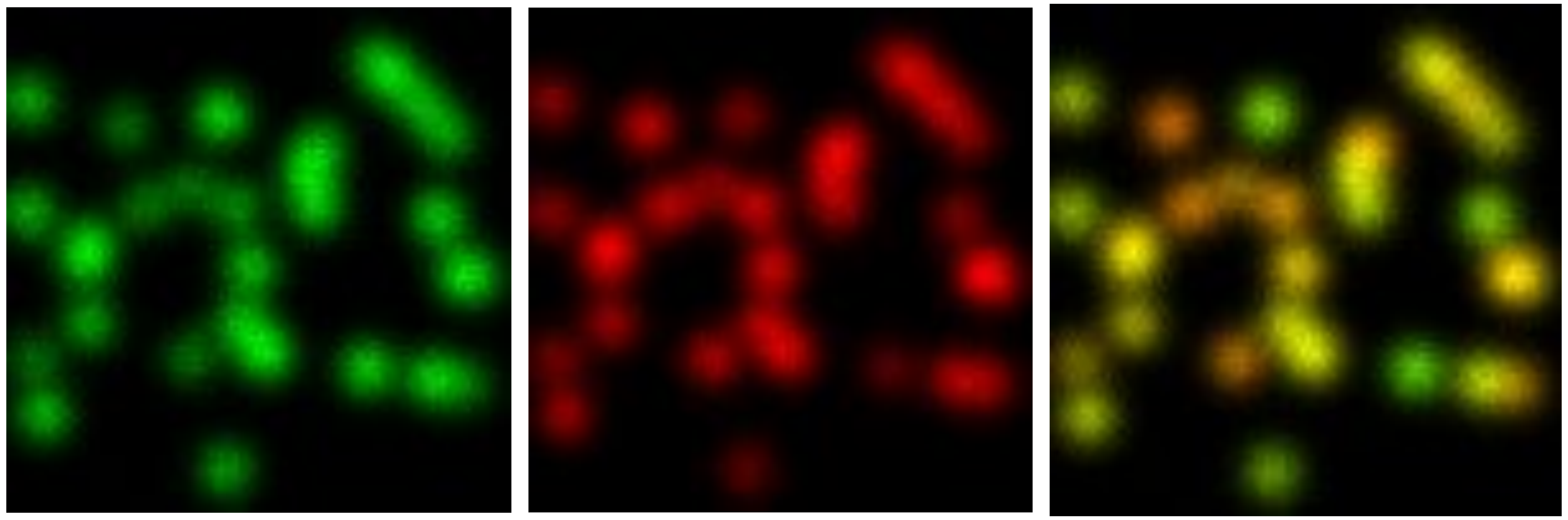
BoBiAC

Boston Bioimage Analysis Course | 2025

Introduction to Colocalizationin Fluorescence Microscopy

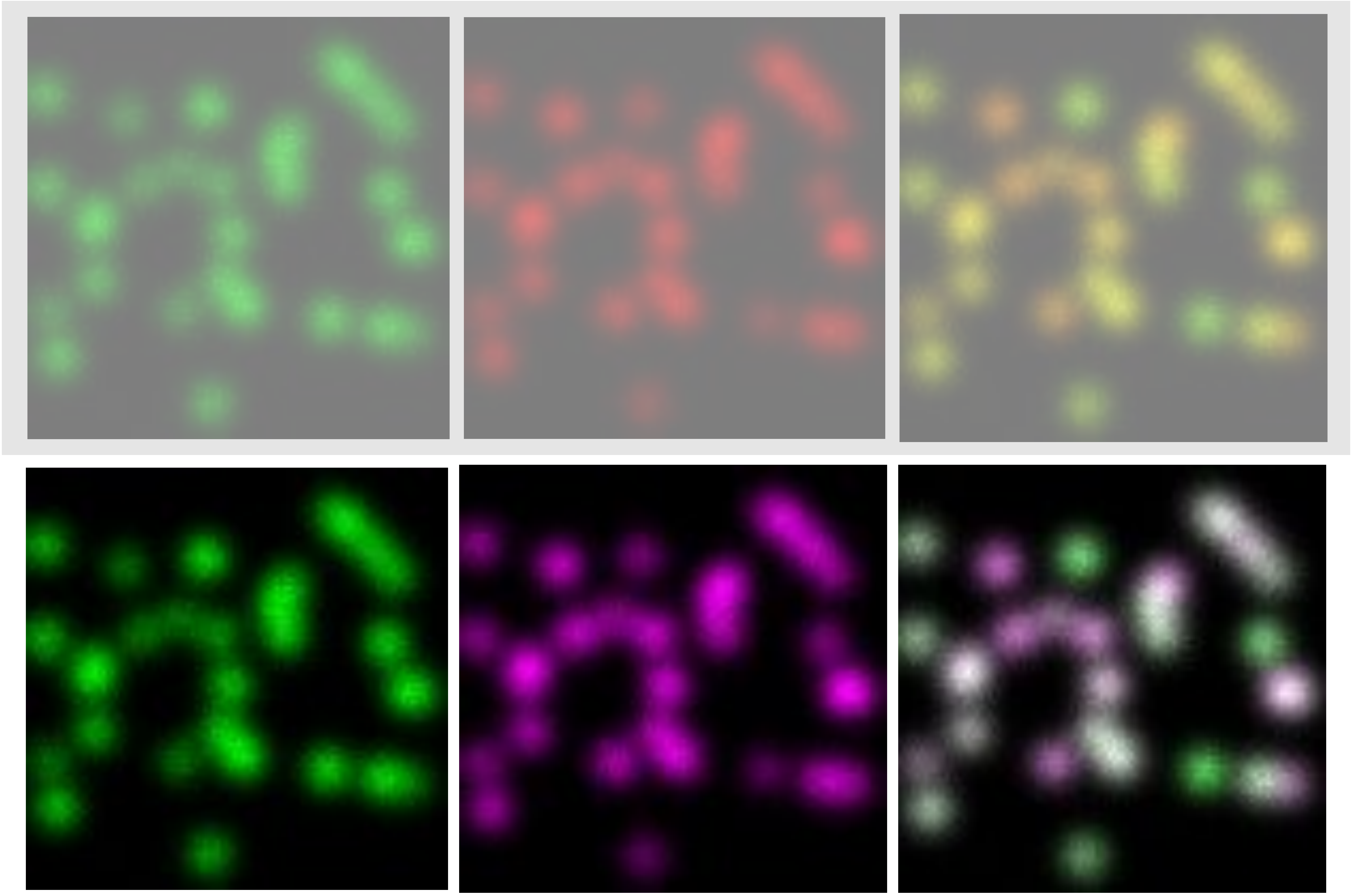
What Colocalization IS NOT in Fluorescence Microscopy



"Yellow" is **not** colocalization

Why?

What Colocalization IS NOT in Fluorescence Microscopy



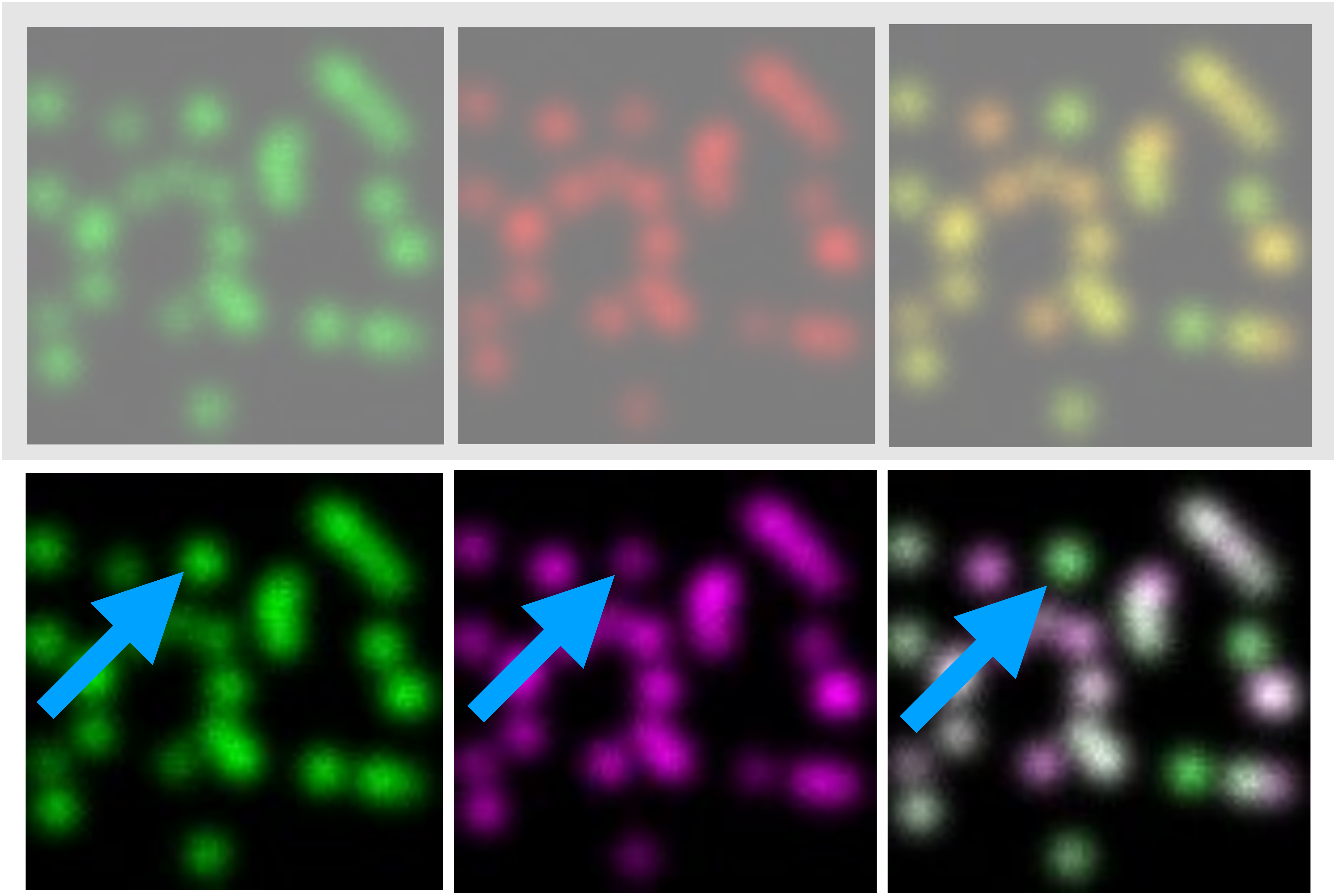
"Yellow" is **not** colocalization

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?

