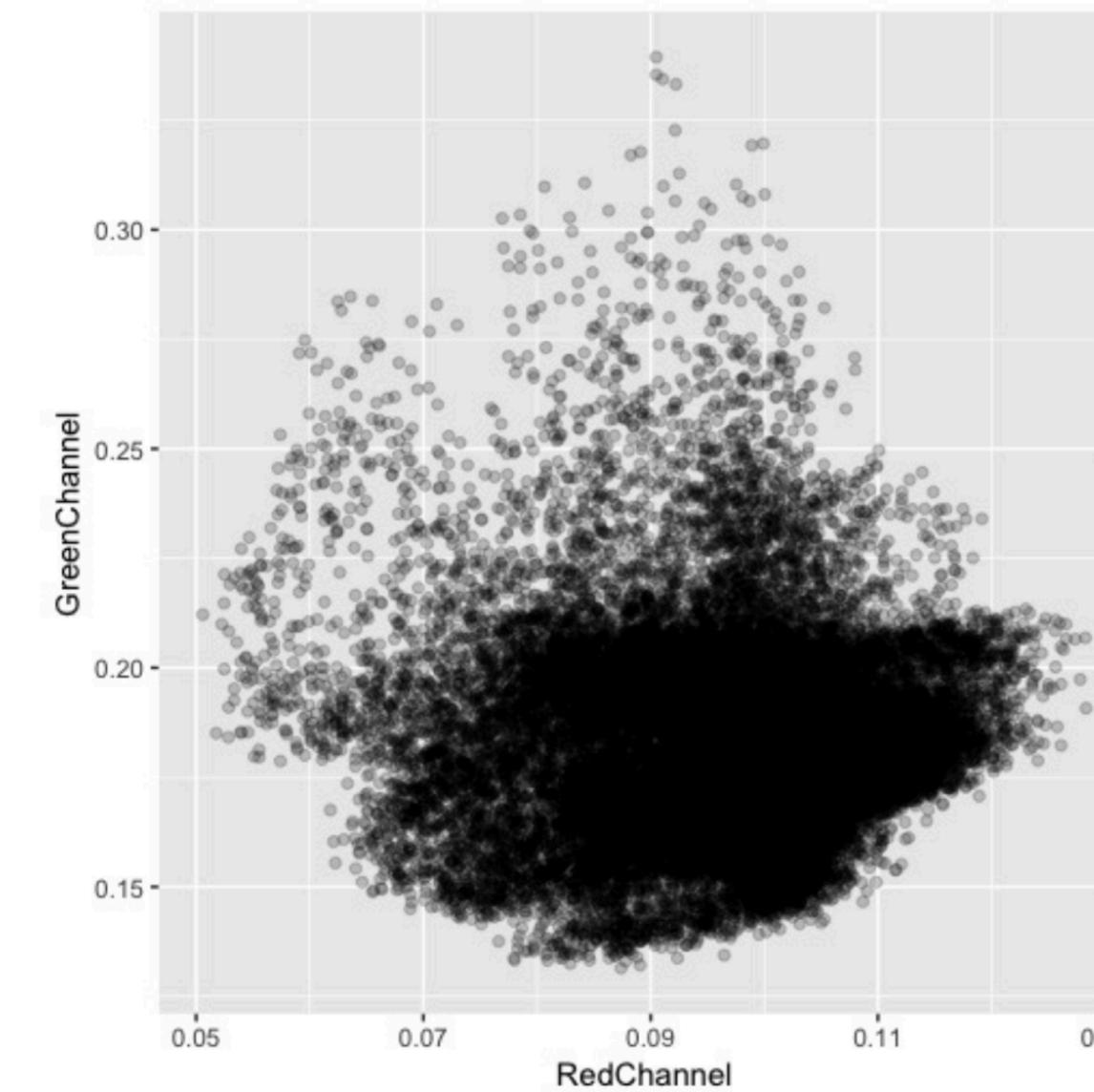
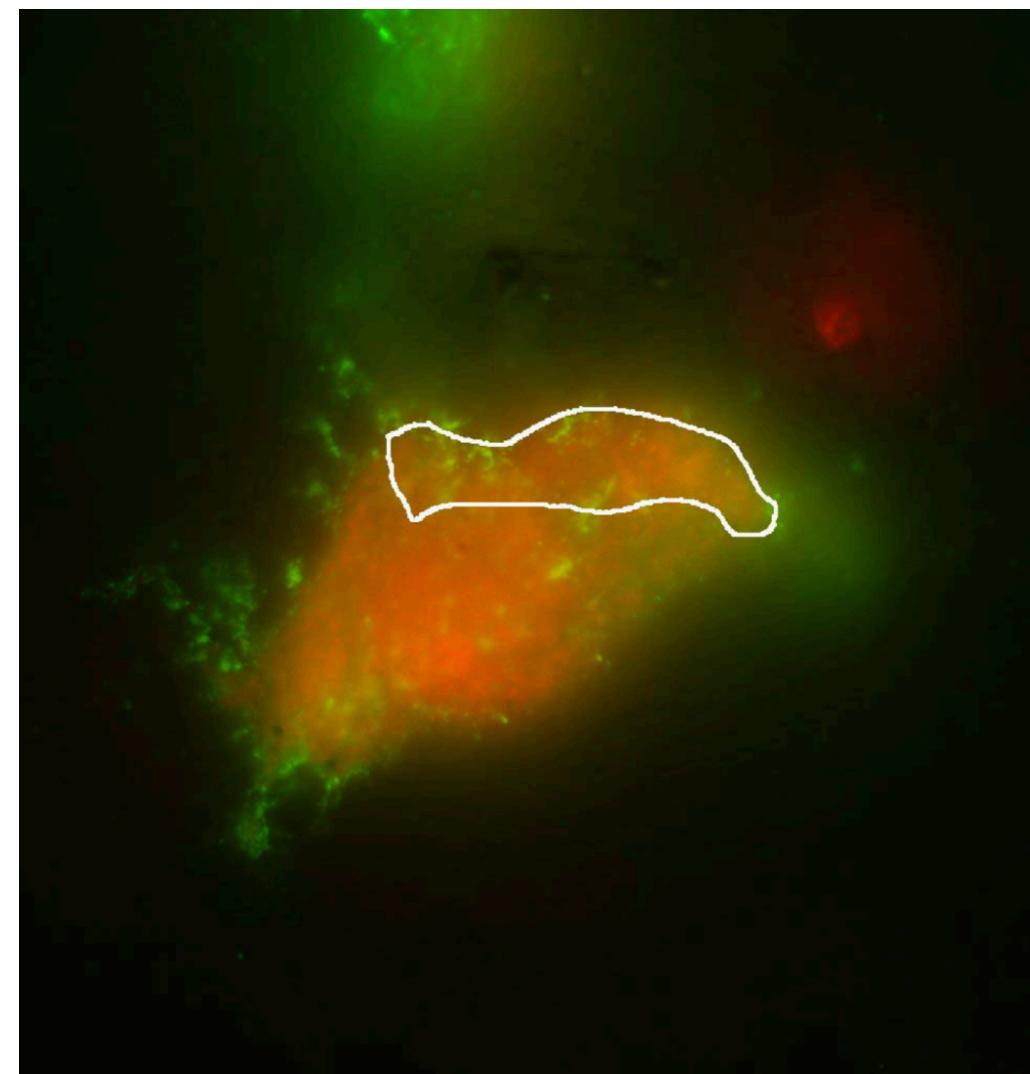
Distance between signalling proteins in SMLM; scale bar B: 200 nm



When to use object-based methods

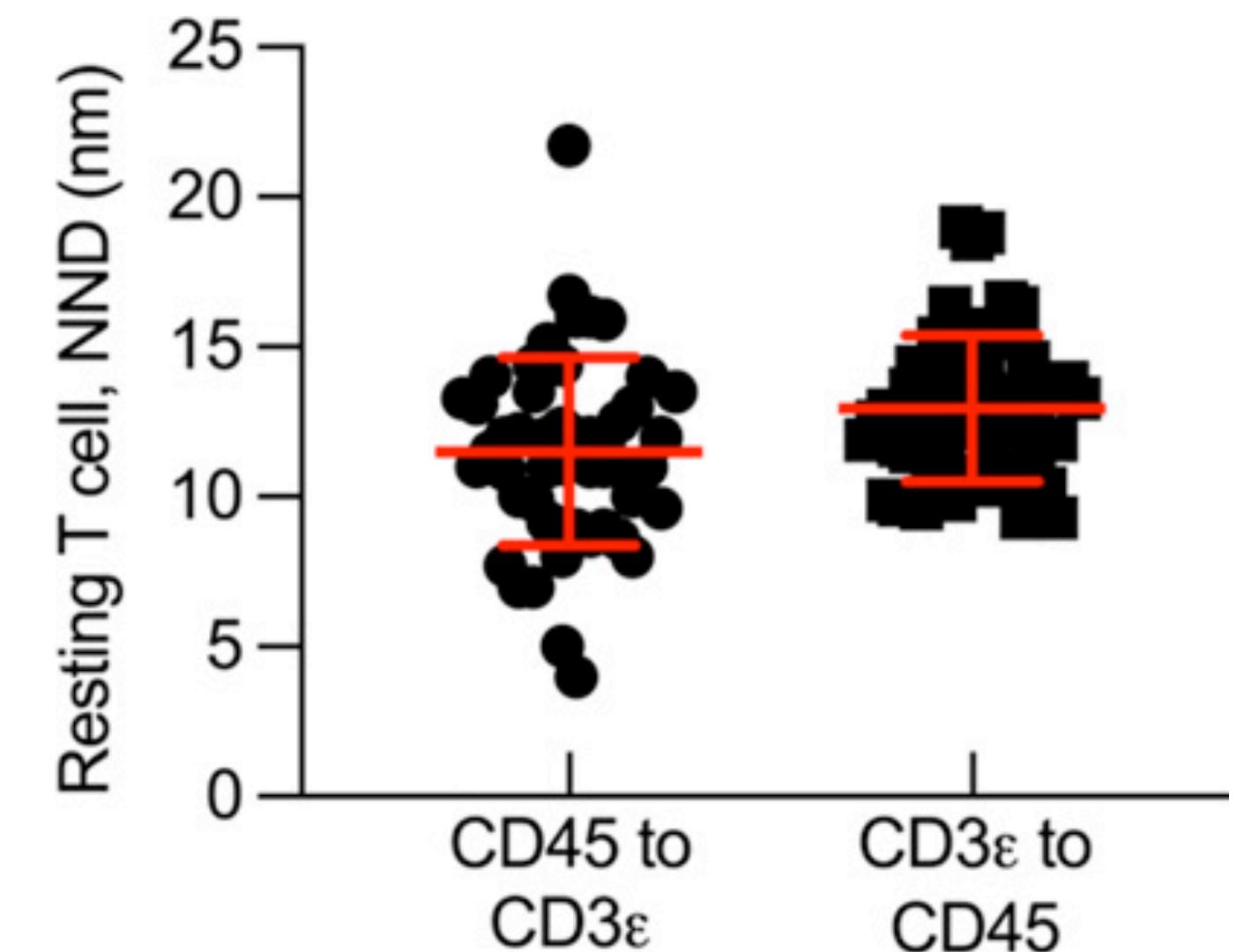
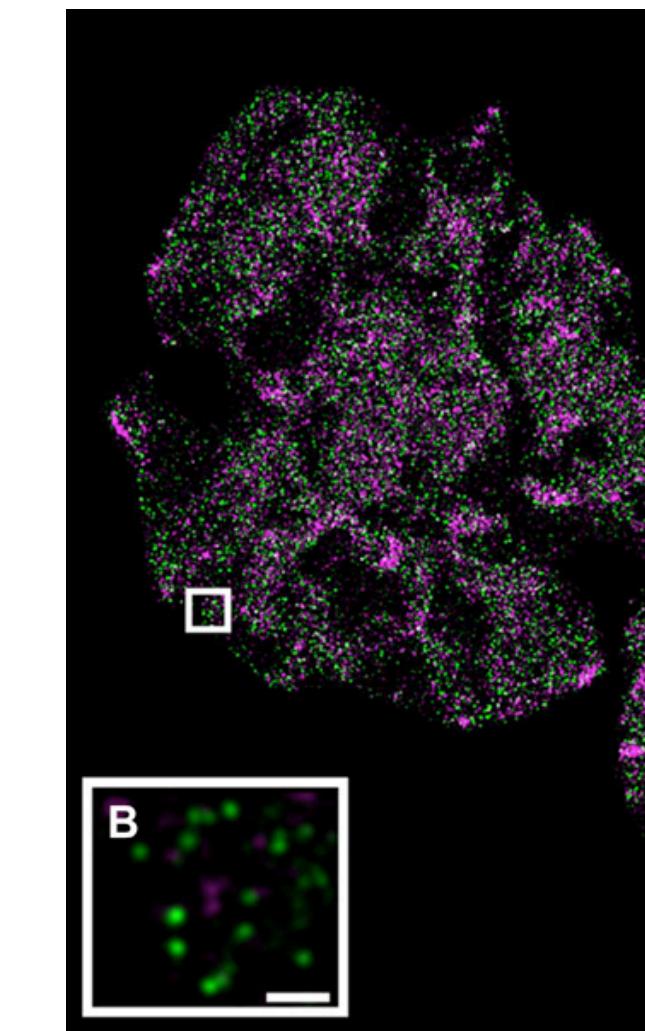
Intensity-based

- Statistical quantities
 - “What correlation”



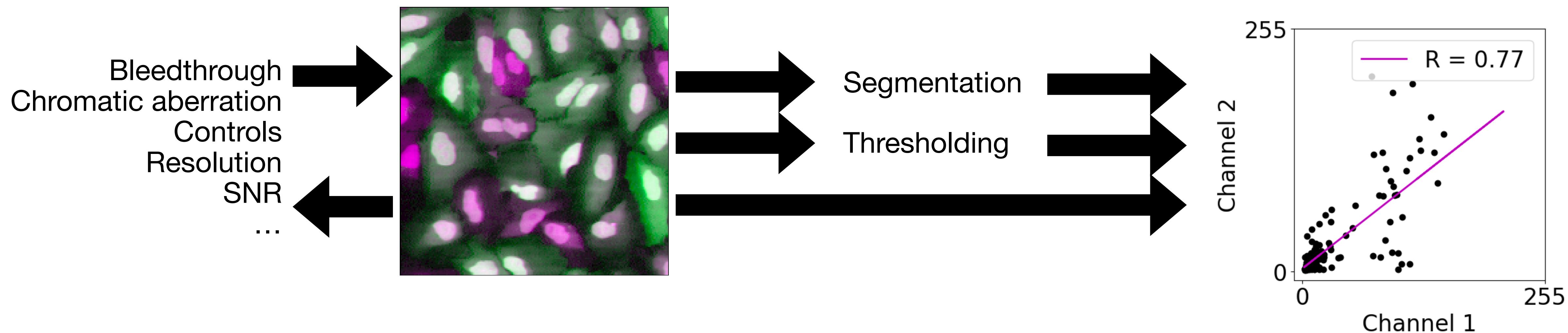
Object-based

- Physical quantities
 - “What distance”, “How many”