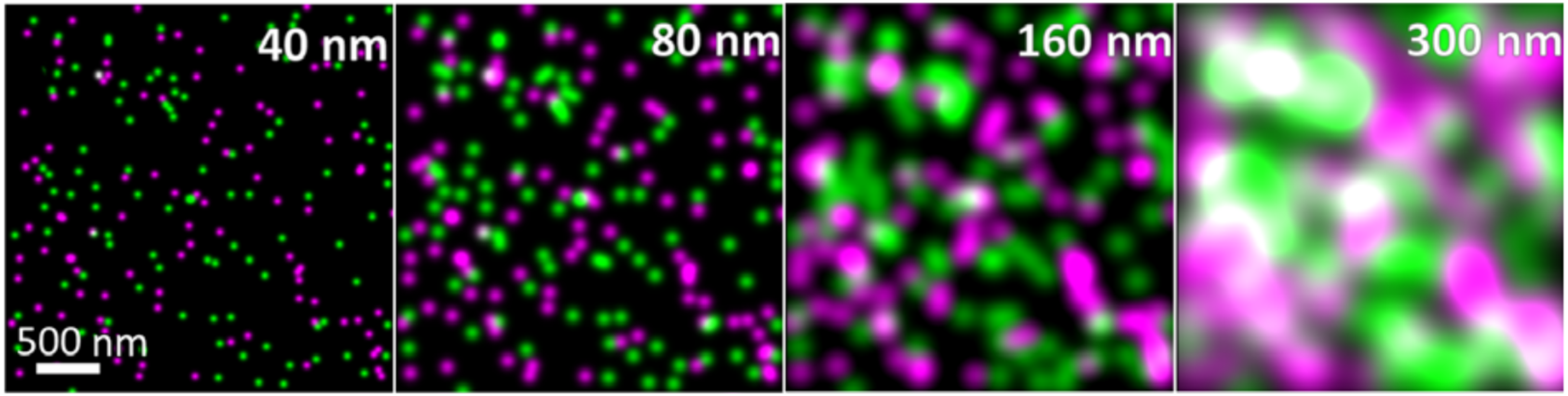
1. you should never see yellow because you should **not use red and green** together.
2. You can visualize overlap only if the signal is high in both channels.
3. How to quantify?

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

What Colocalization IS NOT in Fluorescence Microscopy

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

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



Adapted from Xu et al, 2016

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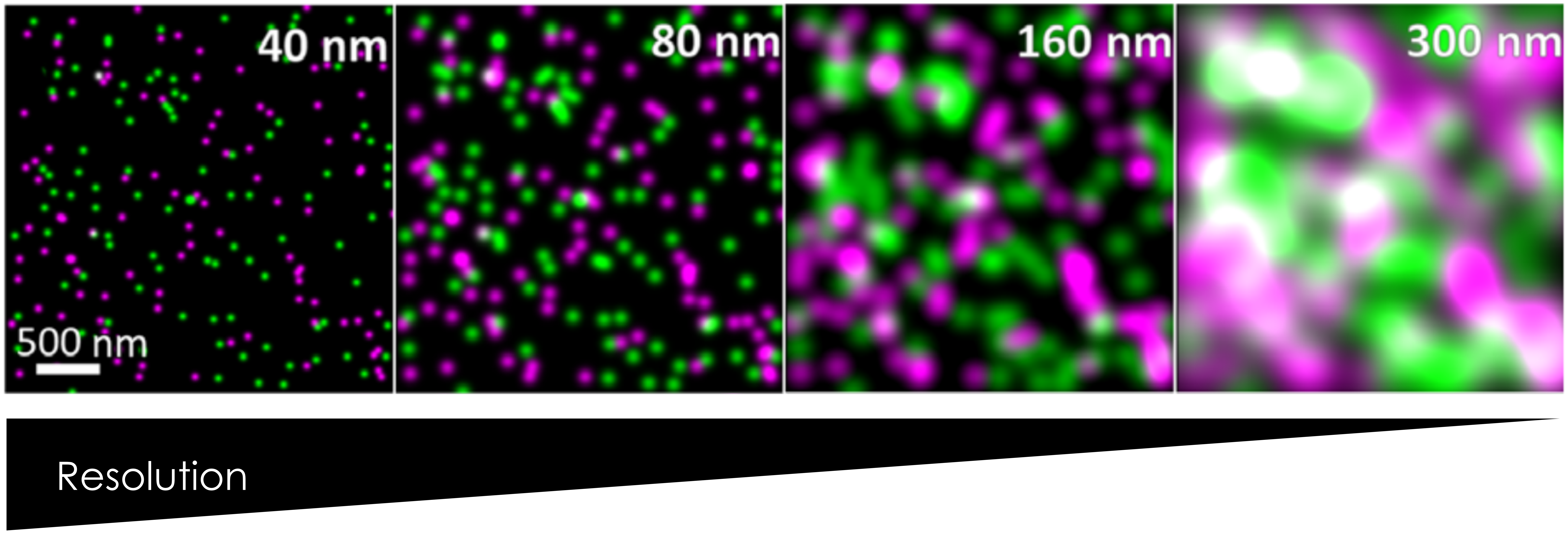
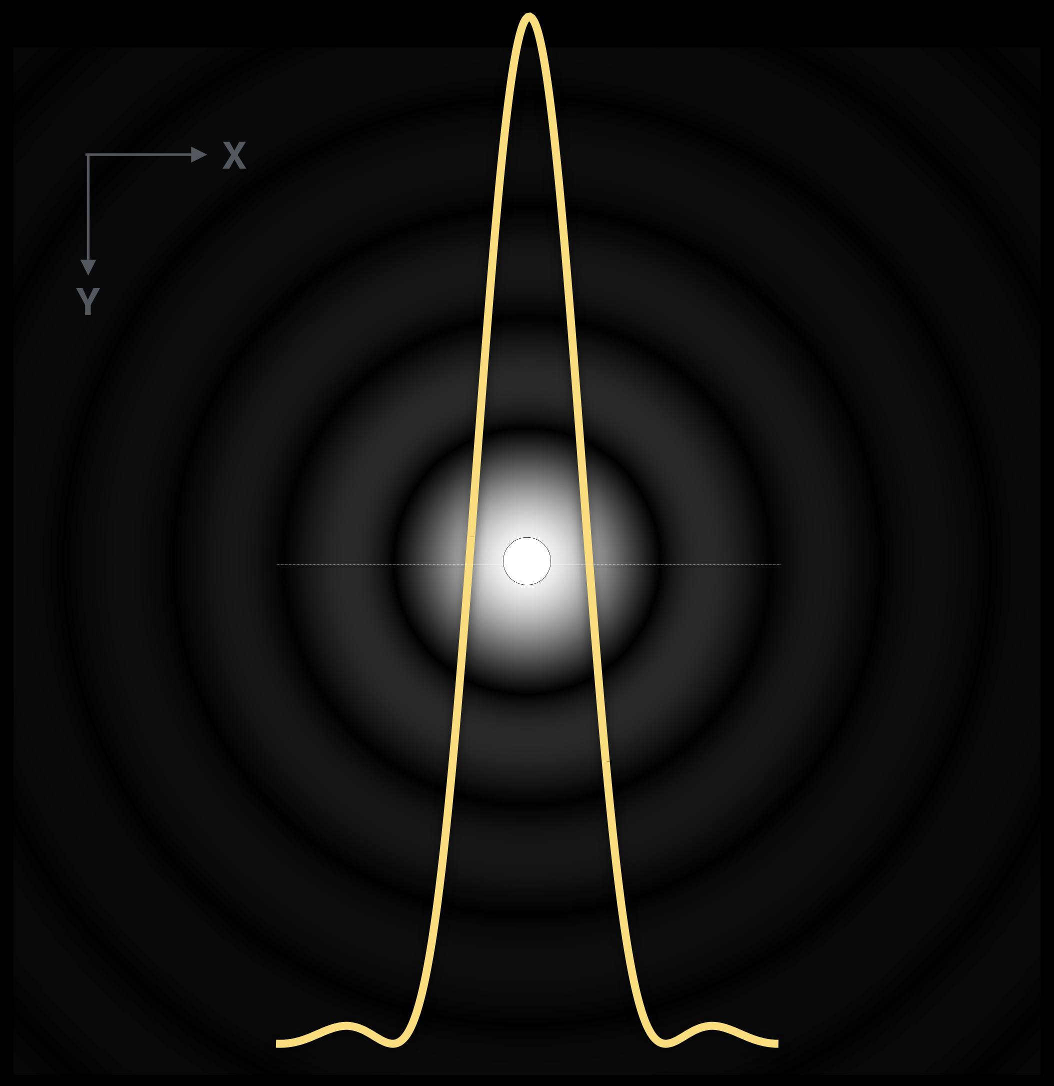


Adapted from Xu et al, 2016

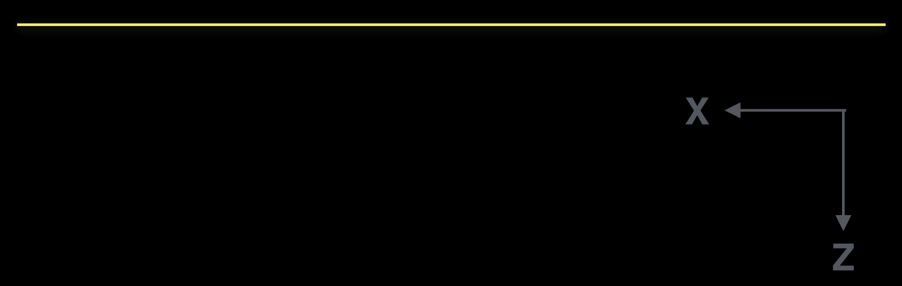
resolution: the ability to distinguish objects that are separate in the sample as separate from one another in the image of the sample

Adapted form Jennifer Waters

The Point Spread Function (PSF)



lateral



axial

Adapted form Jennifer Waters

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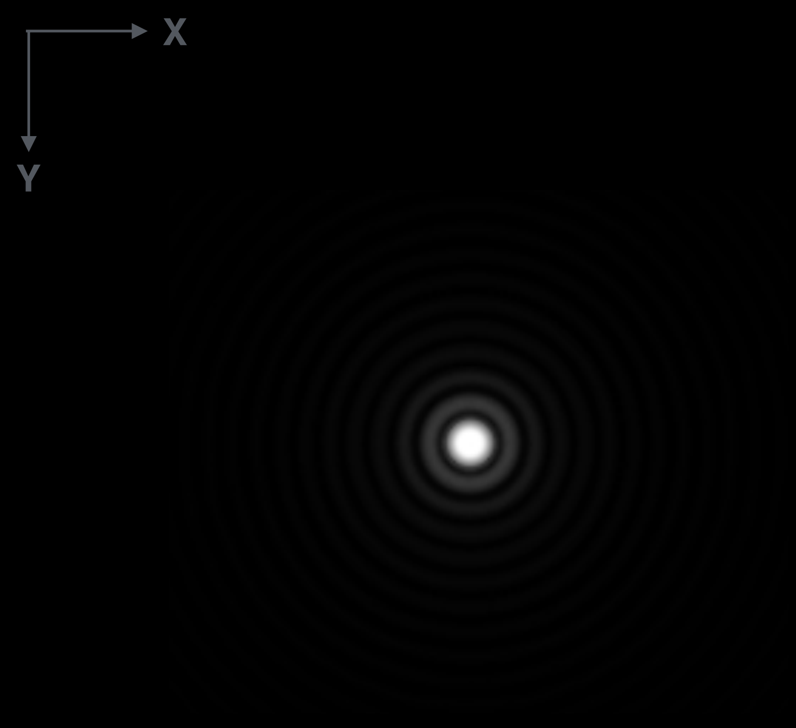
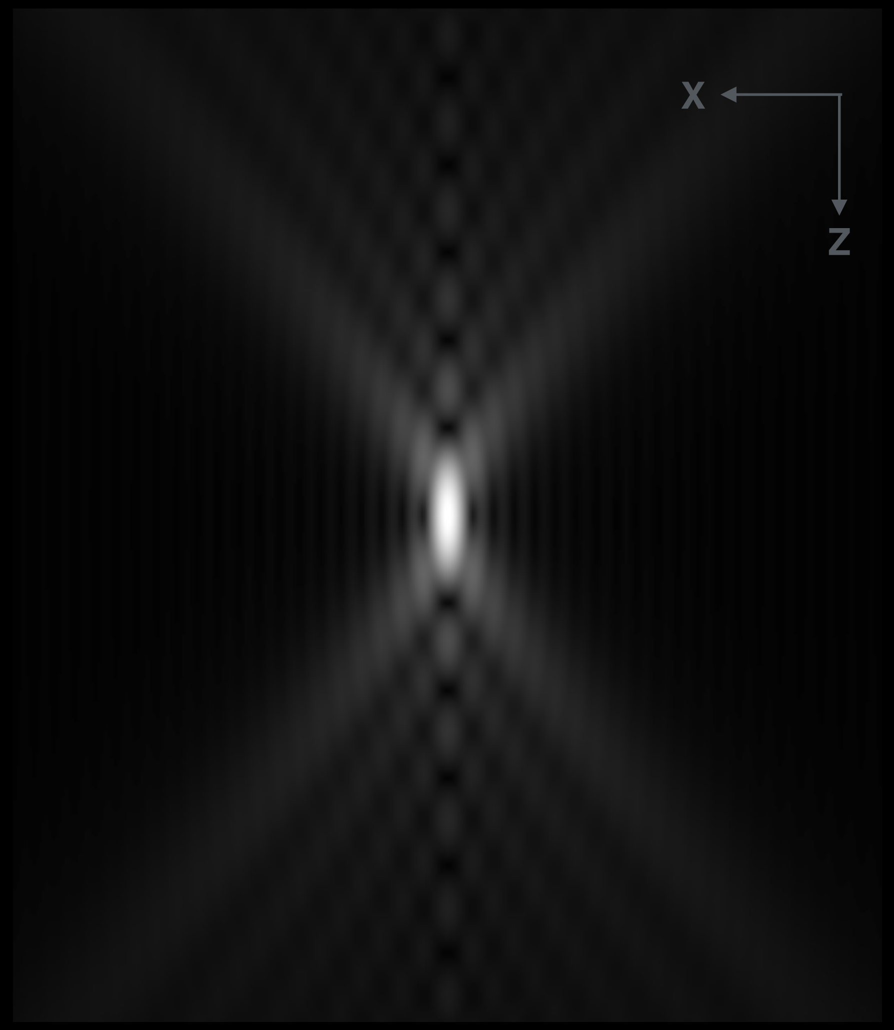


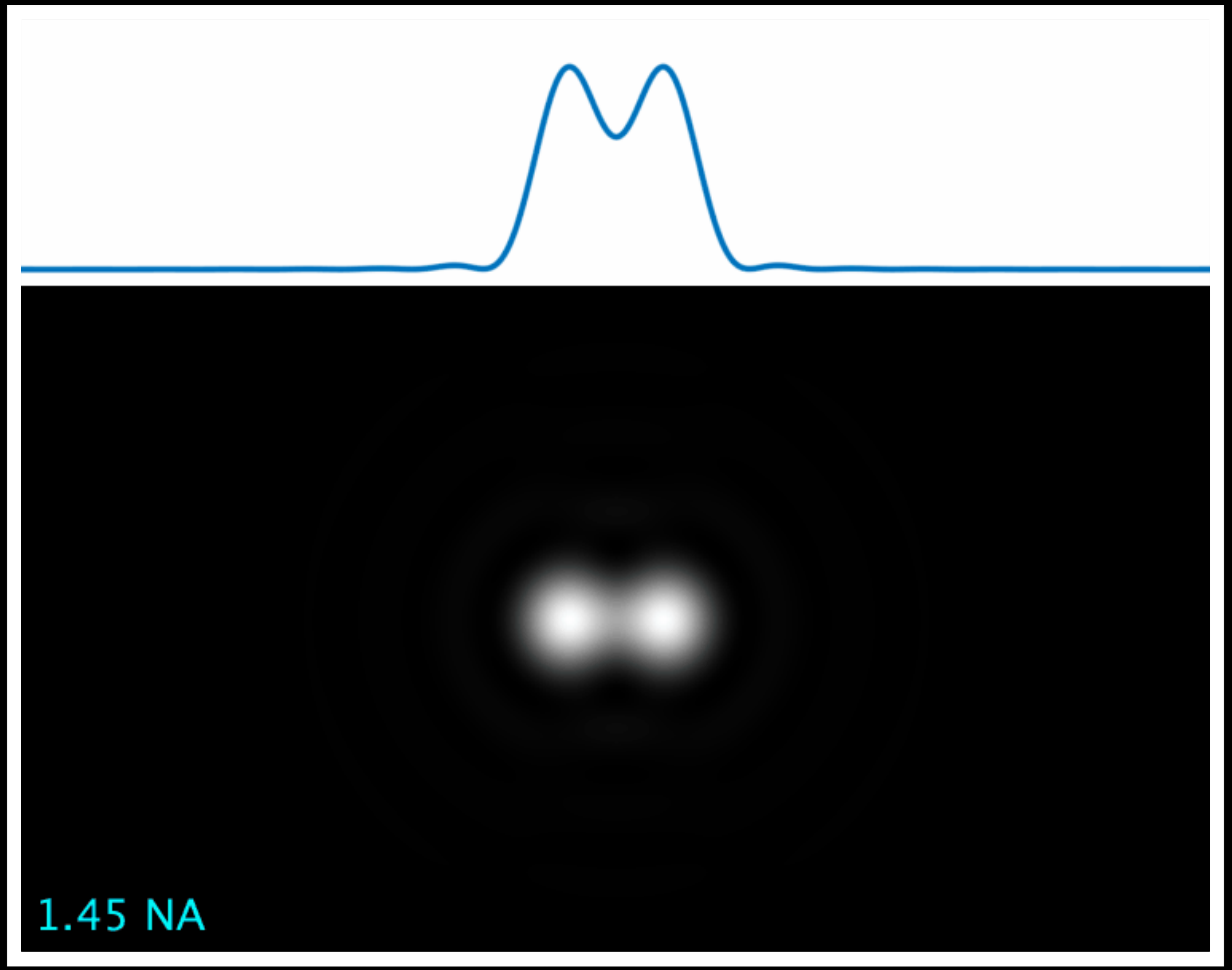
lateral



axial

Adapted form Jennifer Waters

Resolution is limited by the size of the PSF



Adapted form Jennifer Waters

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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