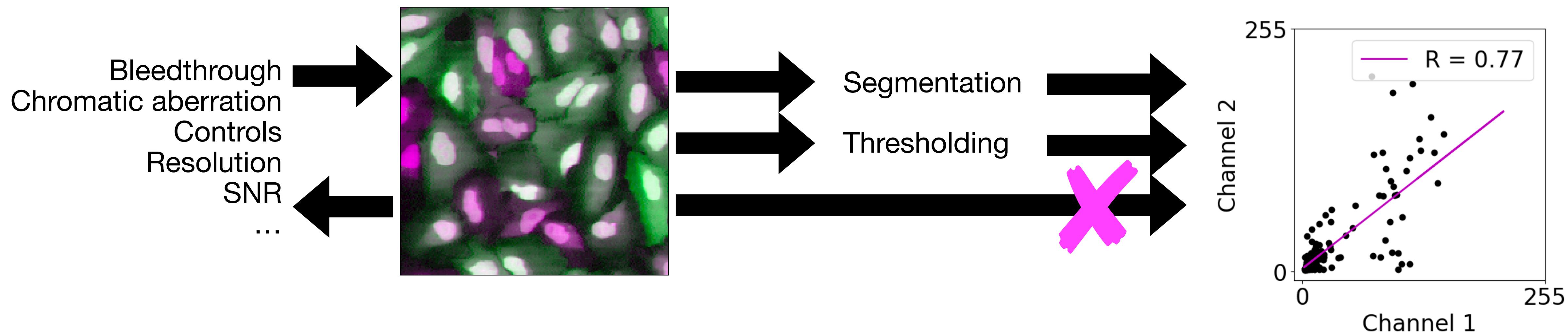
Object-based coloc: workflow



Data: Image set [BBBC014v1](#) provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [[dx.doi.org/10.1101/2083](https://doi.org/10.1101/2083)].



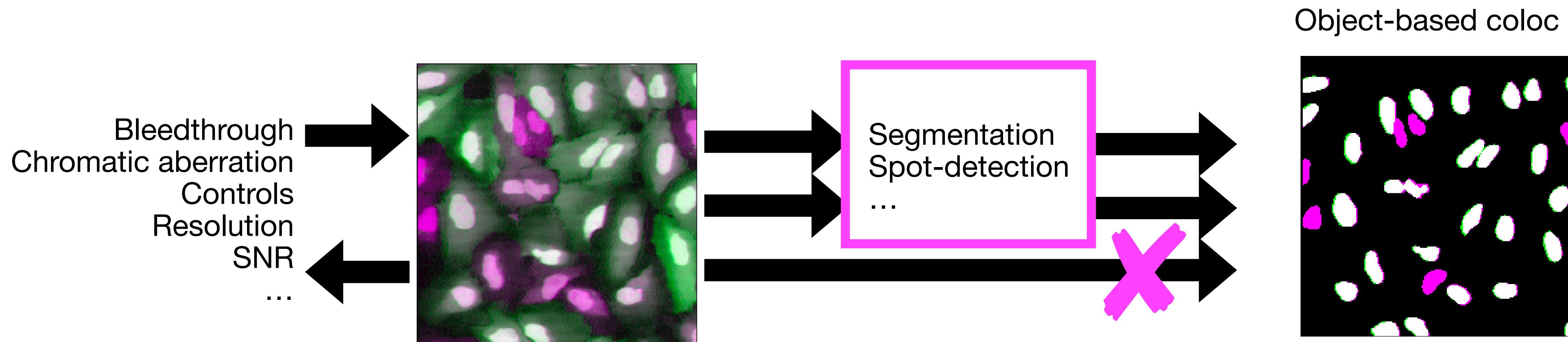
Object-based coloc: workflow



Data: Image set [BBBC014v1](#) provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [[dx.doi.org/10.1101/2083](https://doi.org/10.1101/2083)].



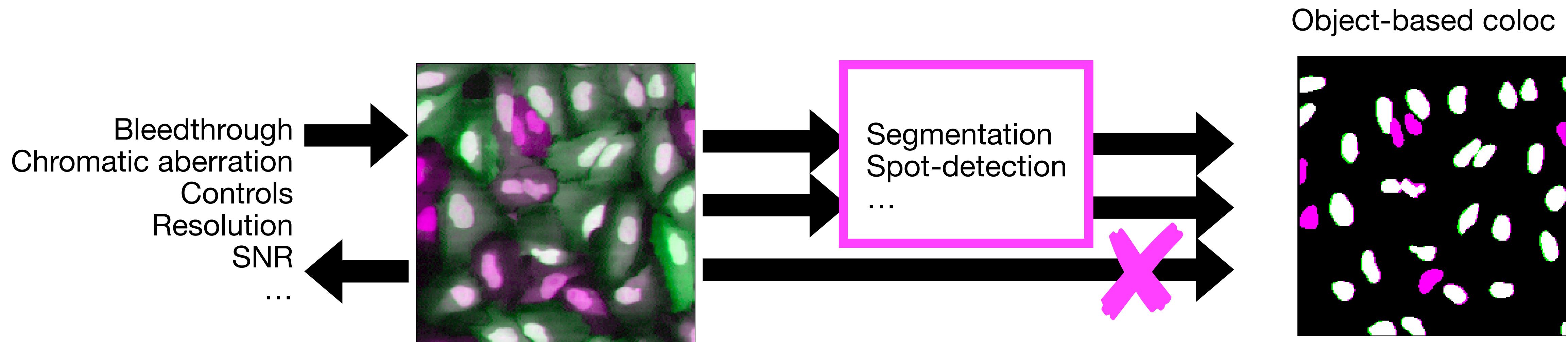
Object-based coloc: workflow



Data: Image set [BBBC014v1](#) provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [[dx.doi.org/10.1101/2083](https://doi.org/10.1101/2083)].



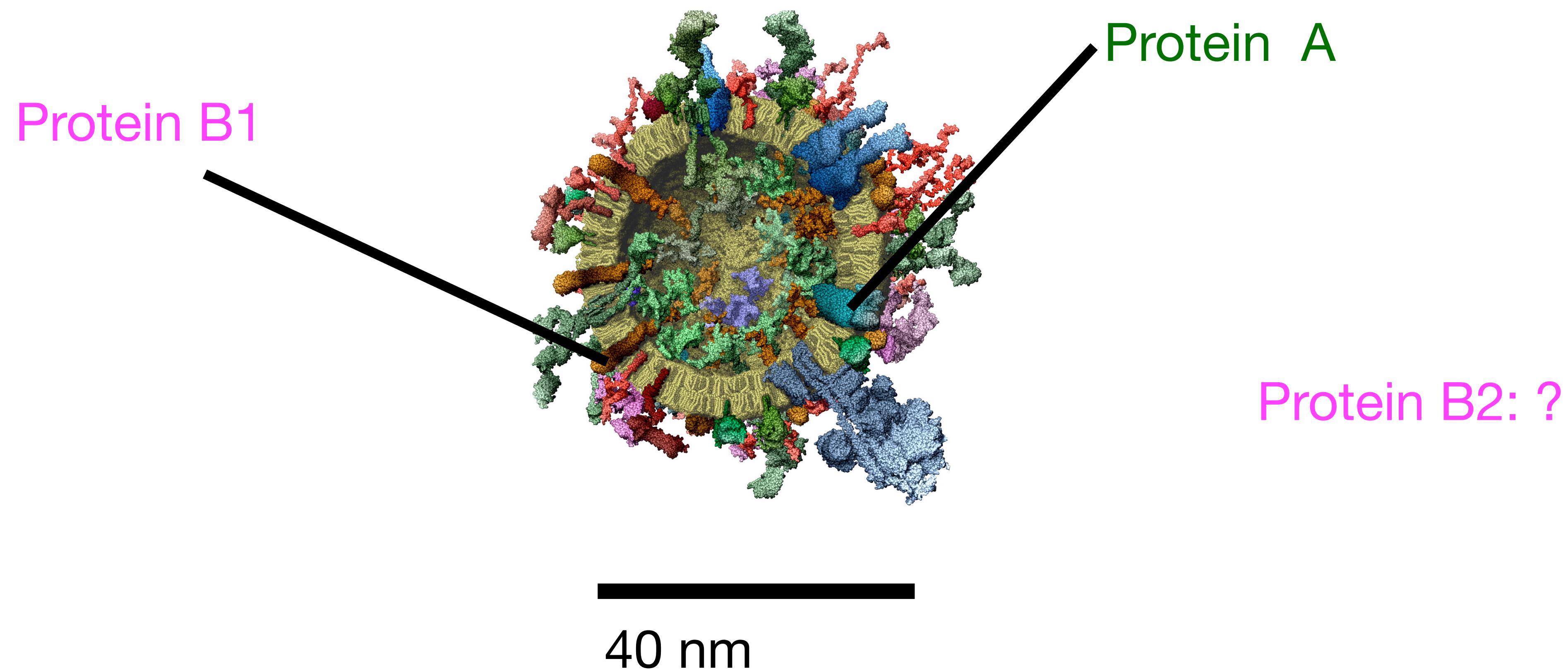
Object-based coloc: workflow



Object-based colocalization analysis also relies on initial image quality!