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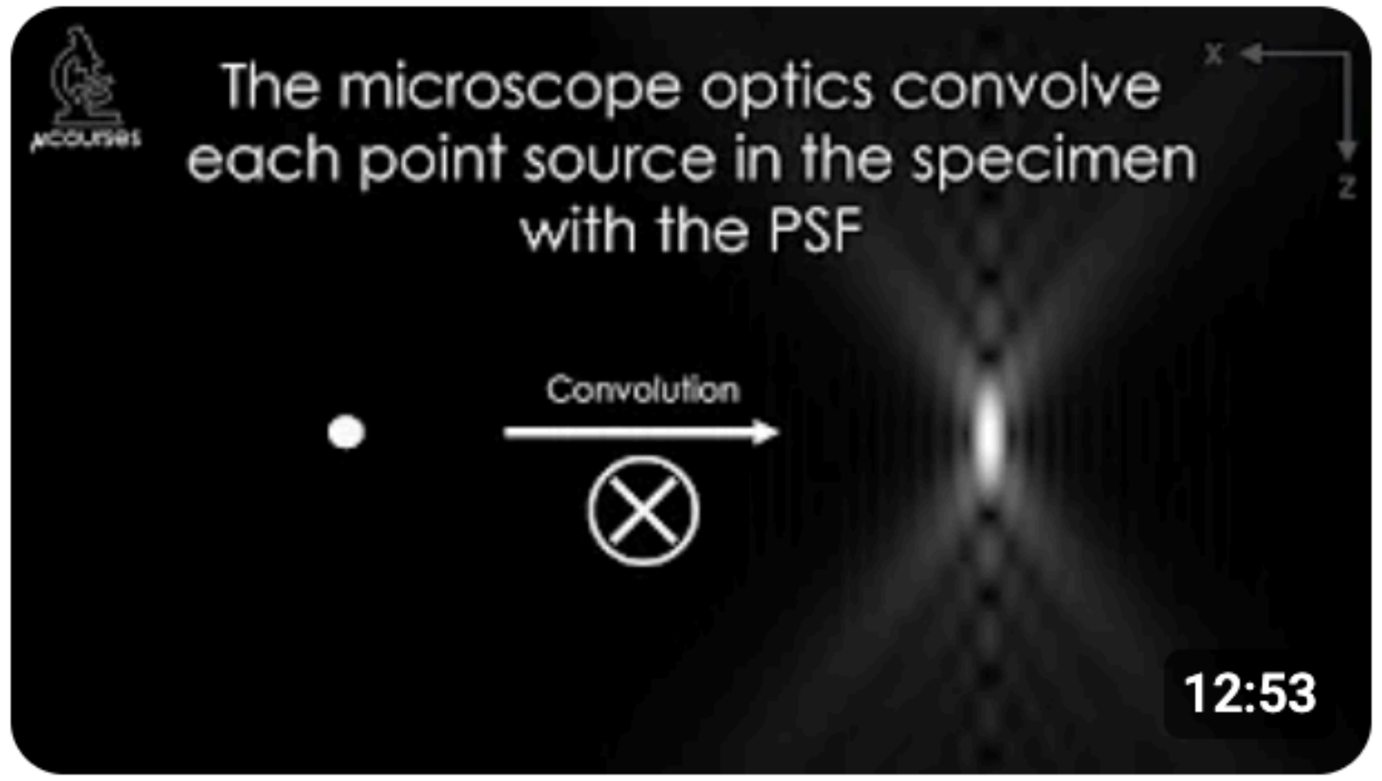
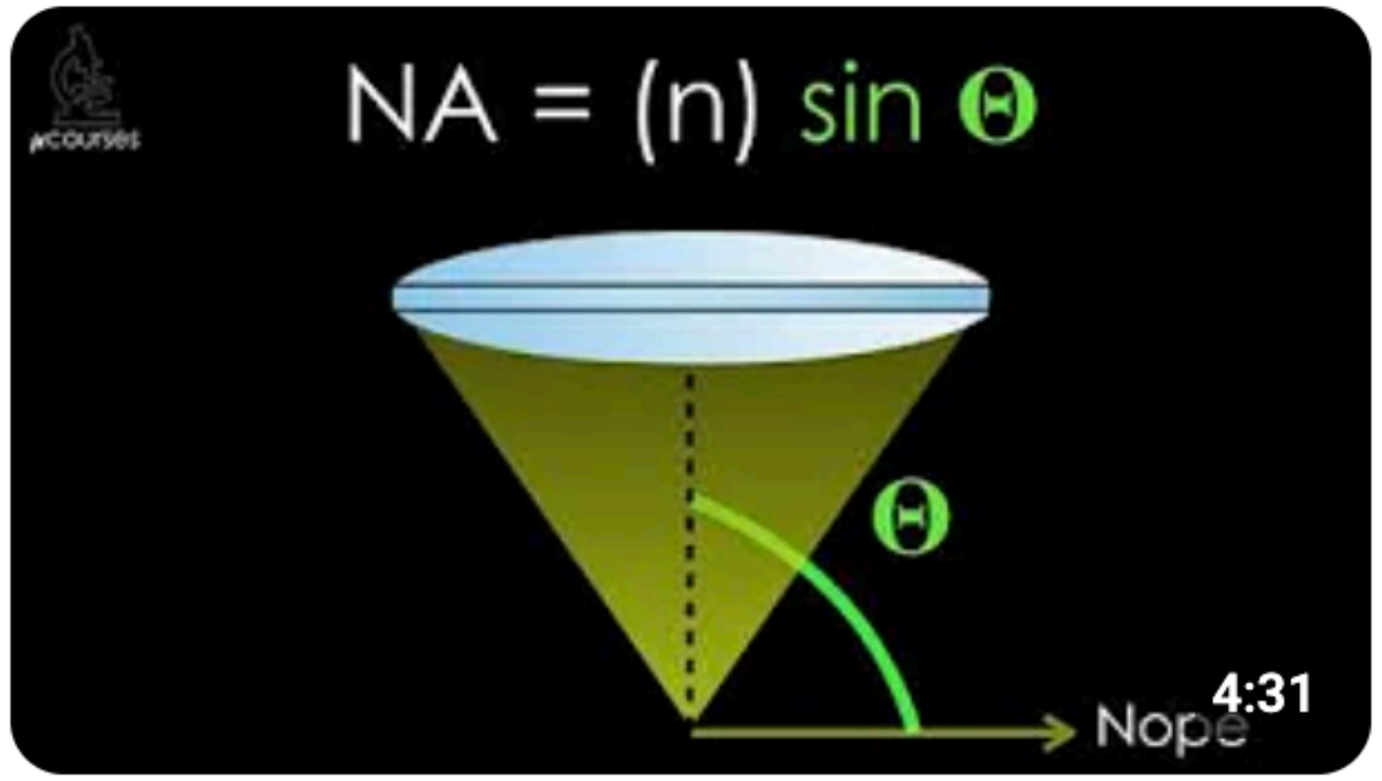


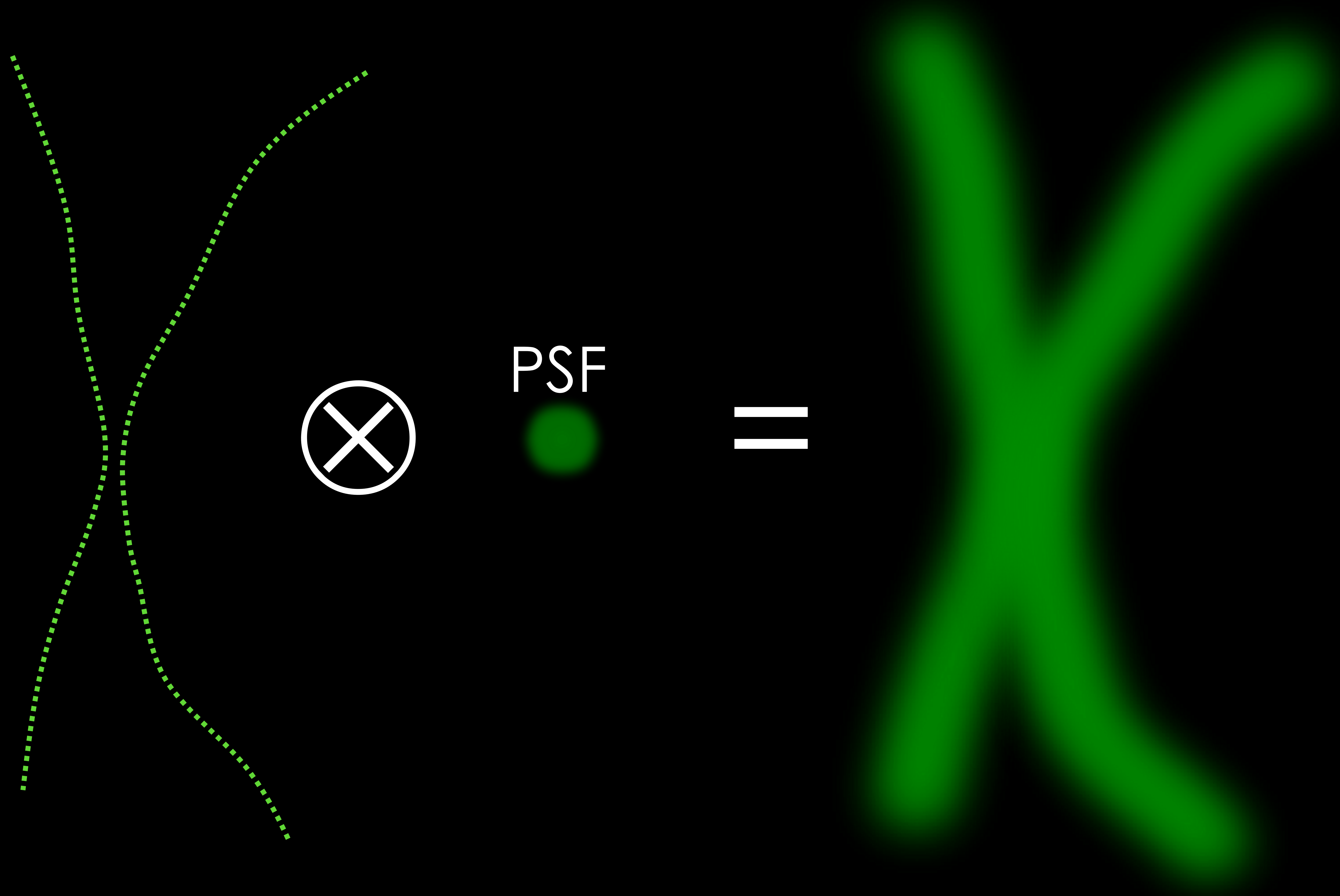
The Point Spread Function

70K views • 5 years ago



Numerical Aperture

82K views • 5 years ago



Adapted form Jennifer Waters

What Colocalization IS NOT in Fluorescence Microscopy

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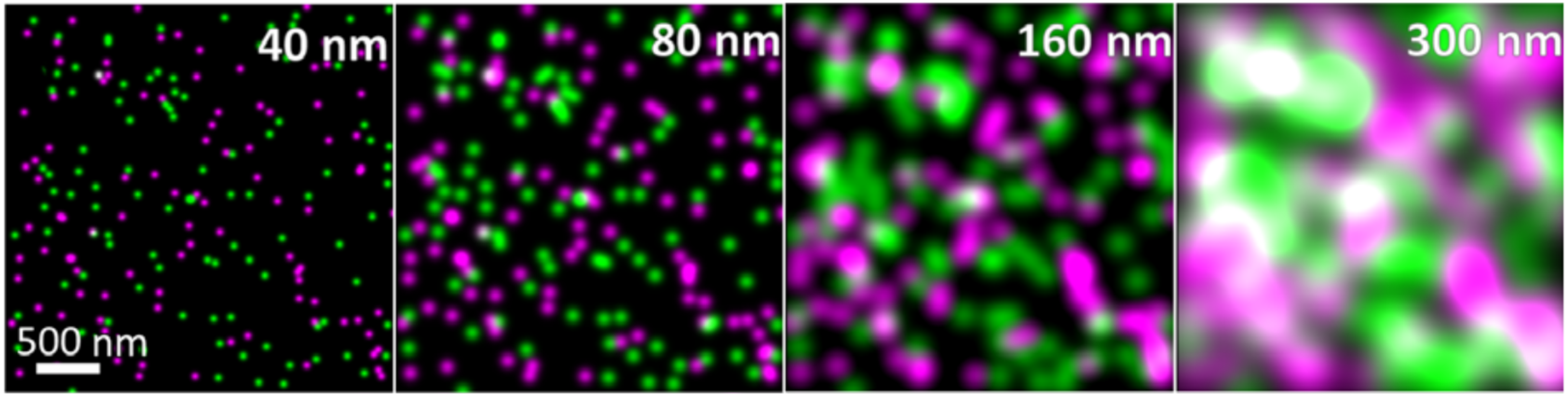
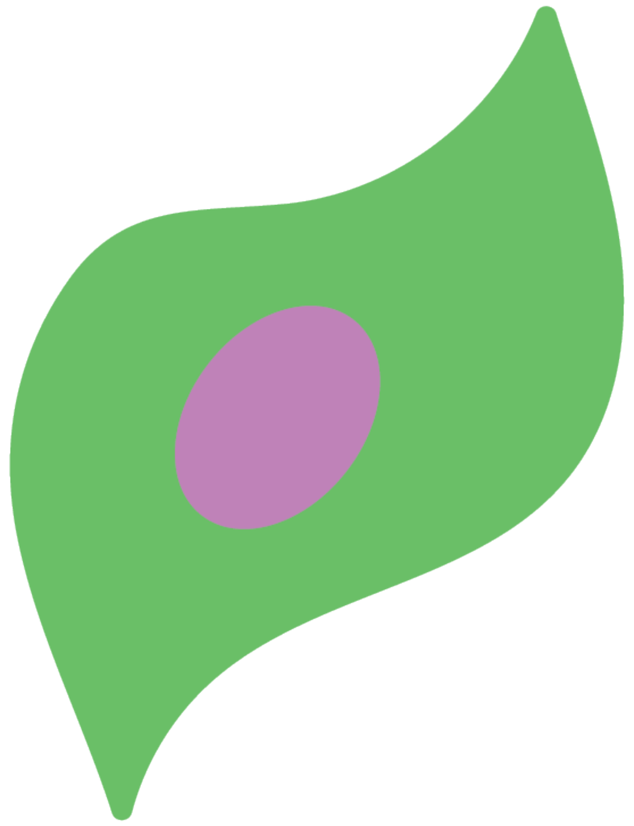
We can detect **where** the fluorescence signal is



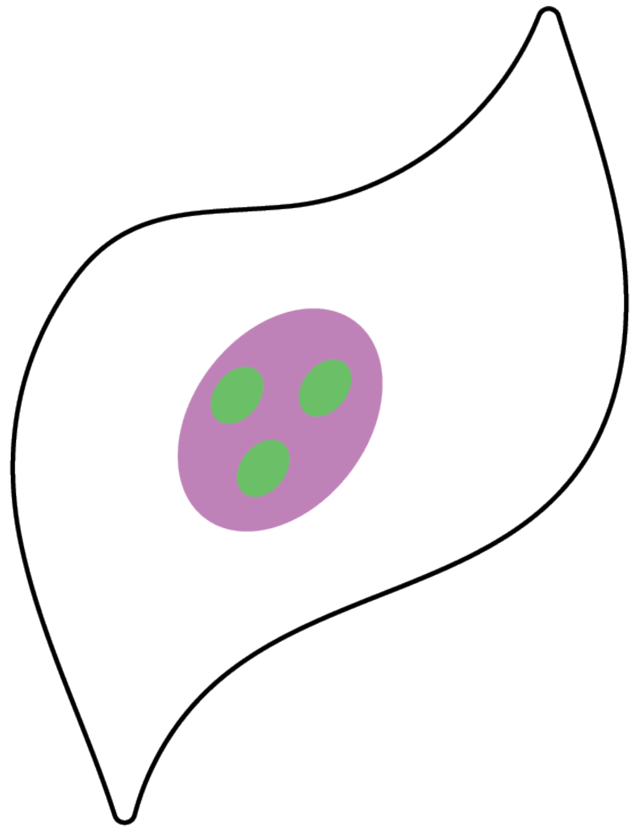
Resolution

Adapted from Xu et al, 2016

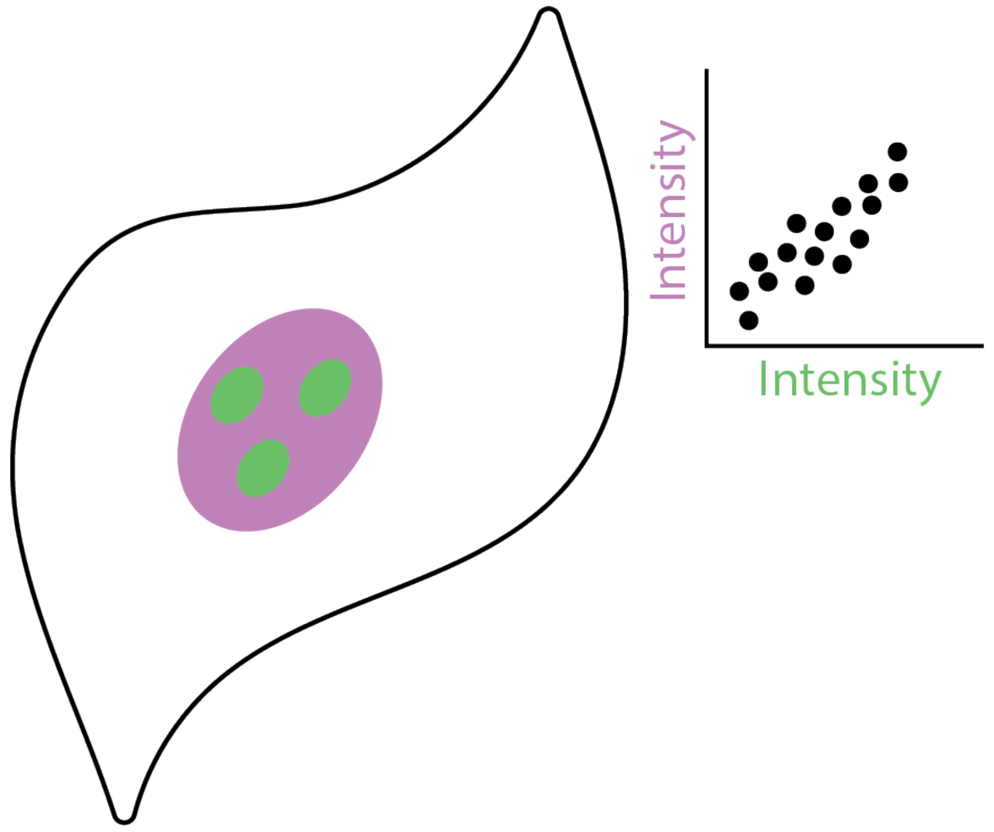
What is Colocalization in Fluorescence Microscopy



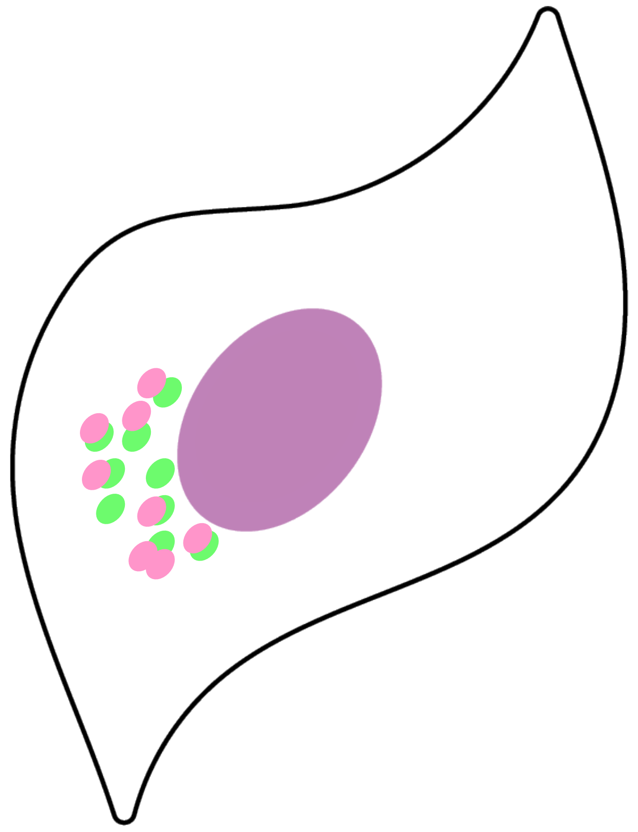
Co-expression: The presence of two or more fluorescent signals in the same cell, indicating that the corresponding proteins or molecules are expressed in the same biological sample.



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.



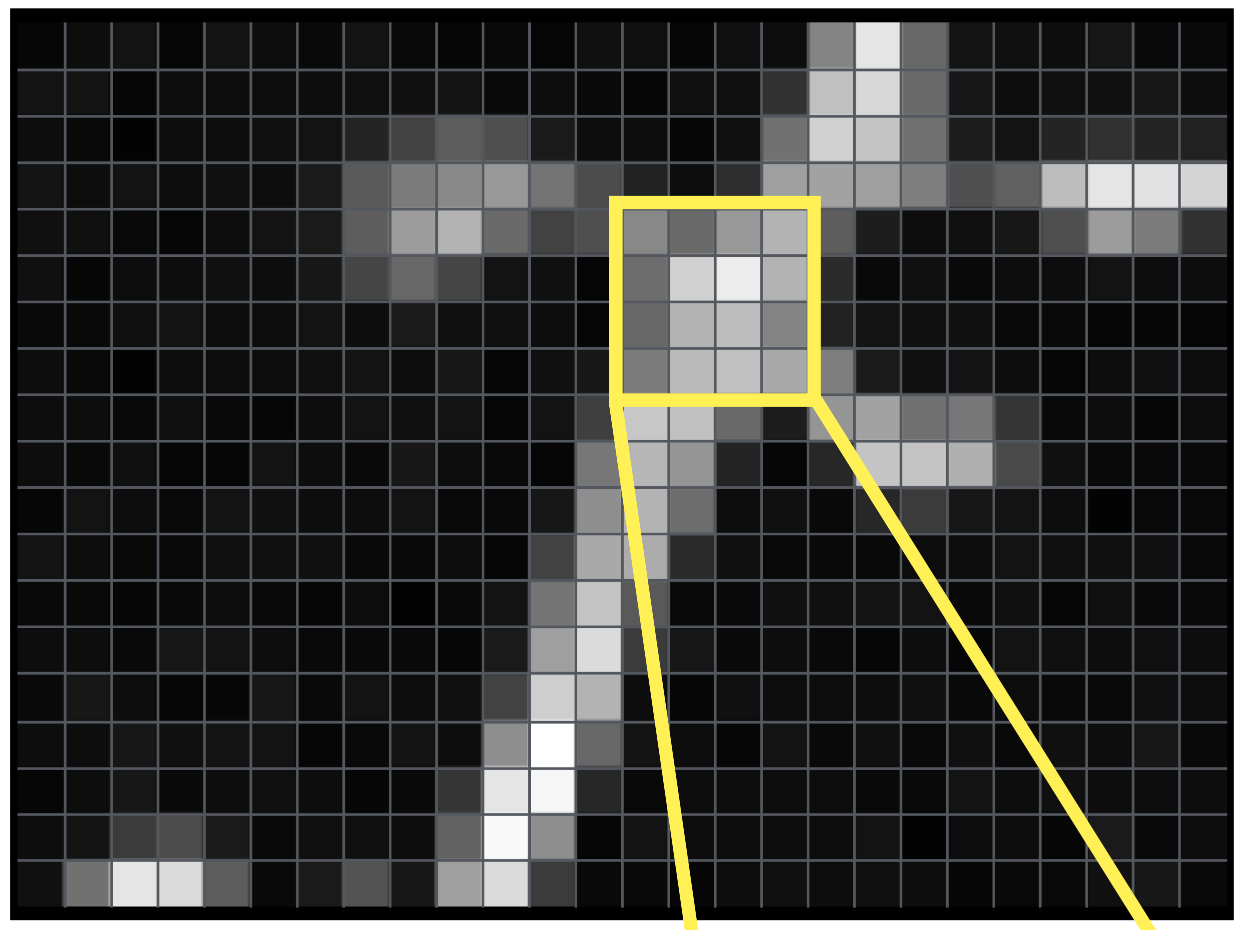
Correlation: A quantitative measure of how the intensity of two fluorescent signals changes together across the sample, helping to determine if their distributions are related.