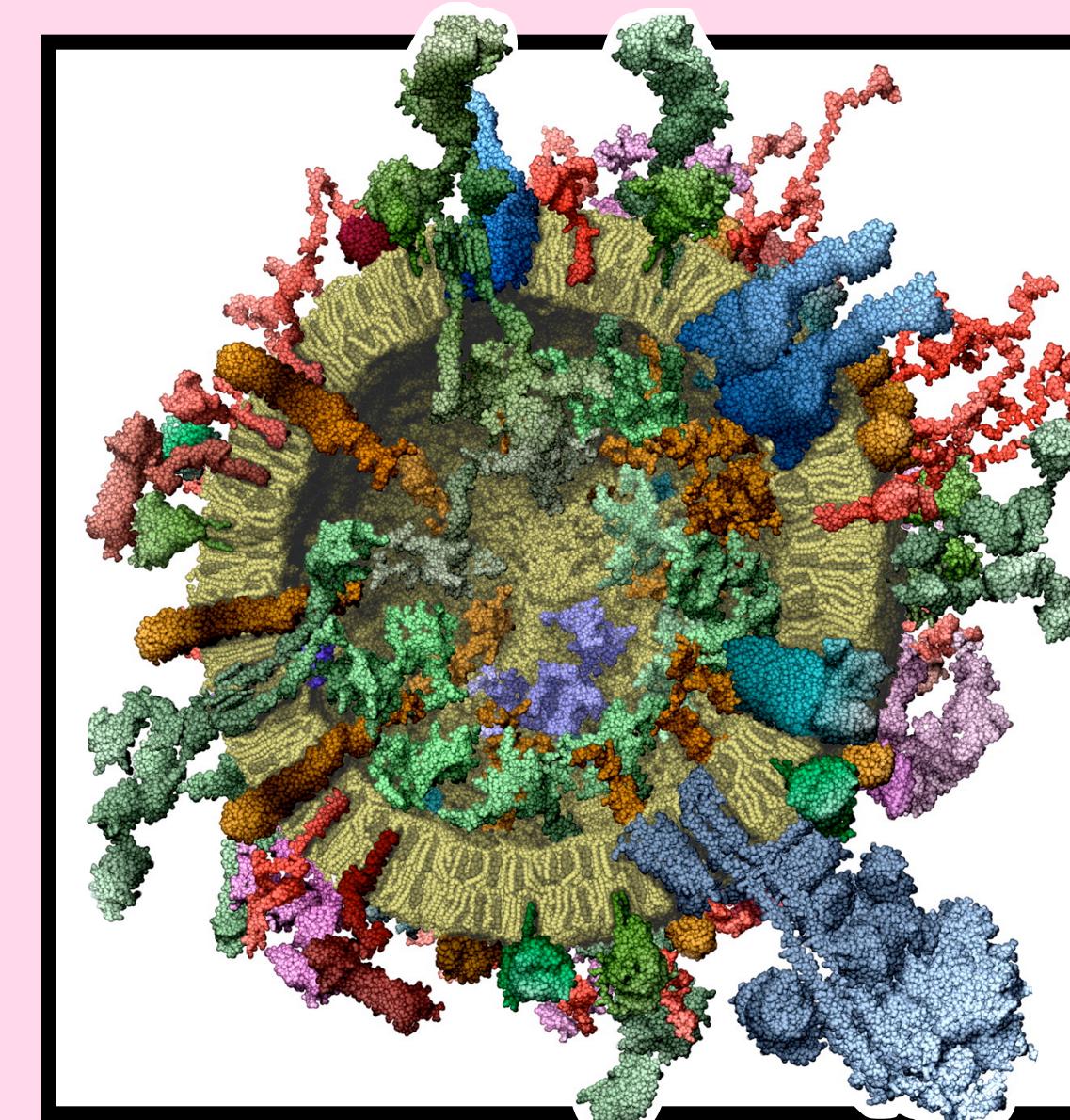


Example: Area



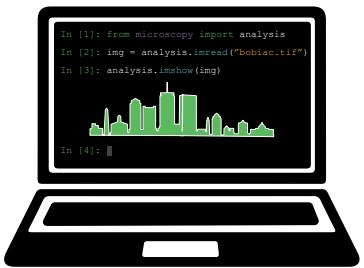


Example: Area



px: 60 nm

~ PSF

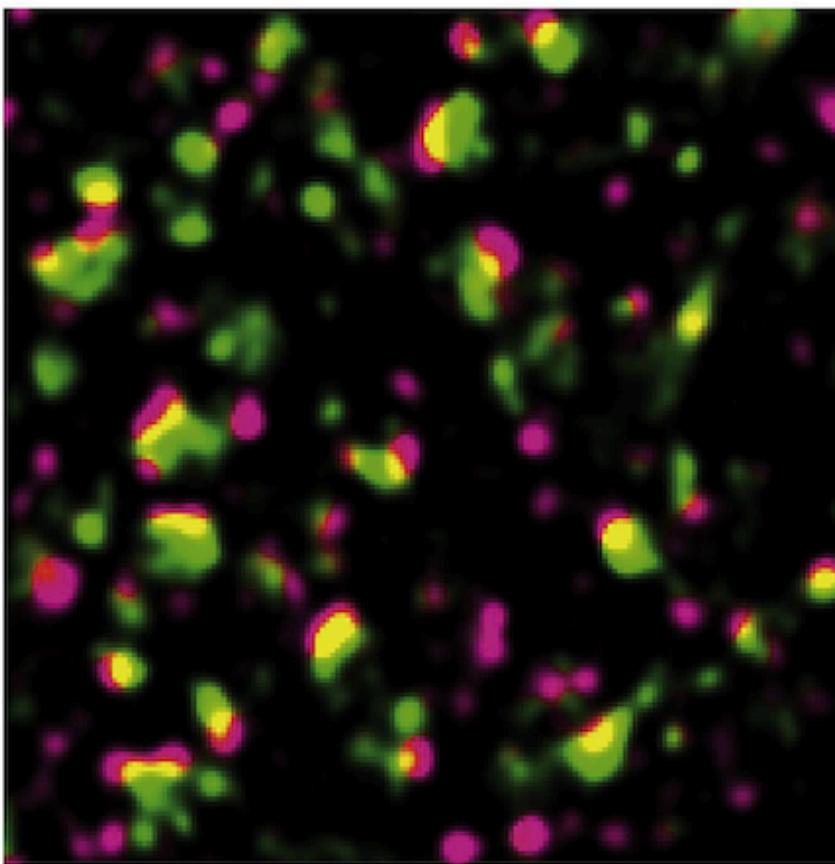


Example: Area

Original

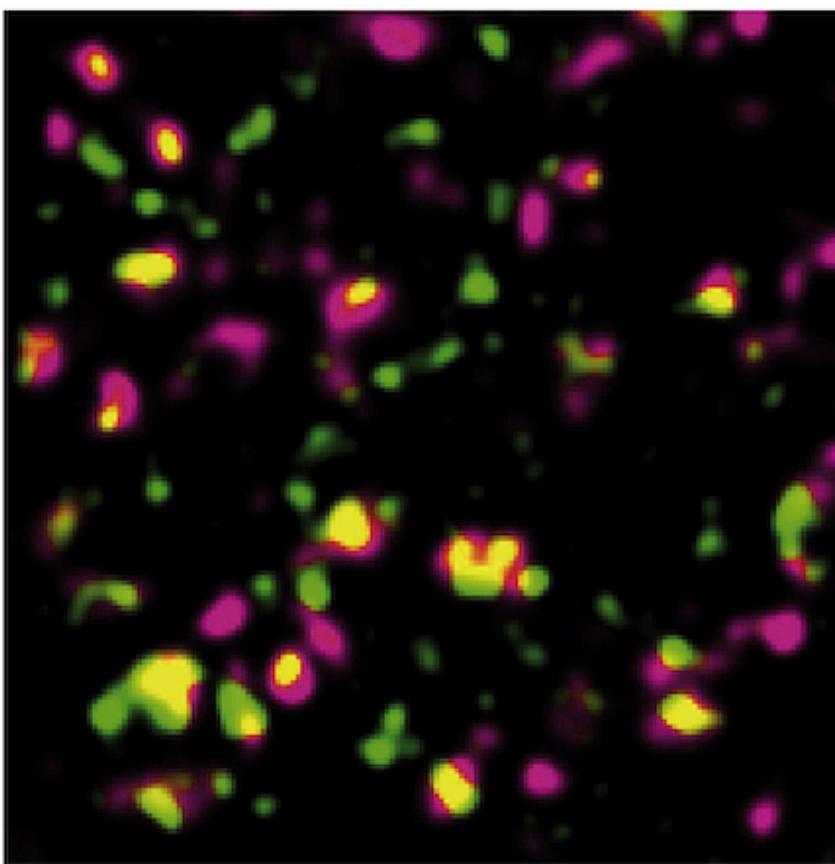
A

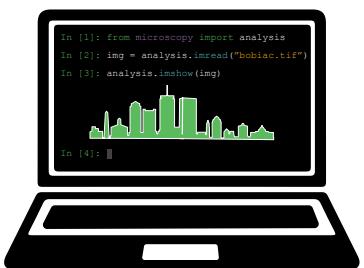
B2



A

B1

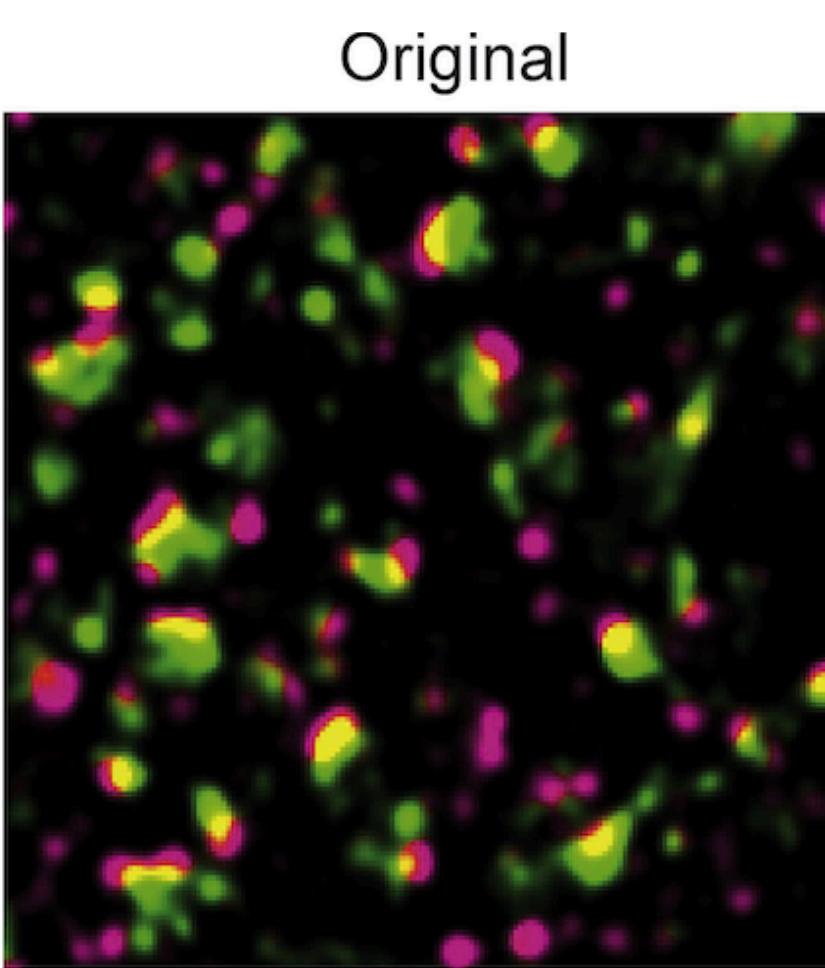




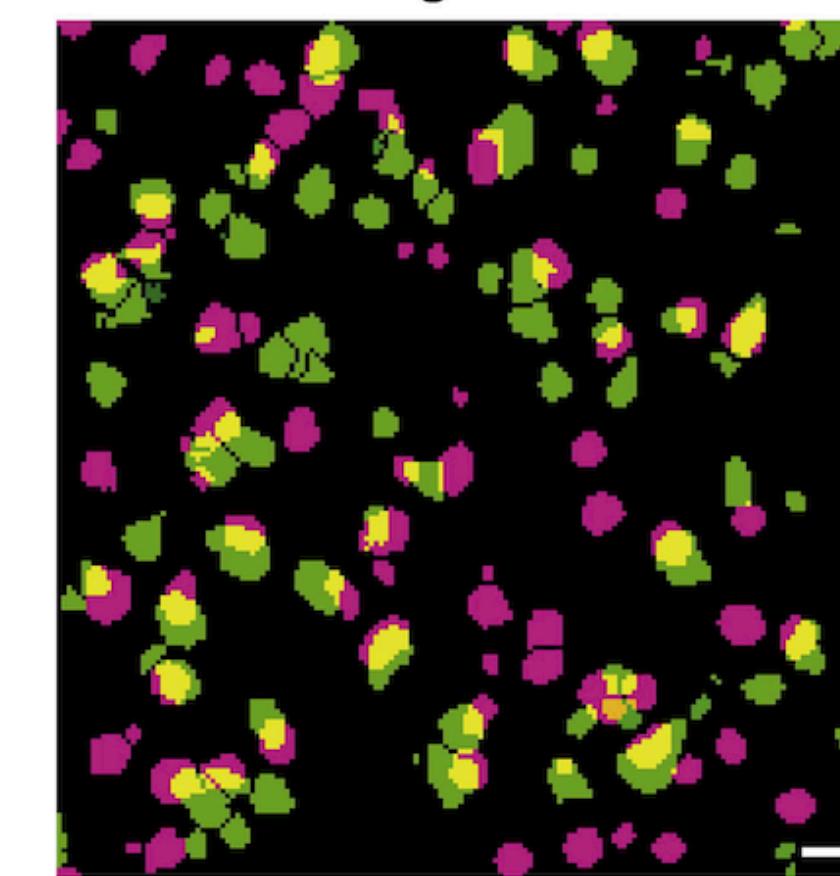
Example: Area

A

B2

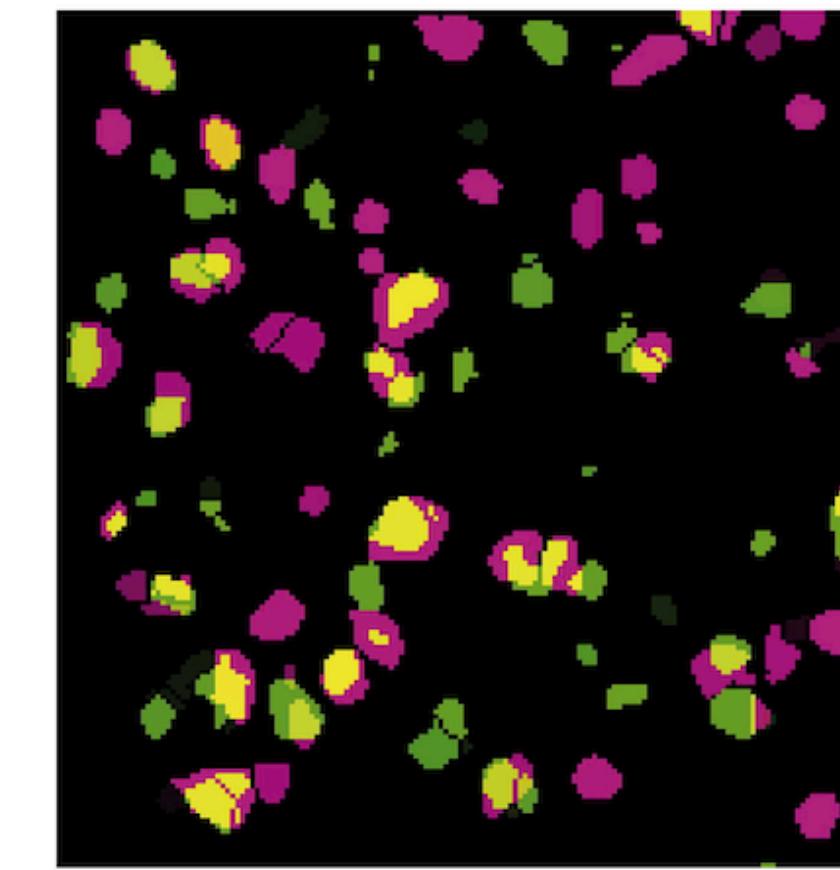
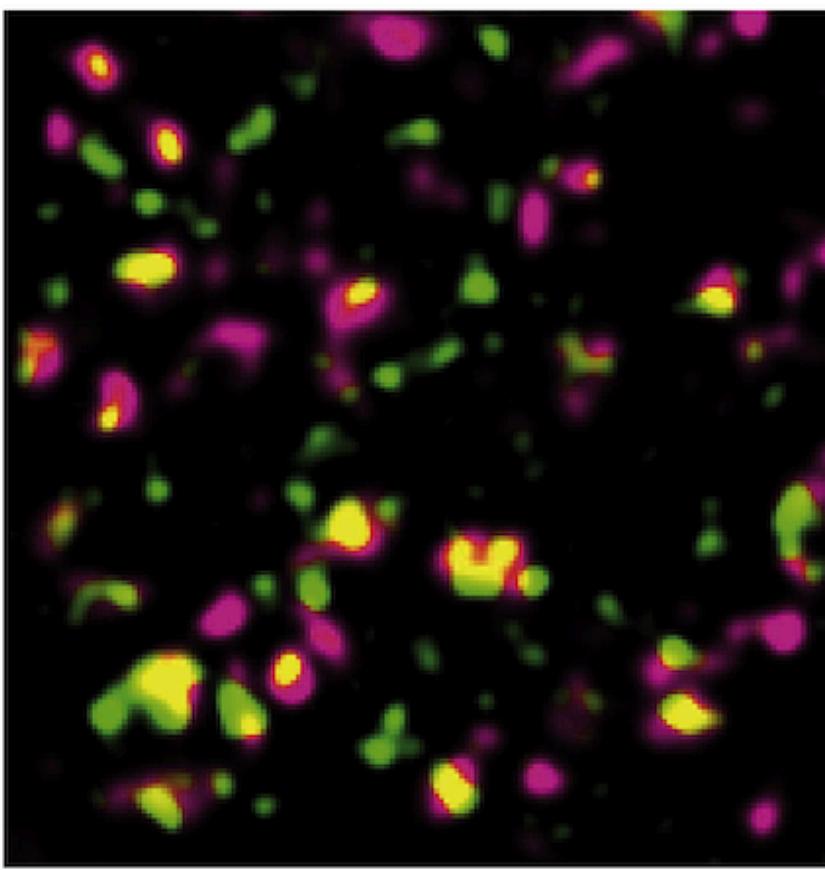