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

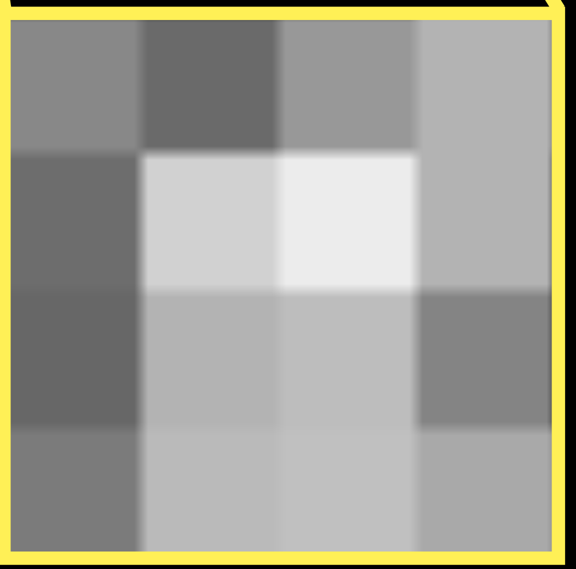
Eva De La Serna

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



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

Pixel = Picture Element

How can we Measure Colocalization?

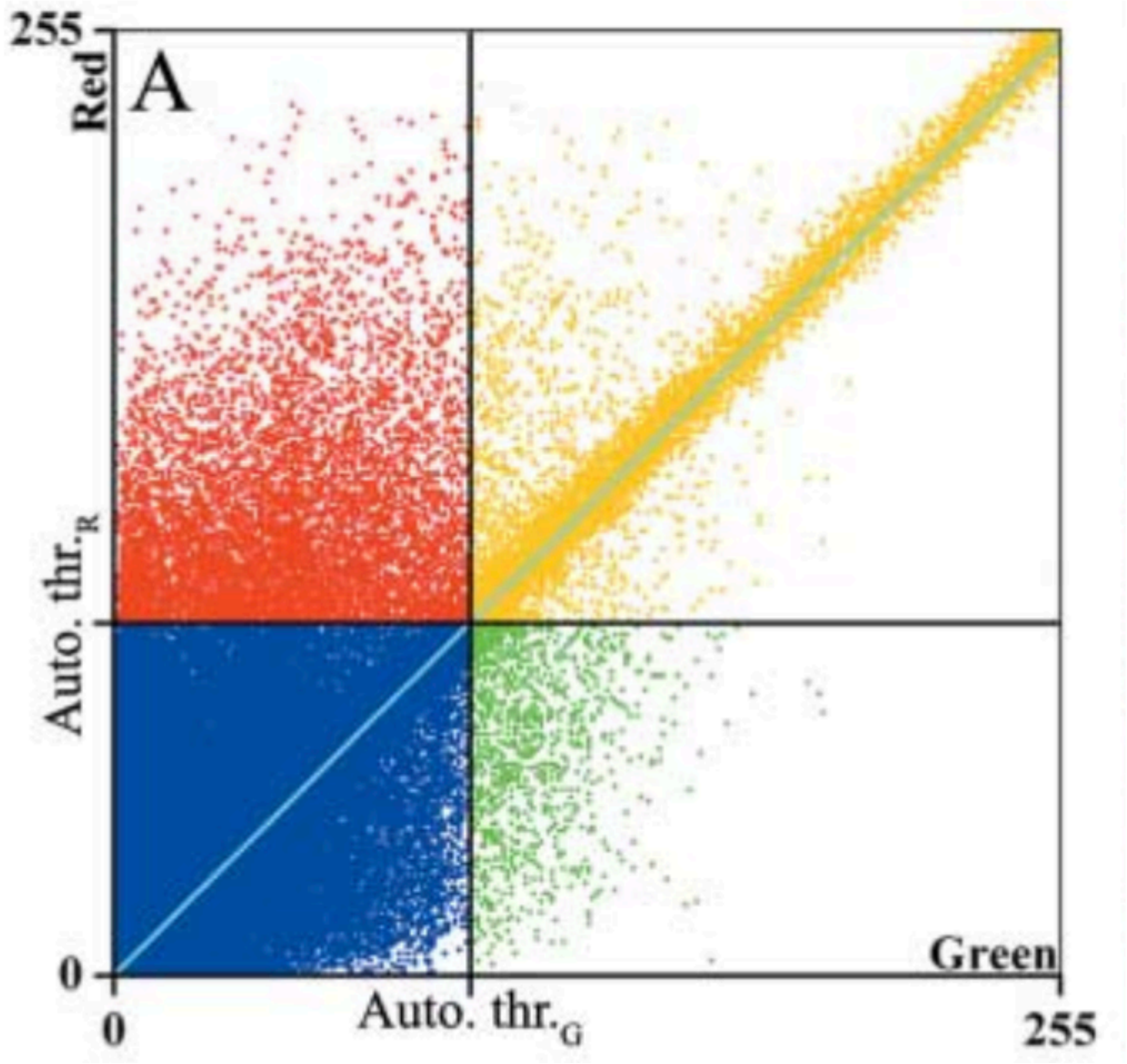
- <u>Pixel Intensity-based methods</u> for co-occurrence & correlation
- <u>Object-based methods</u> for co-expression & co-distribution (spatial statistics)

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



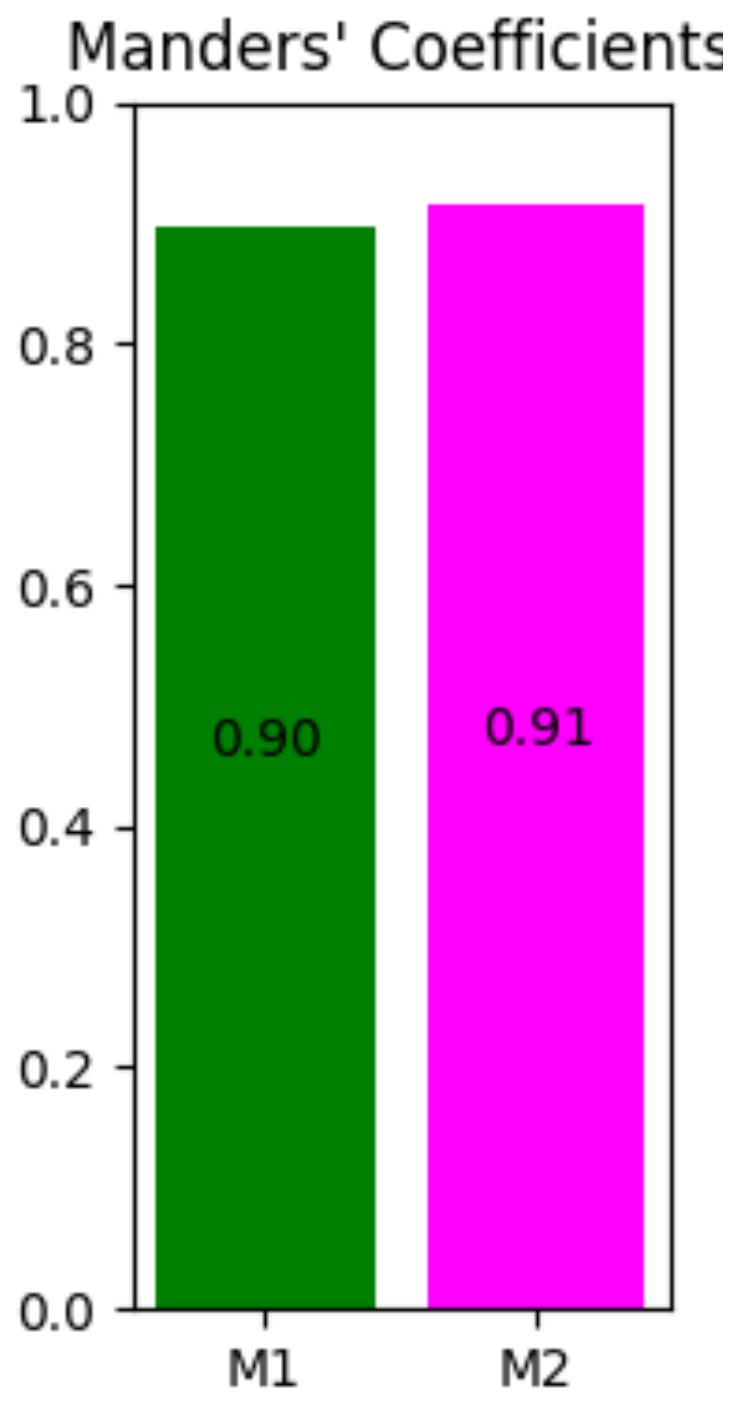
Adapted form S Bolte , F P Cordelières, 2006