Segmented



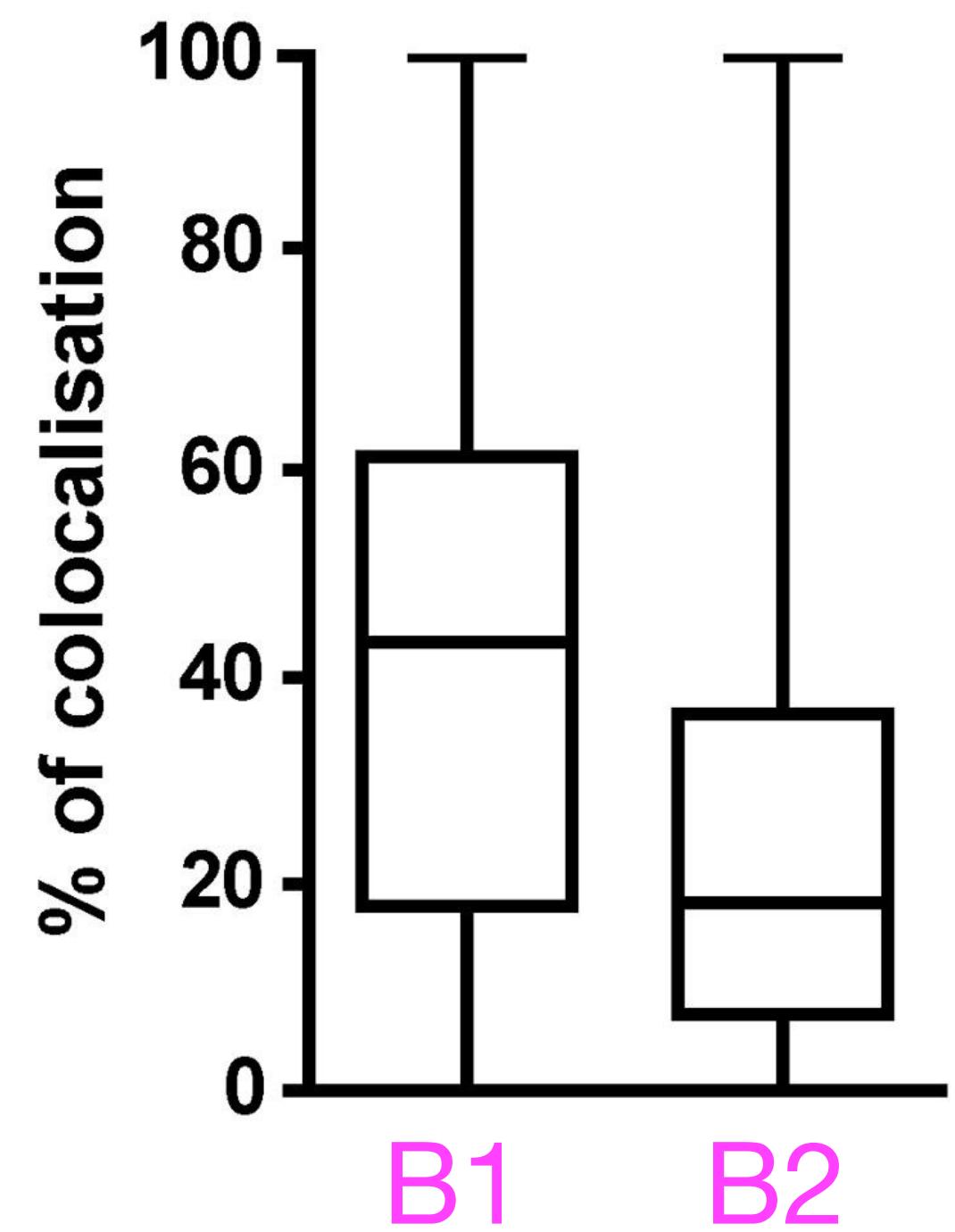
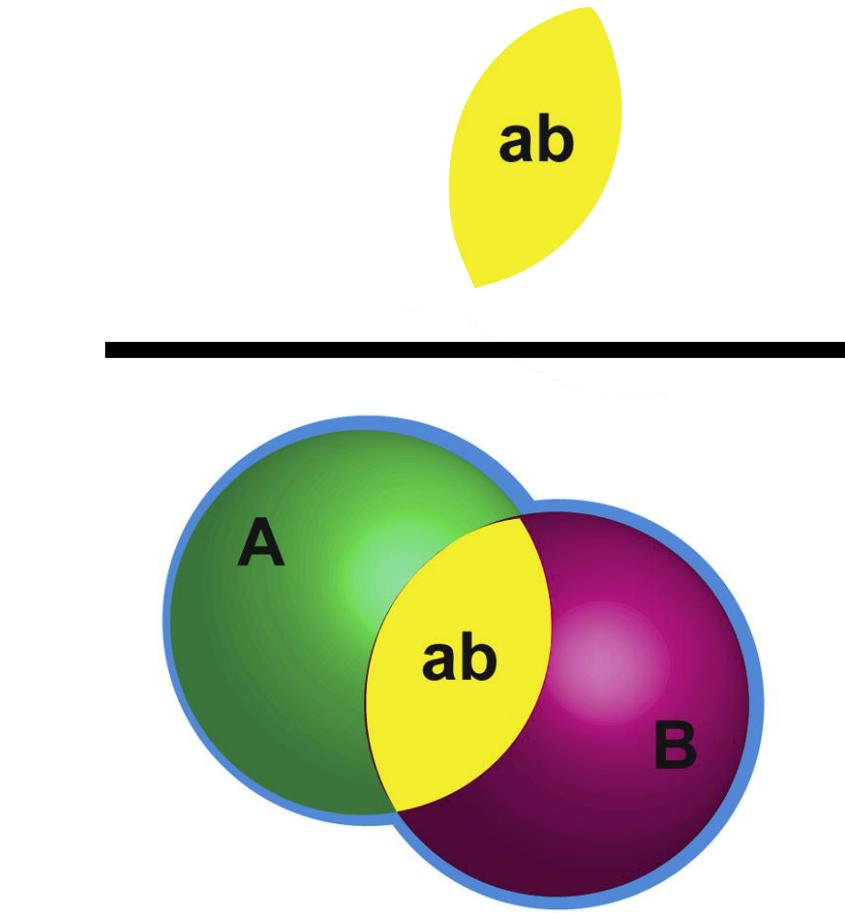
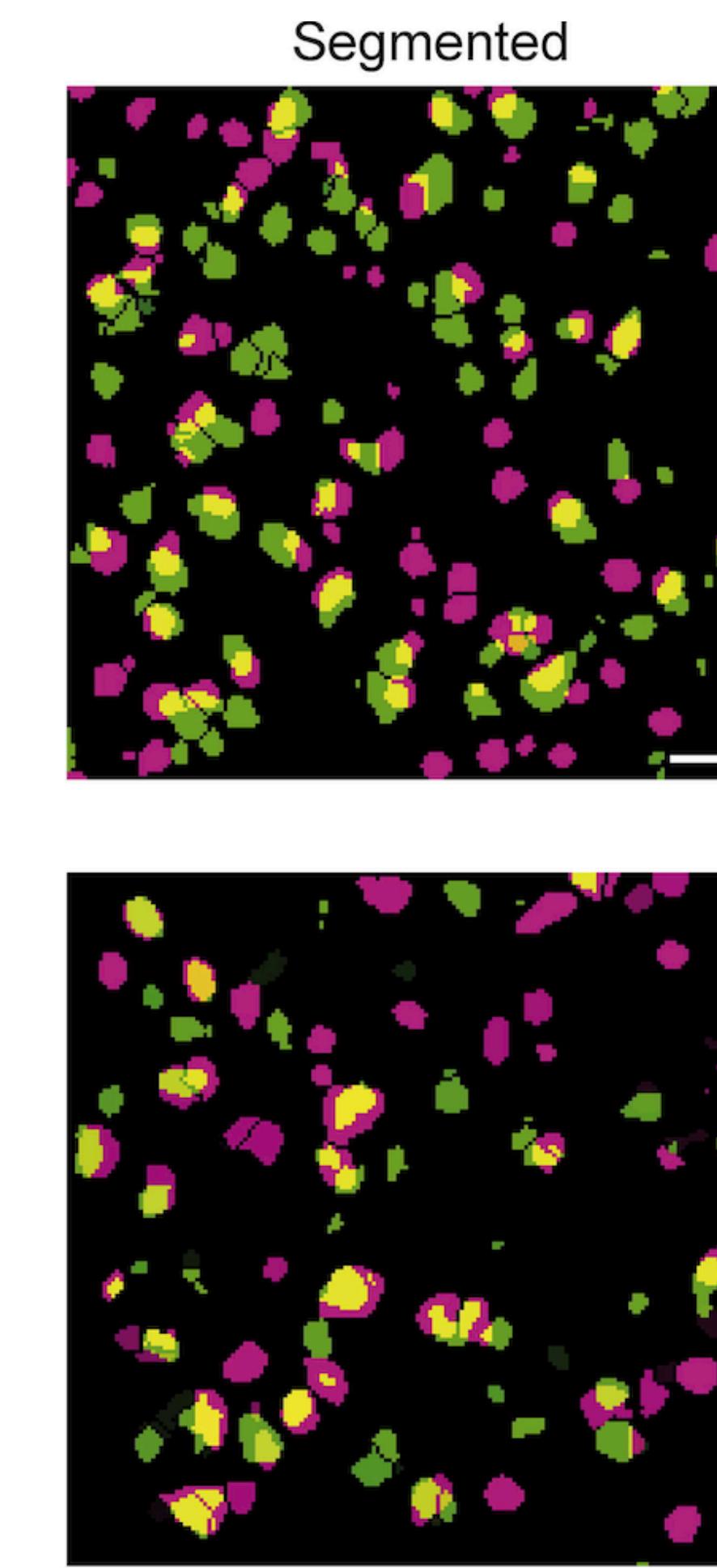
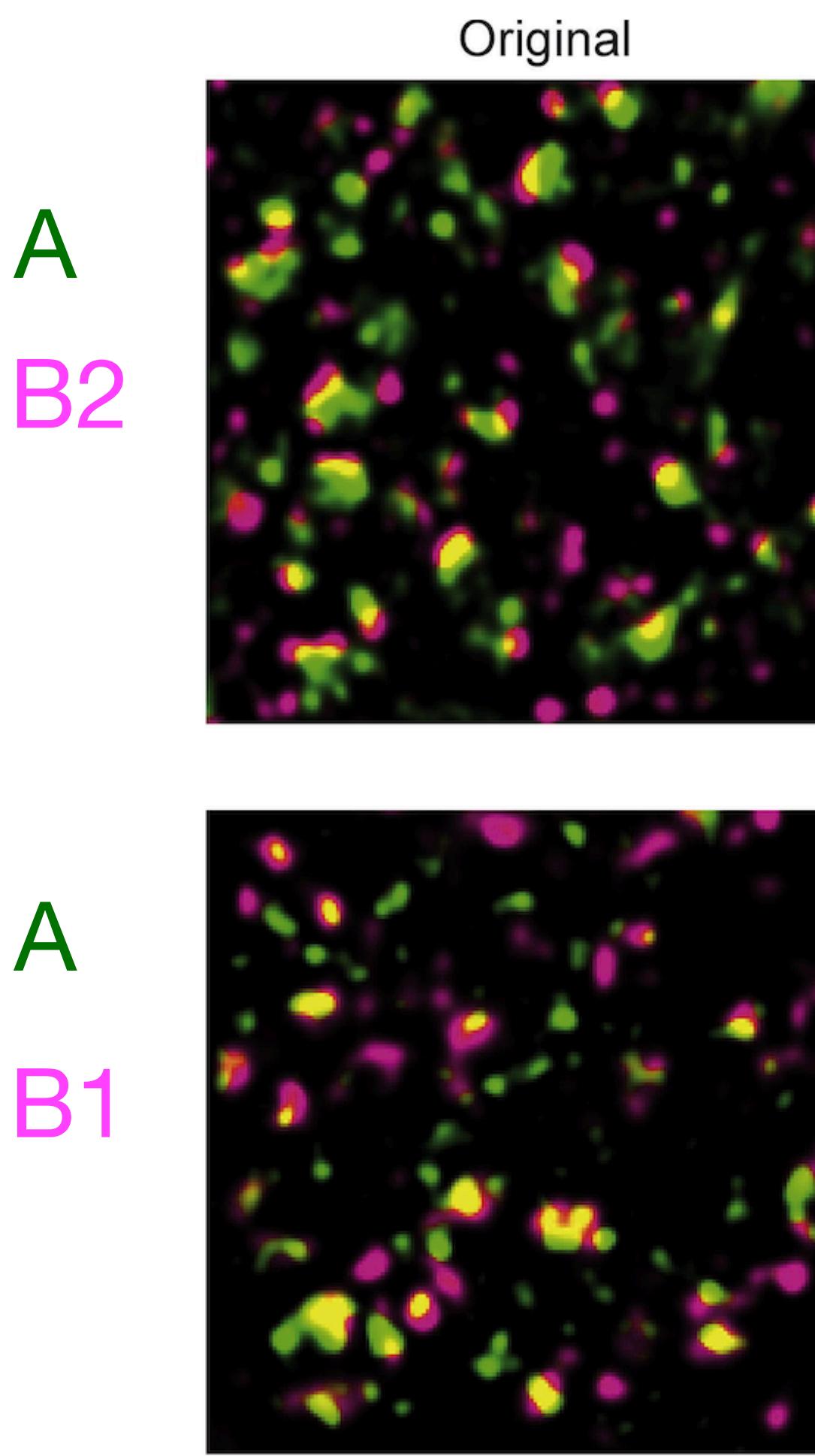
A

B1





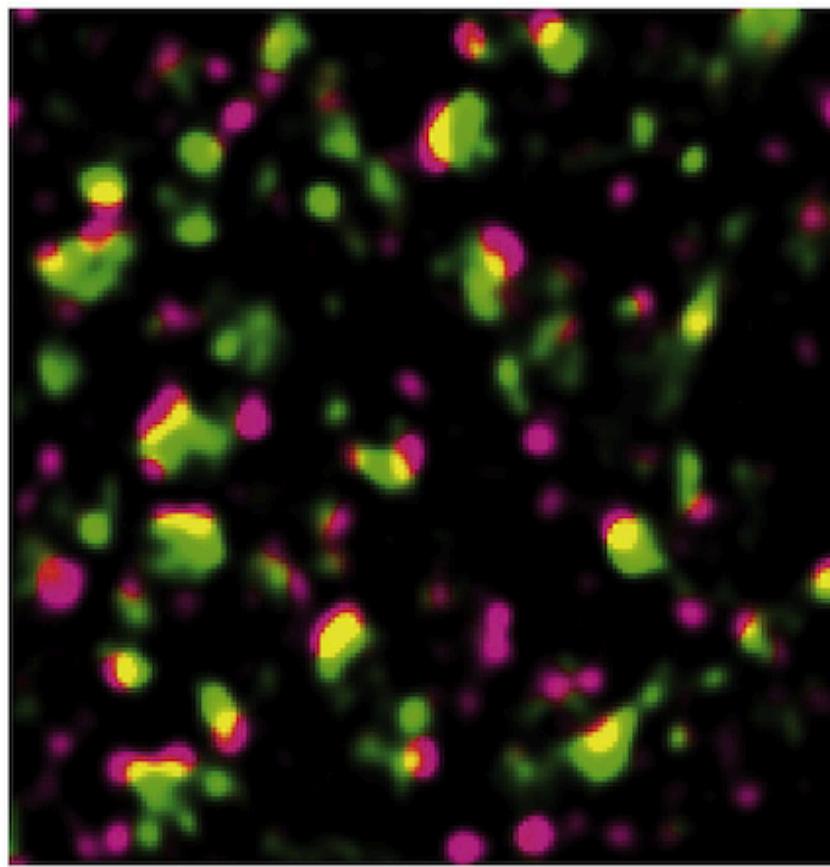
Example: Area



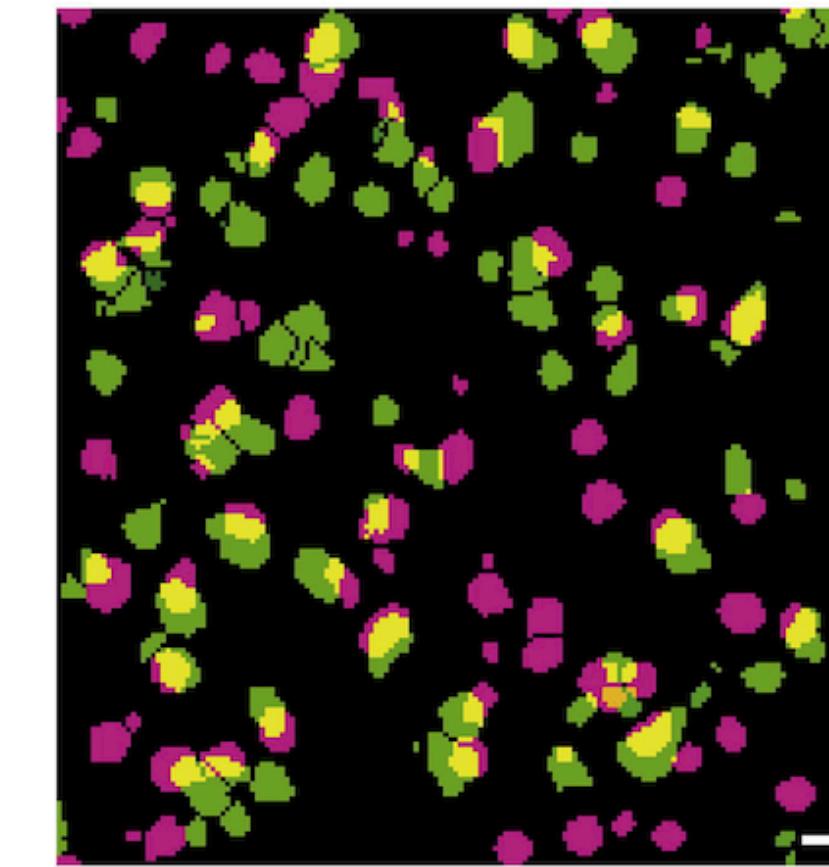


Example: Points

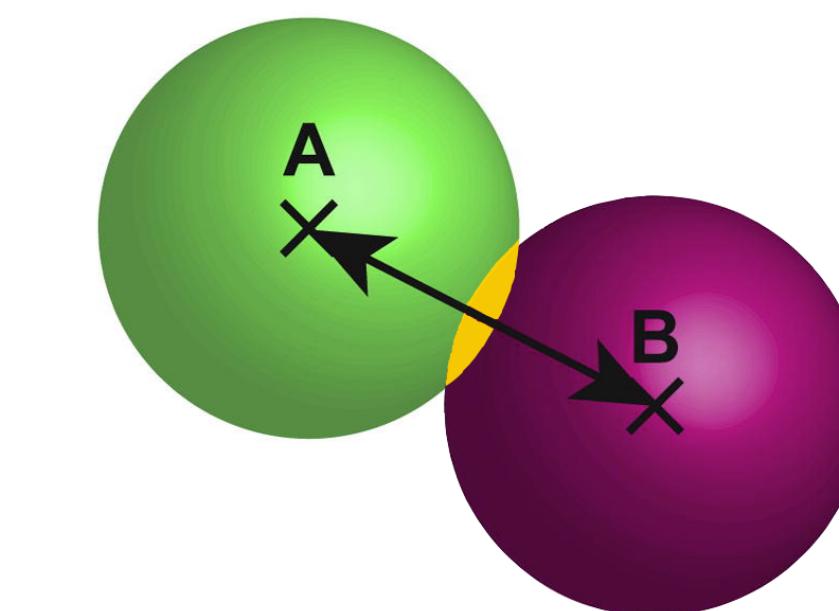
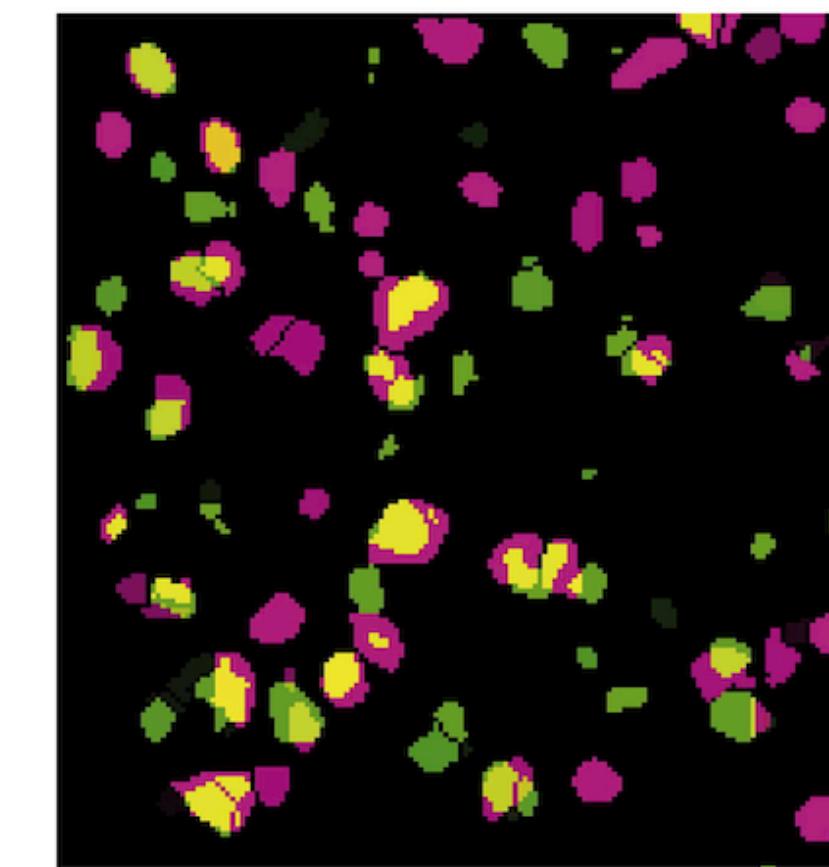
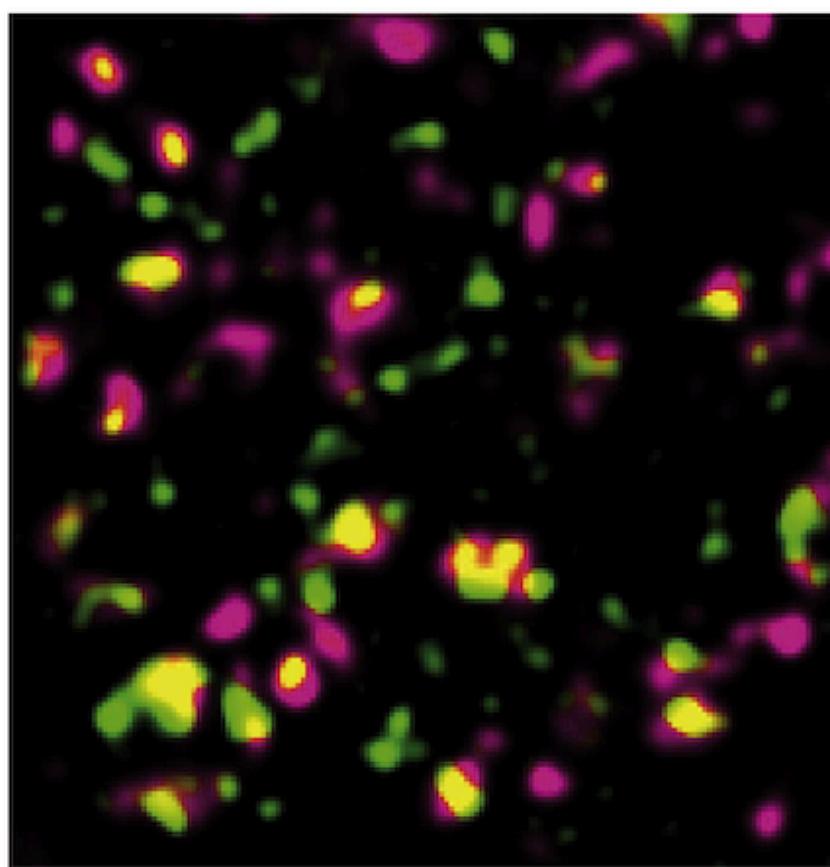
A
B2