Manders' correlation coefficients

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

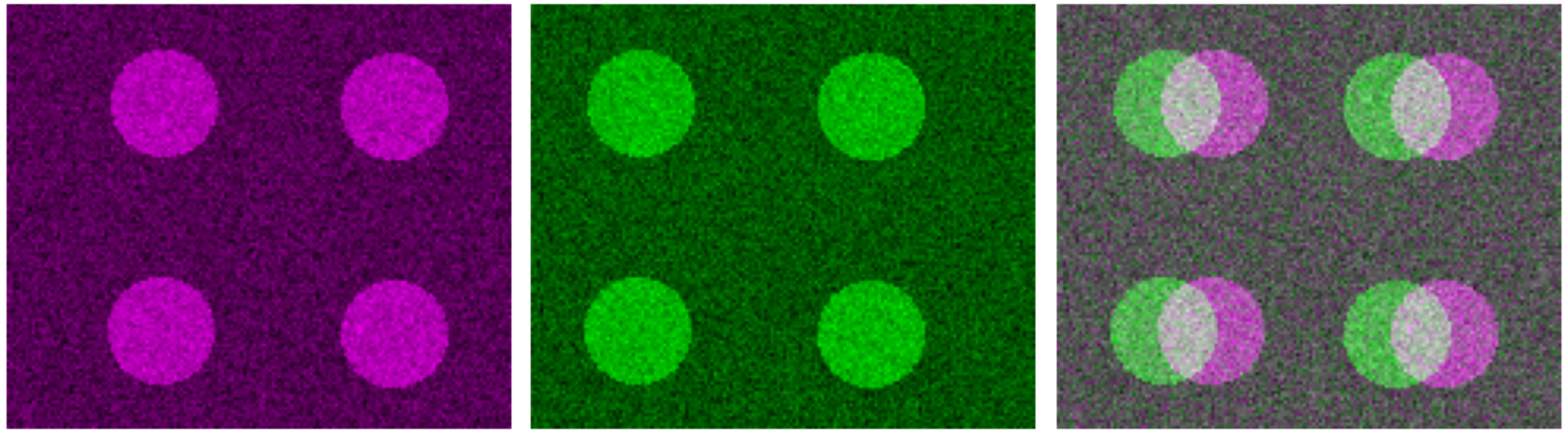
Pearson's correlation coefficient

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



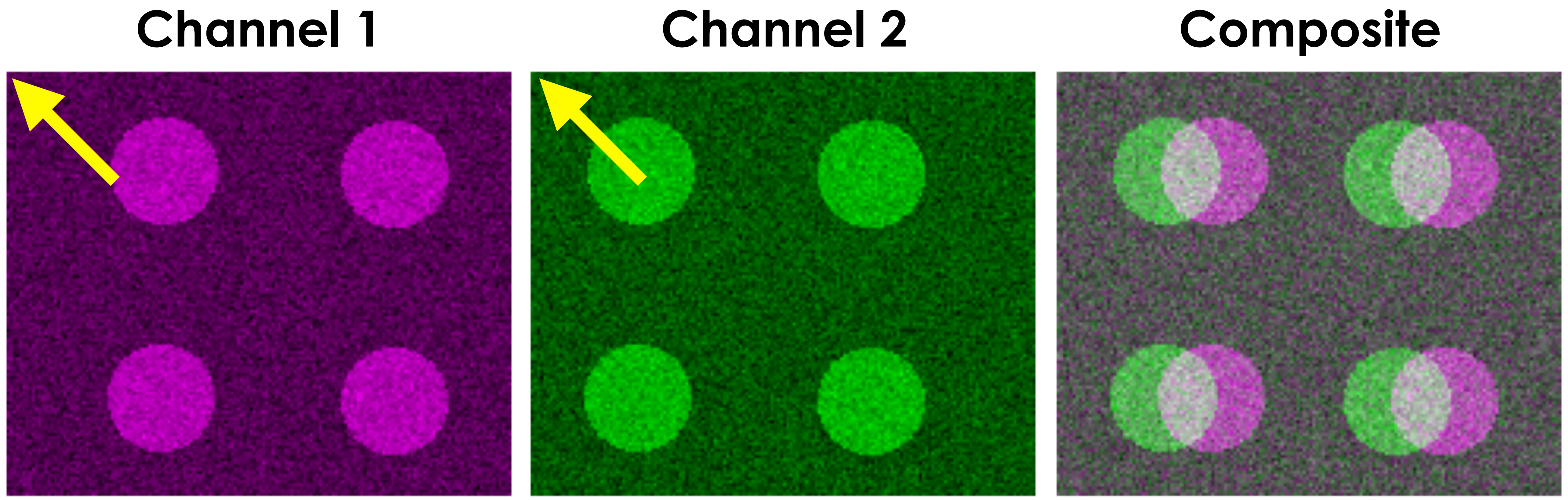
Scatter Plot

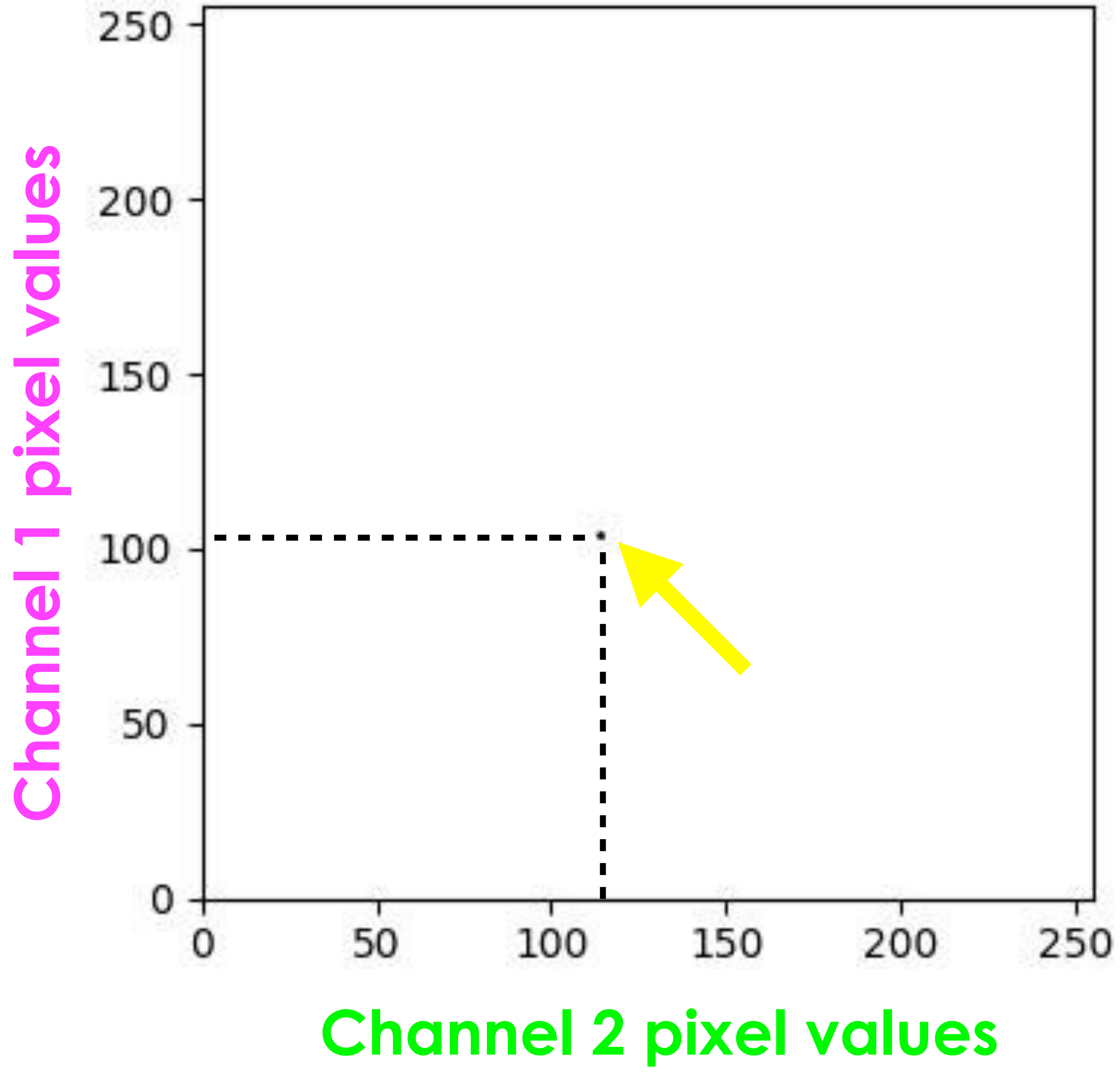