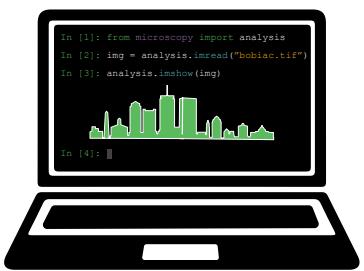


Segmented

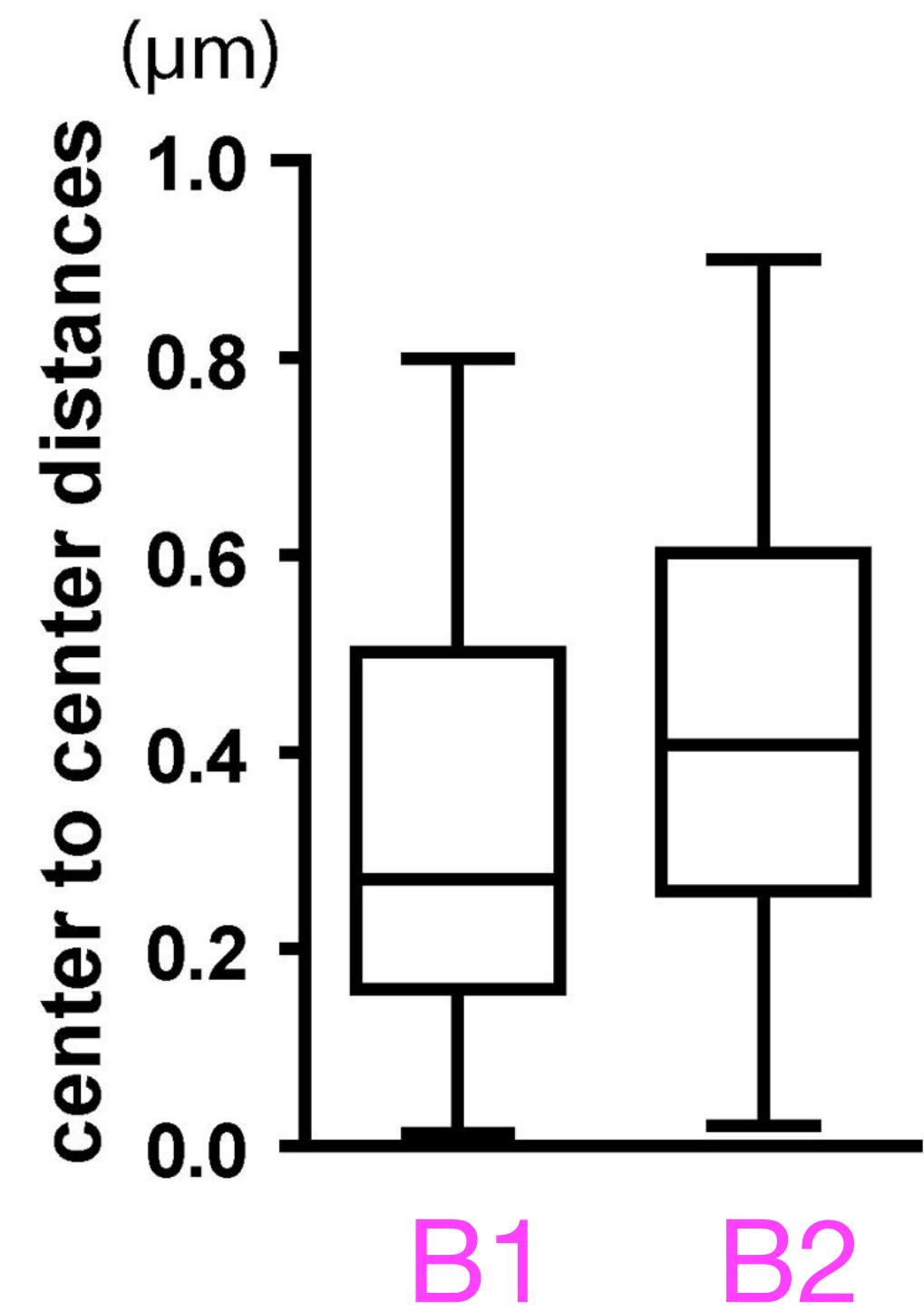
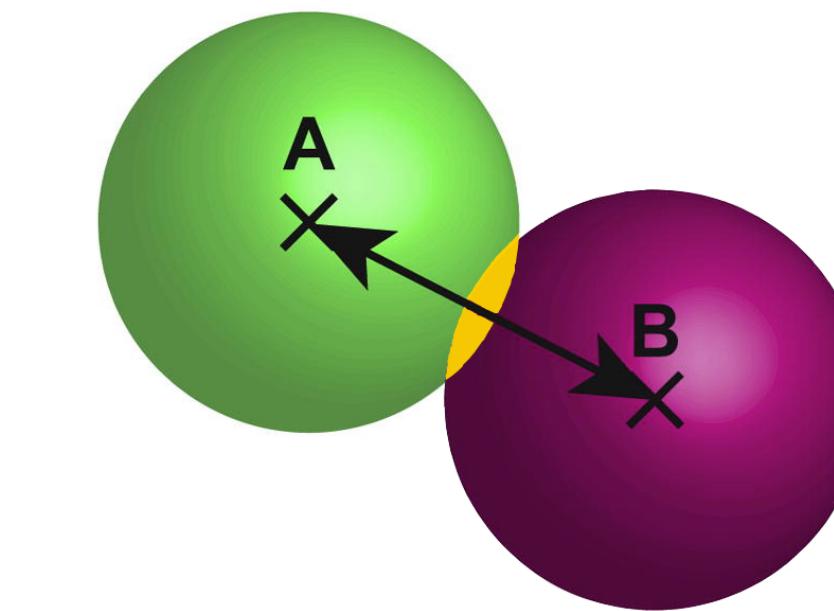
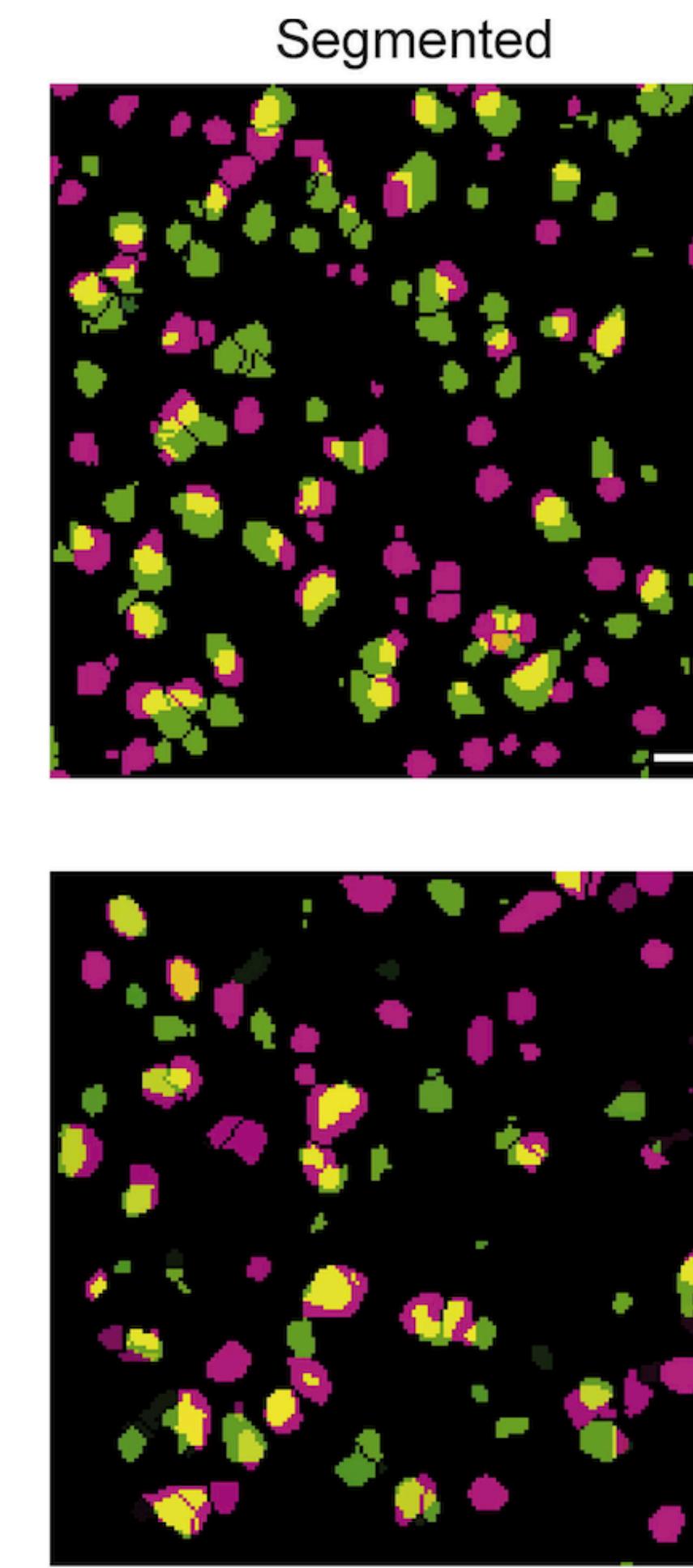
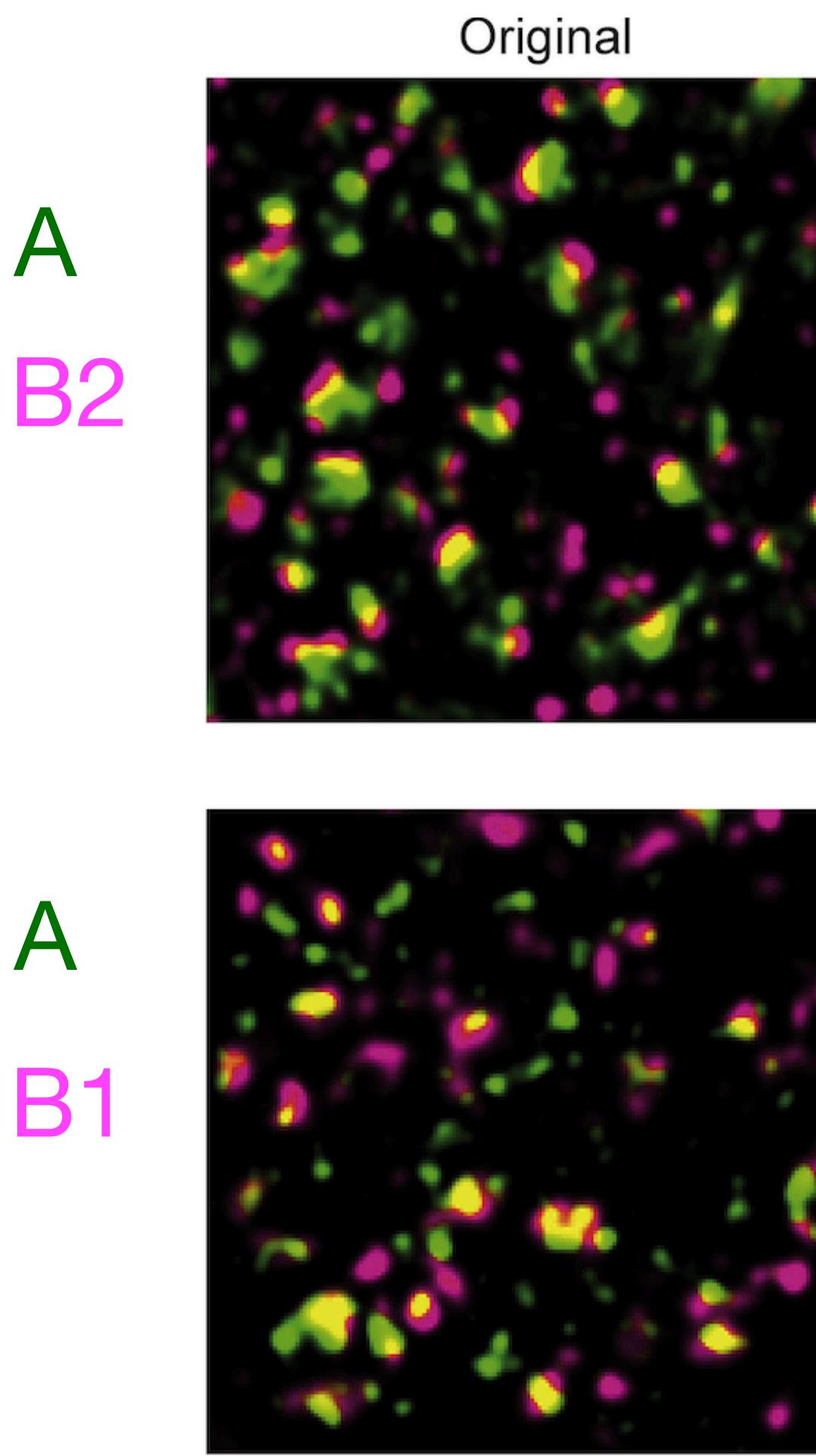


A
B1



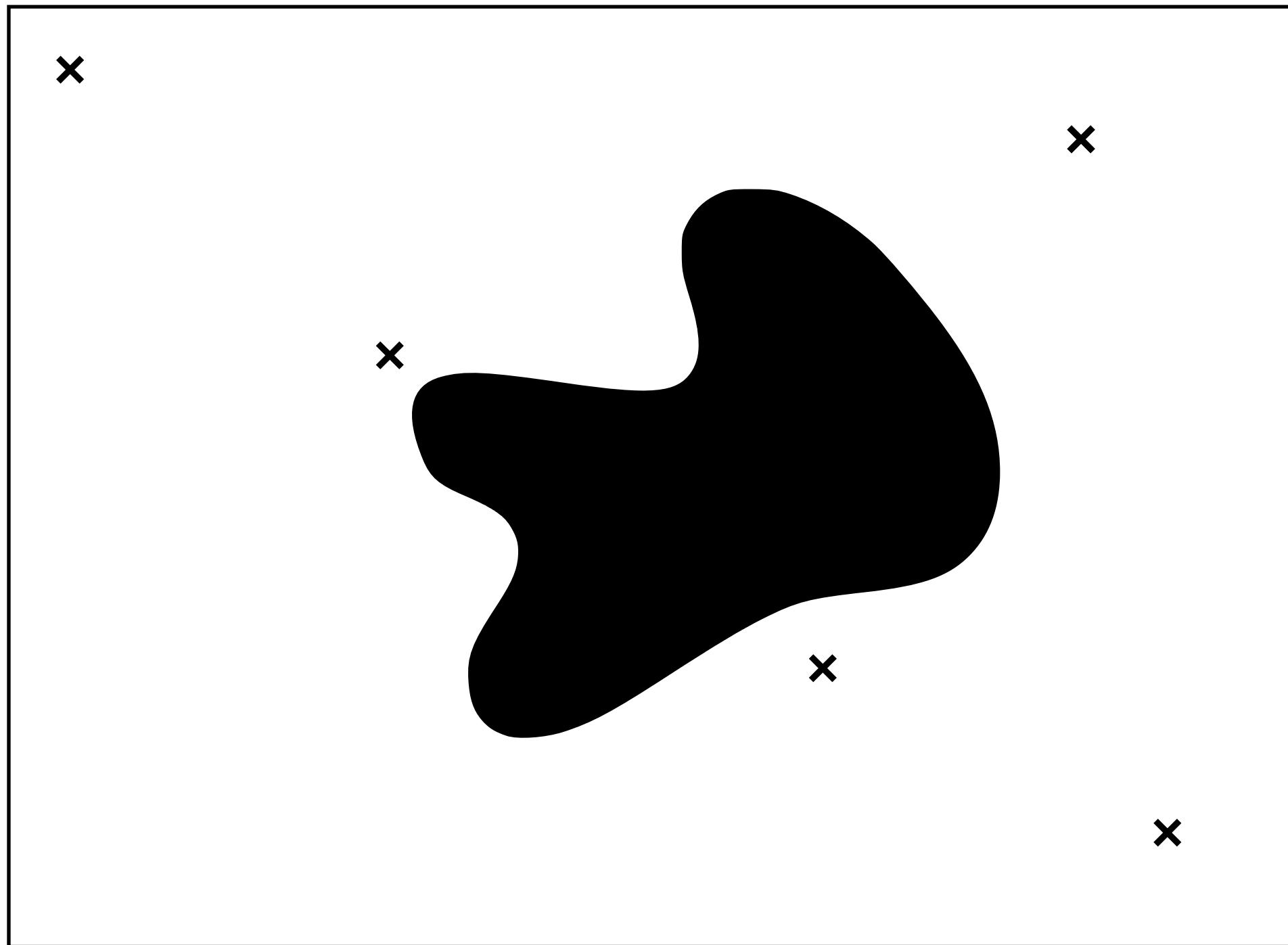


Example: Points



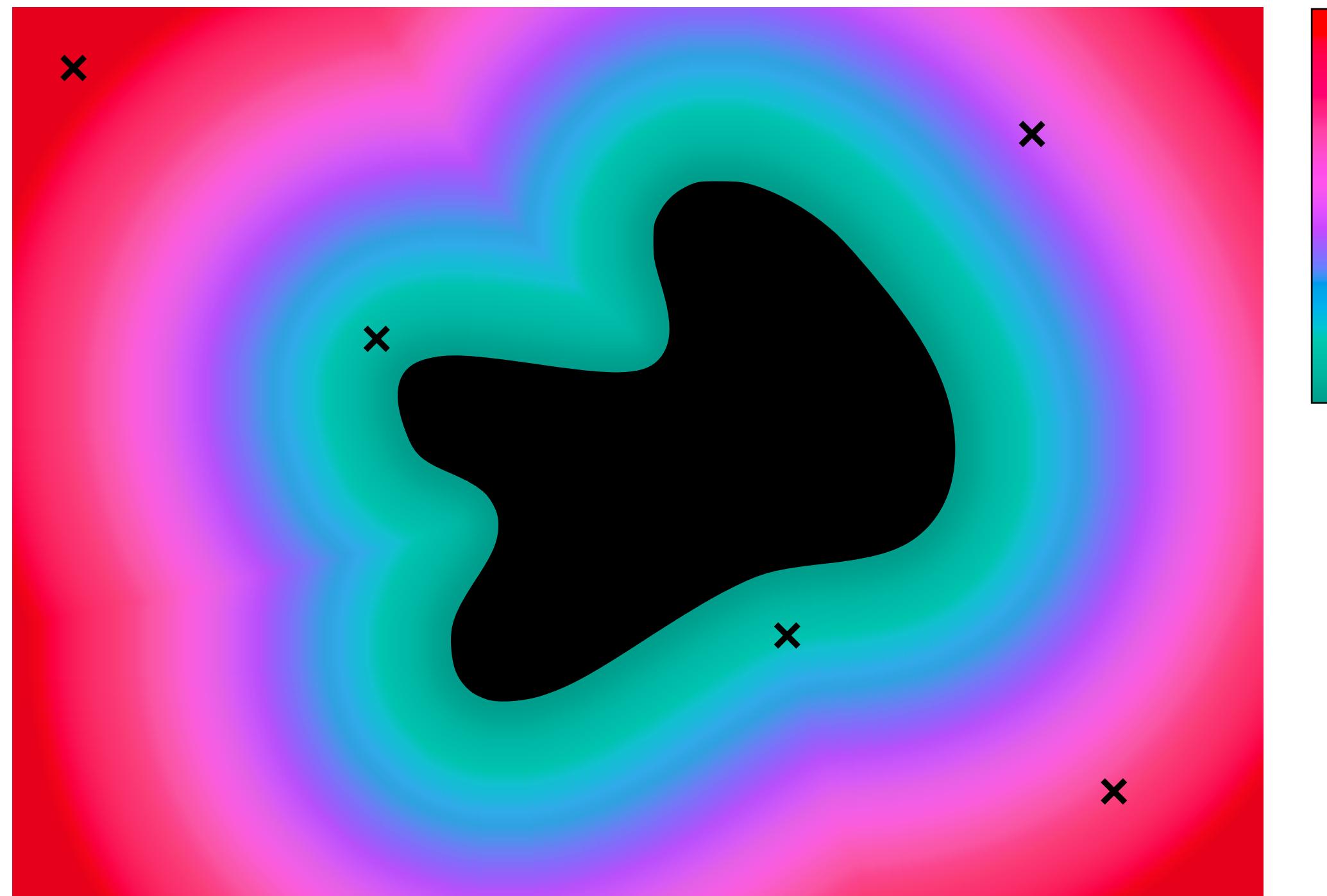


Example: Points to Edges



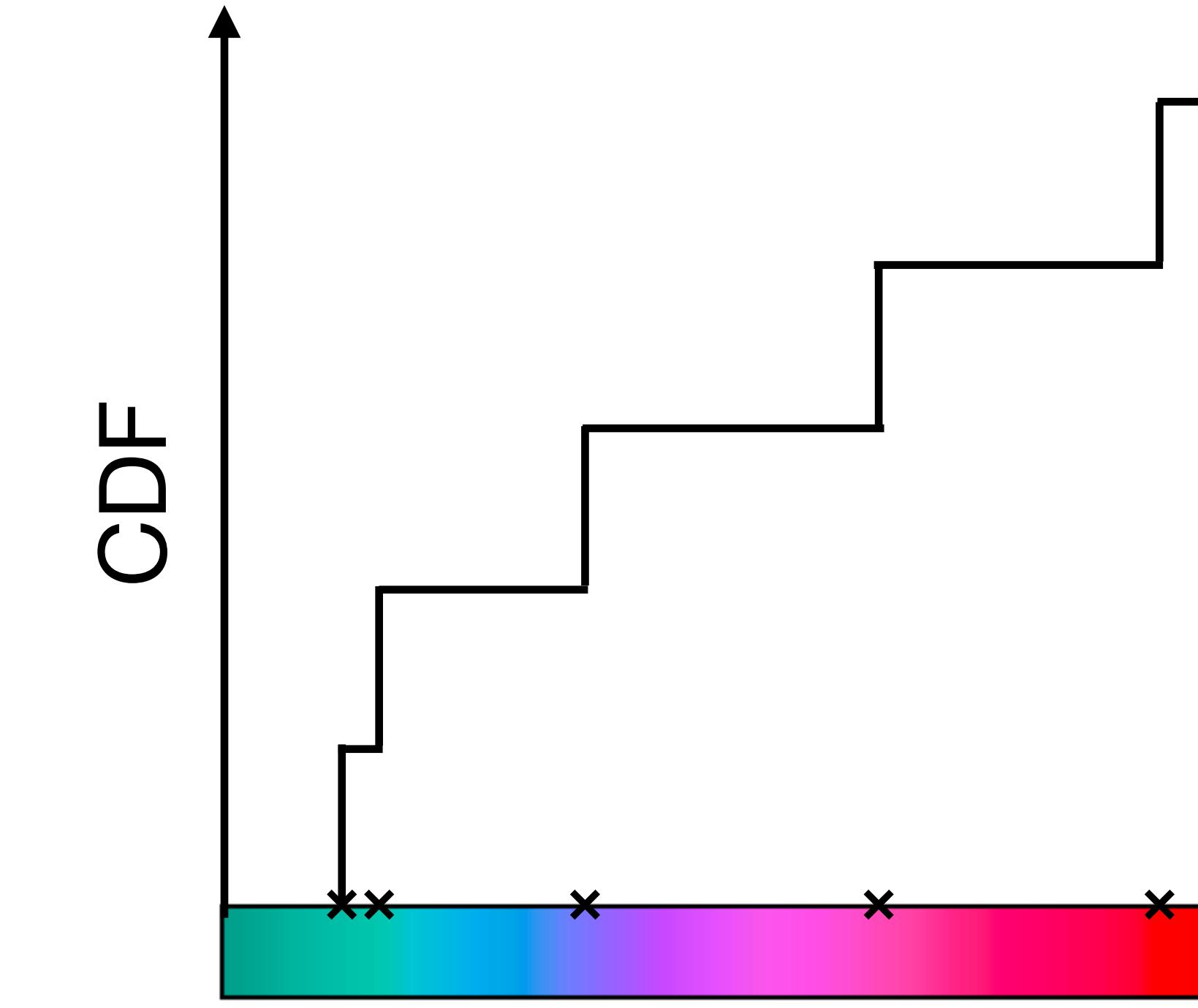
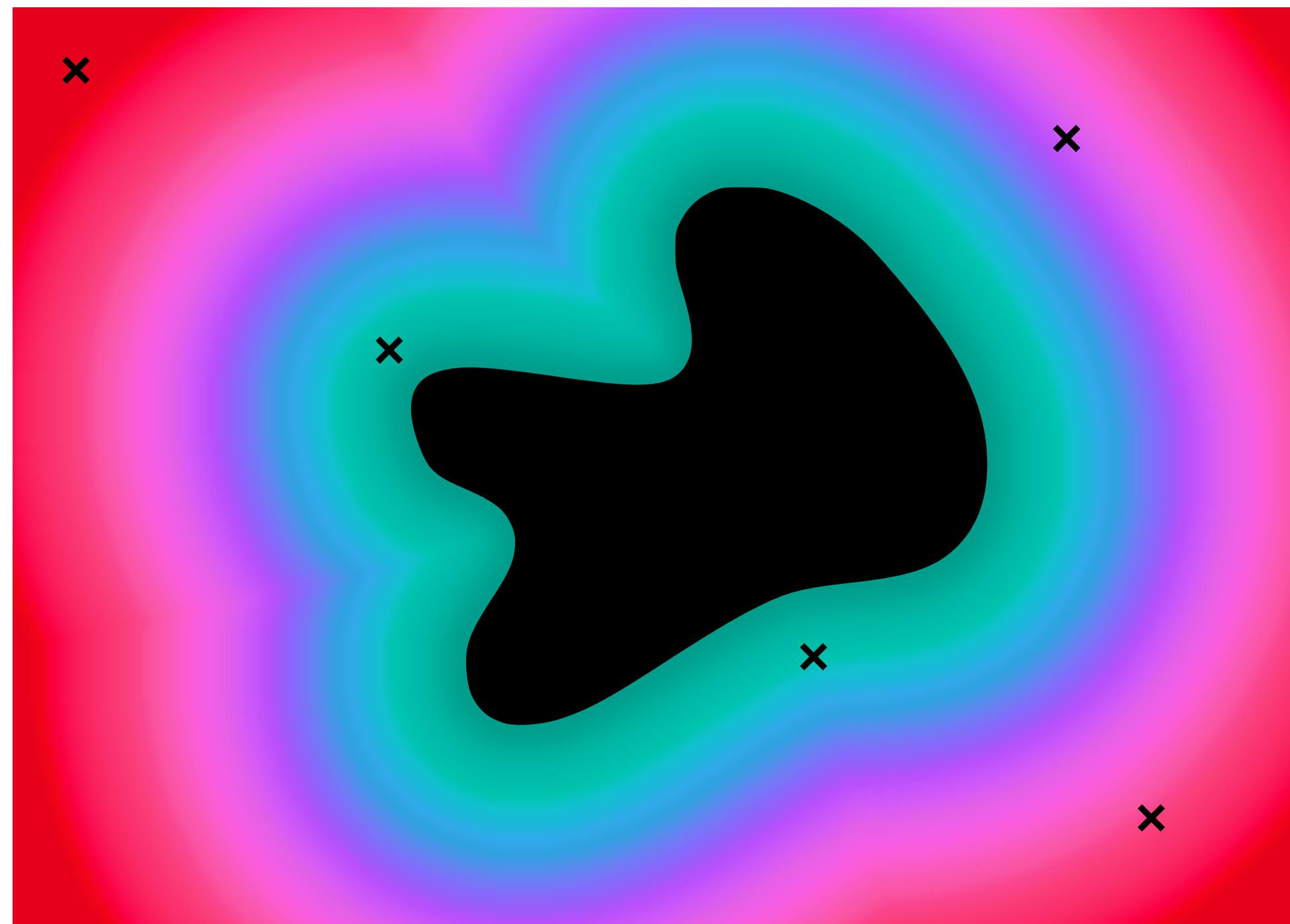


Example: Points to Edges





Example: Points to Edges



Dist to edge

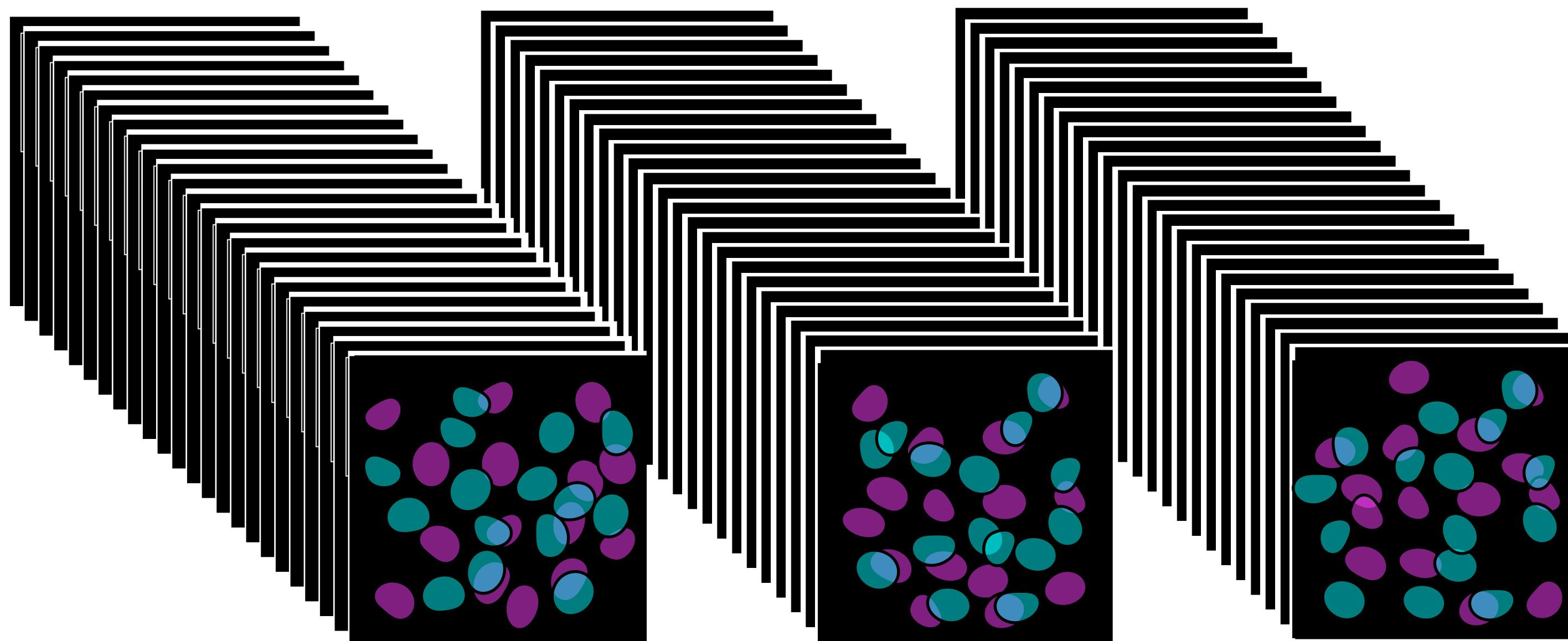


Statistical validation: Comparison to known controls

A large enough sample size allows us to robustly compare samples:

Which hypothesis explains the condition better:

- + CTRL: Hypothesis A
- CTRL: Hypothesis B



Condition

+ CTRL

- CTRL

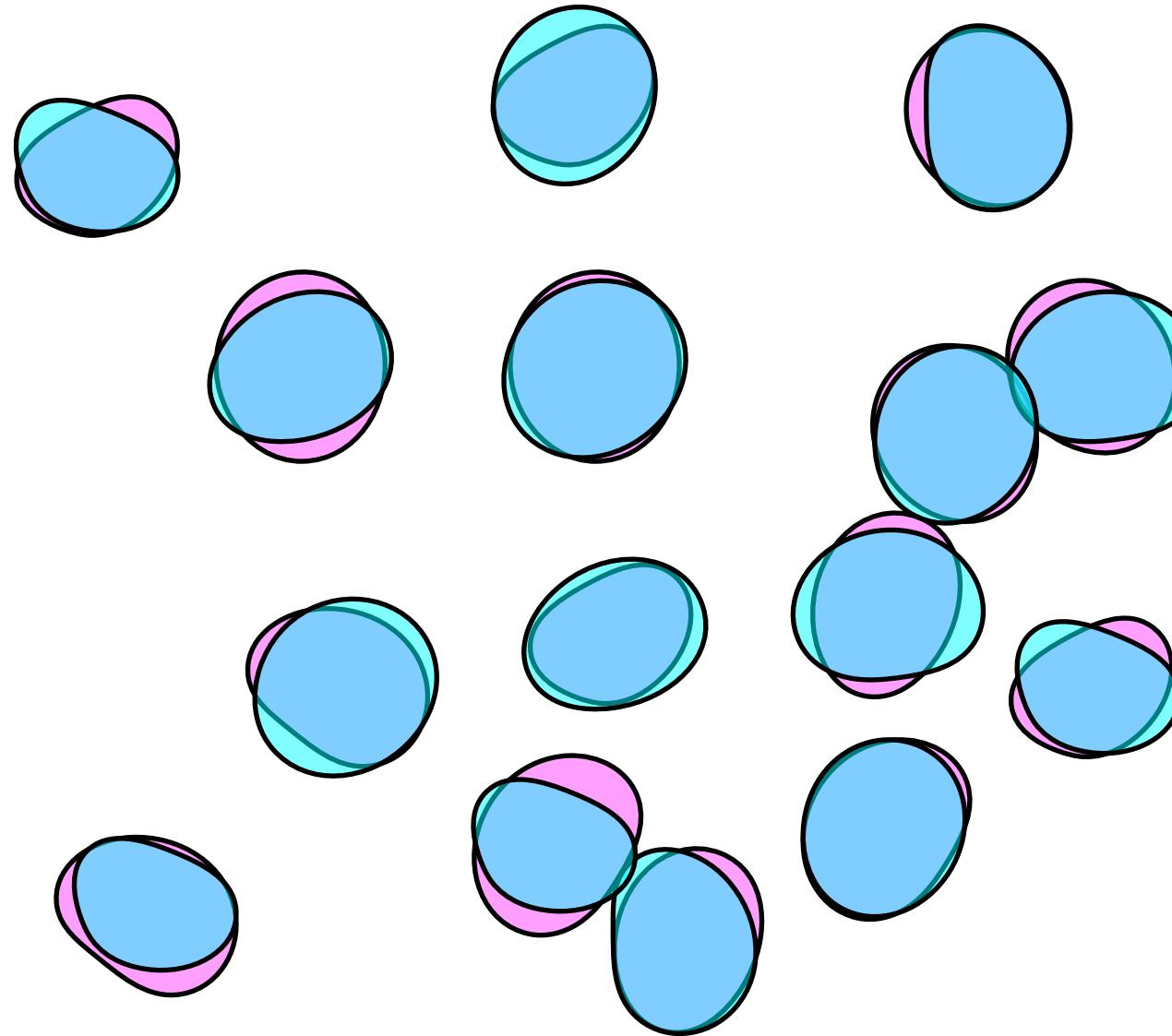


Statistical validation - Comparison to Null

- Question: Is the overlap happening by pure chance?
- Formulate H0: Any overlap we're seeing is a coincidence
- Use -CTRL, where overlap is a coincidence
- And/OR: Simulate what H0 would look like



Statistical validation

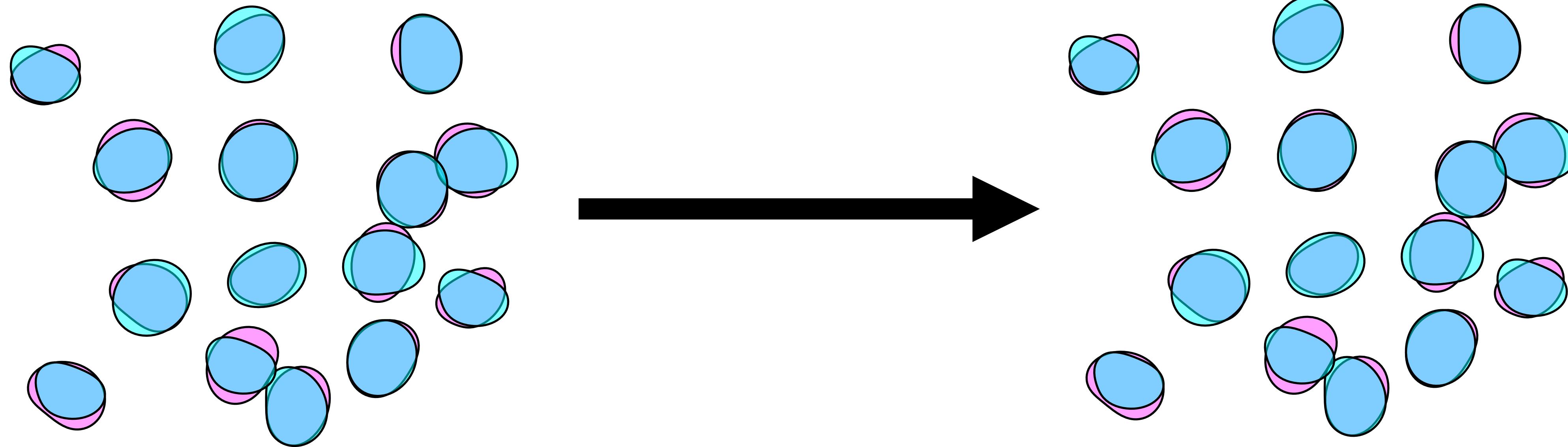


Q: Is the overlap between **cyan** and **magenta** pure chance?

H0: Any overlap between **cyan** and **magenta** we're seeing is a coincidence!



Statistical validation



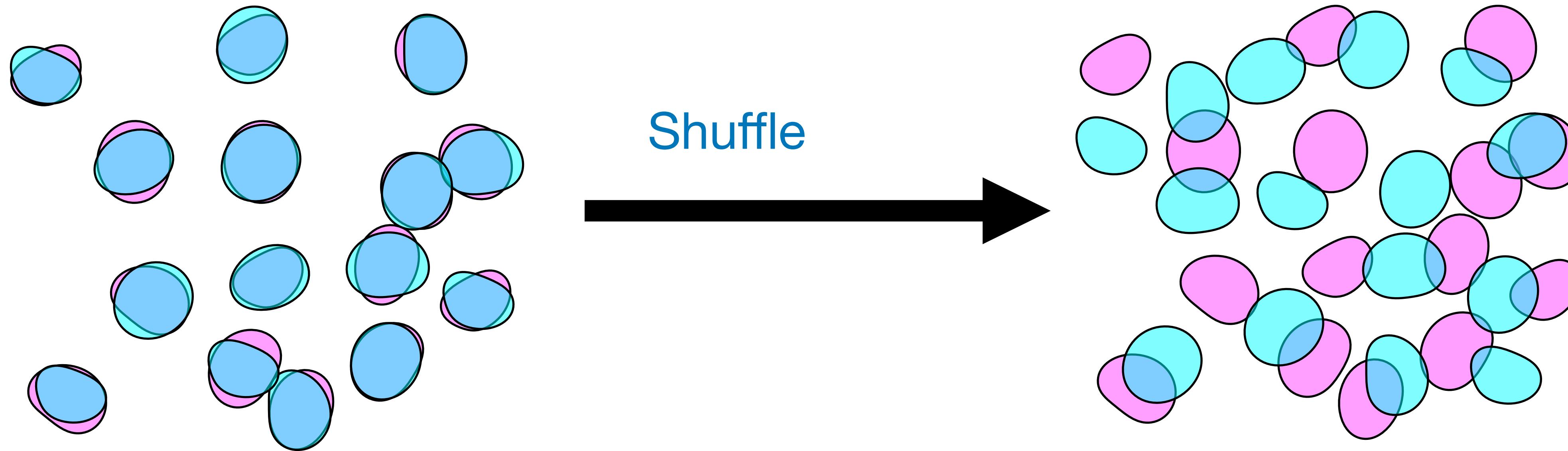
Q: Is the overlap between **cyan** and **magenta** pure chance?

H0: Any overlap between **cyan** and **magenta** we're seeing is a coincidence!

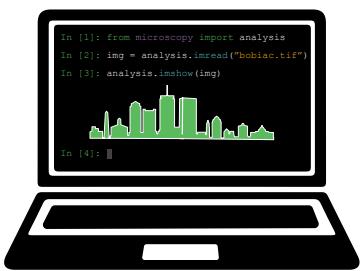
> Simulate what H0 would look like



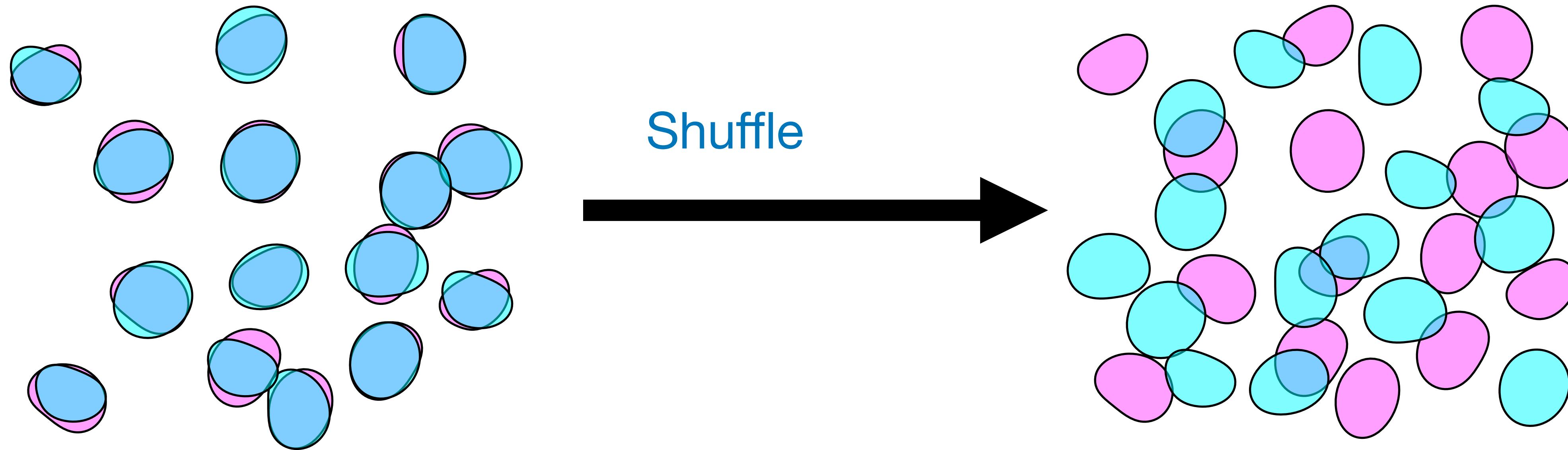
Statistical validation



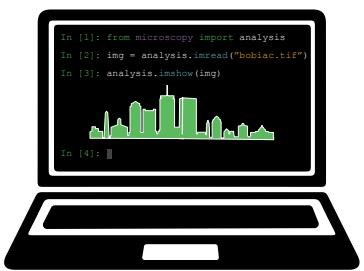
Randomly shuffle blue objects to simulate H0



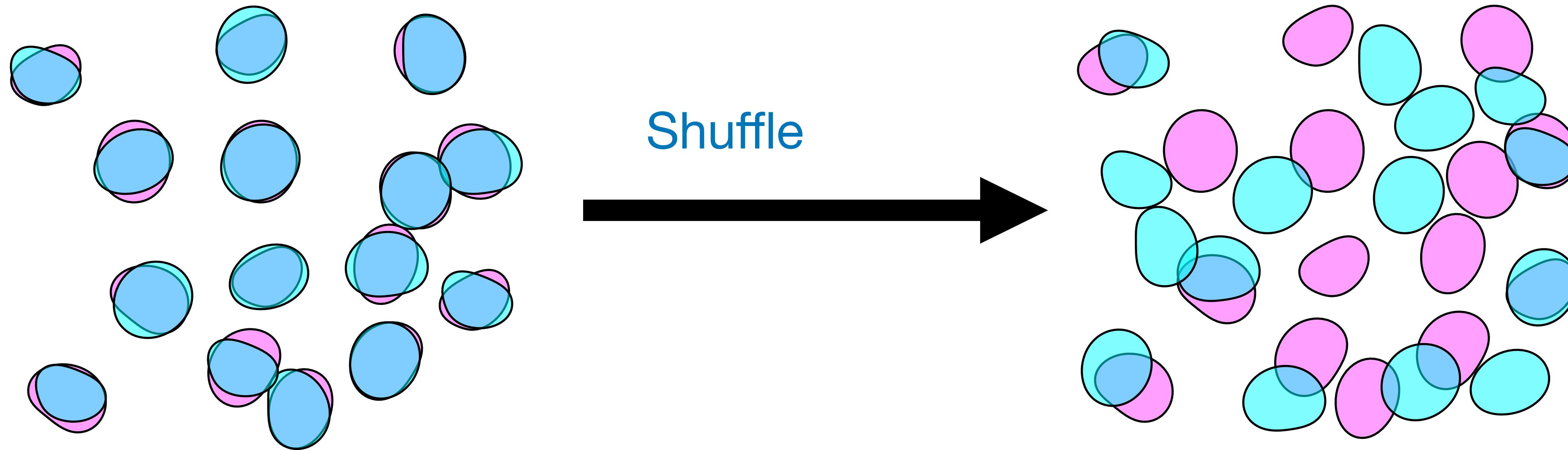
Statistical validation



Randomly shuffle blue objects to simulate H0



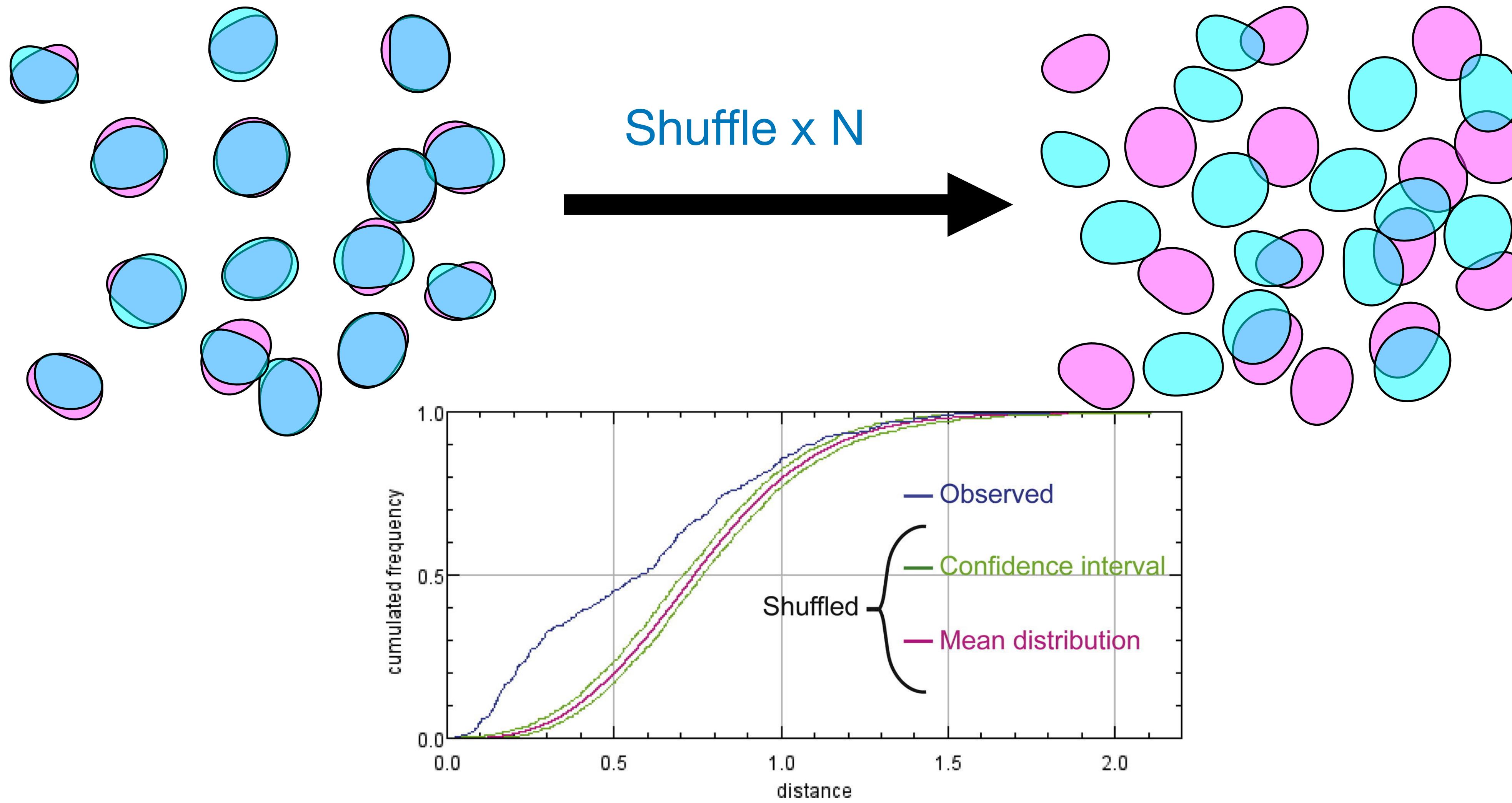
Statistical validation



Randomly shuffle blue objects to simulate H0



Statistical validation





Statistical validation