Channel 1 | Channel 2 | Composite



Scatter Plot

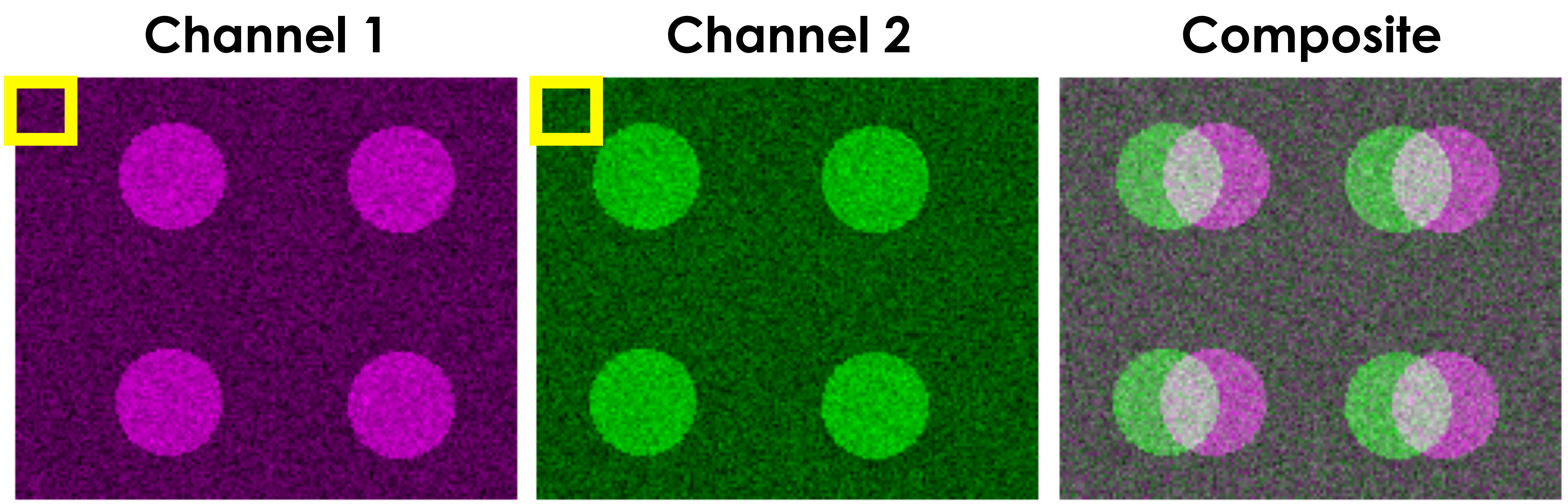
1 pixel

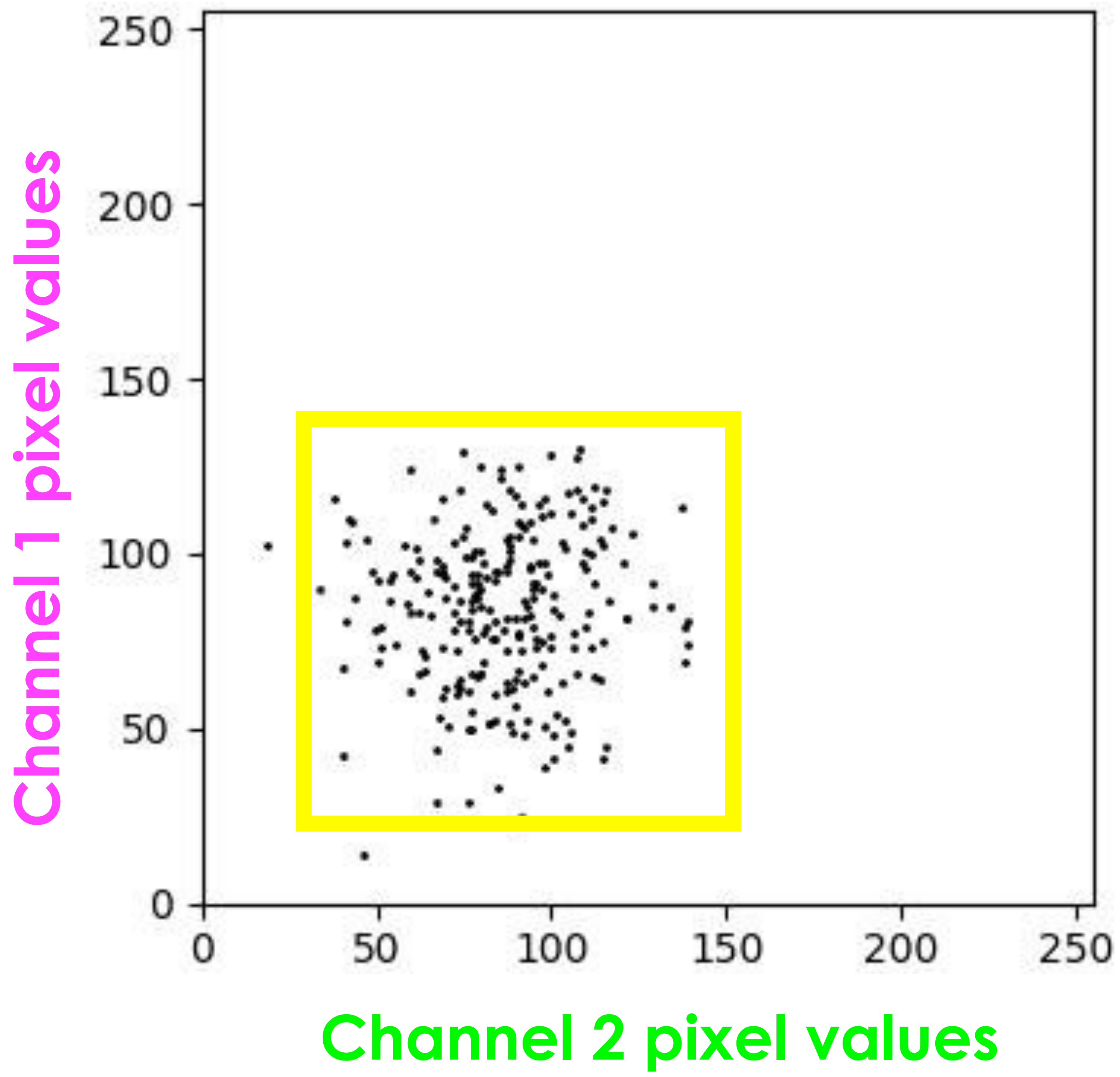




Scatter Plot

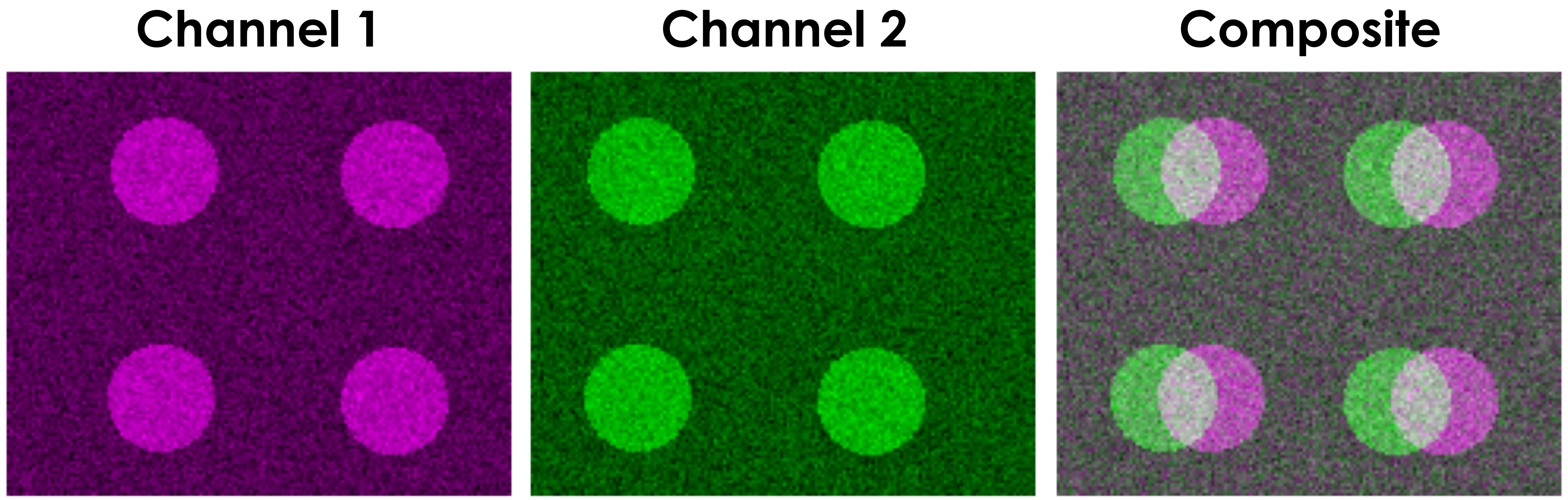
more pixels

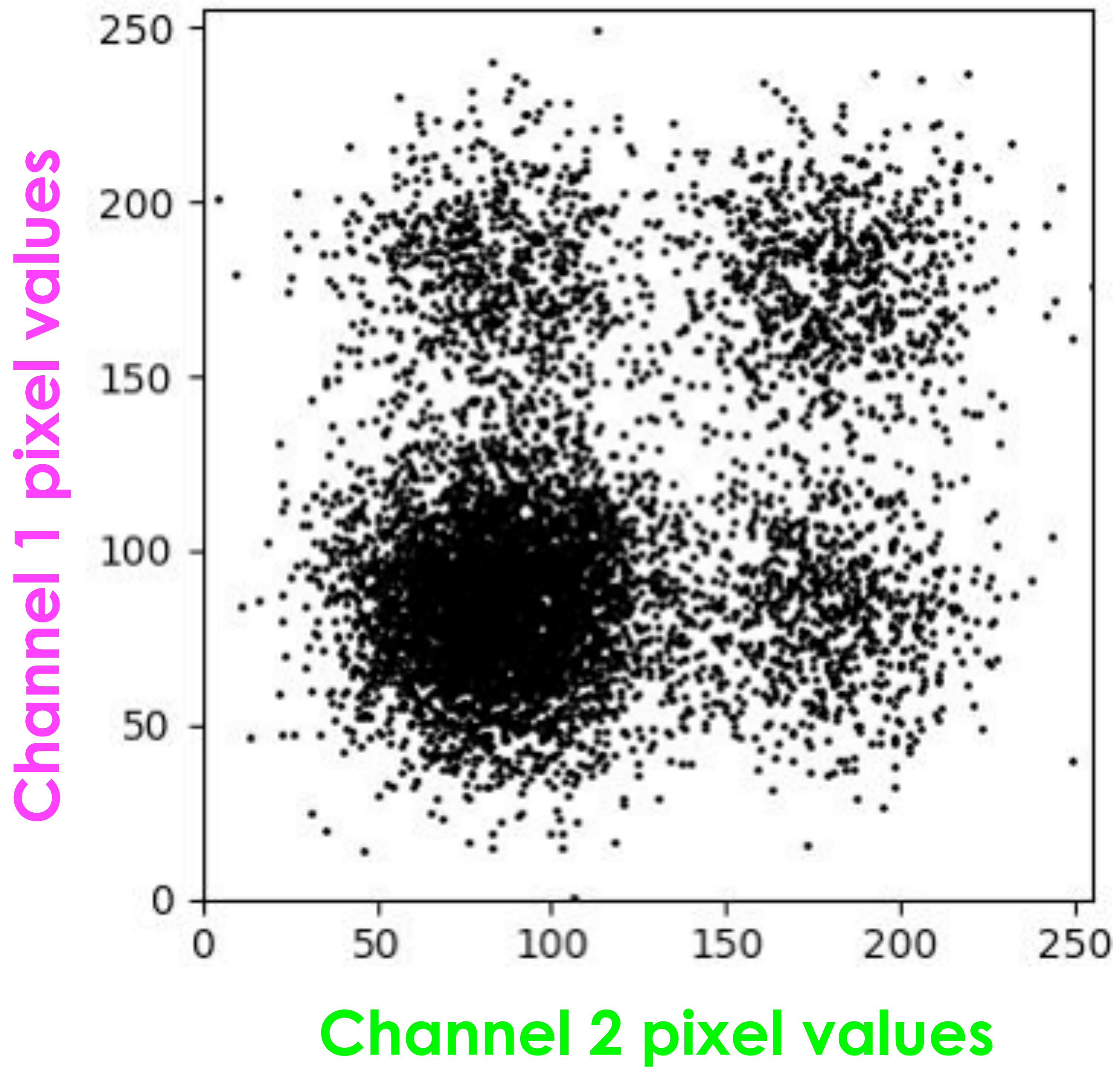




Scatter Plot

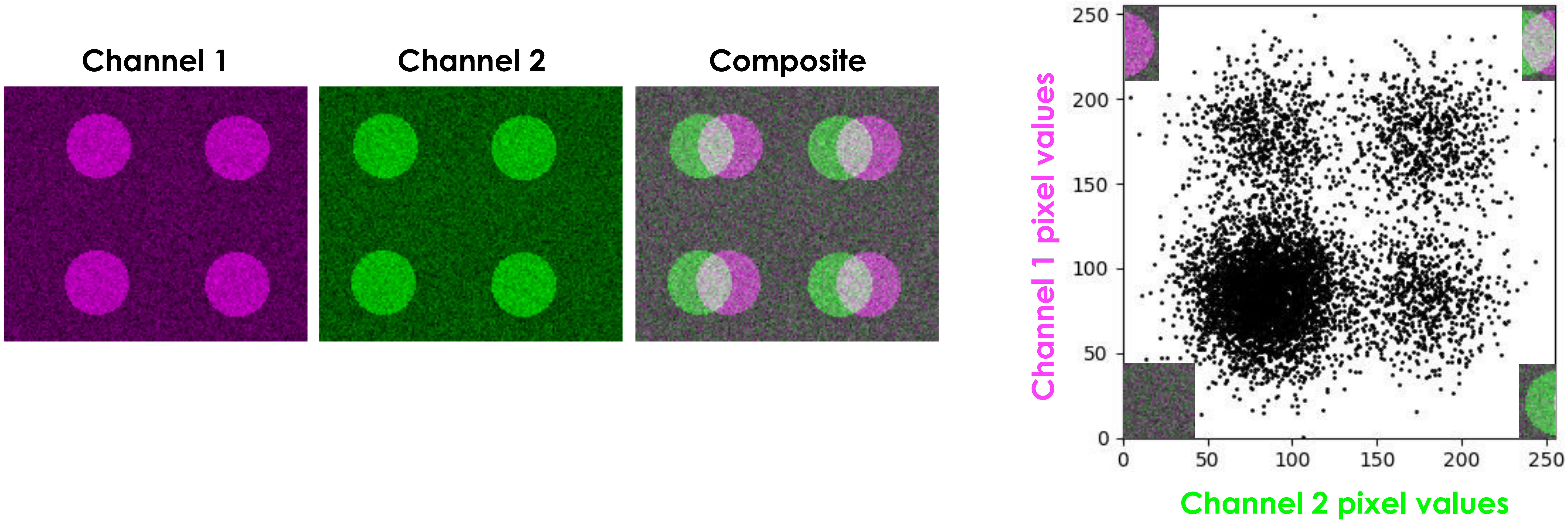
all pixels





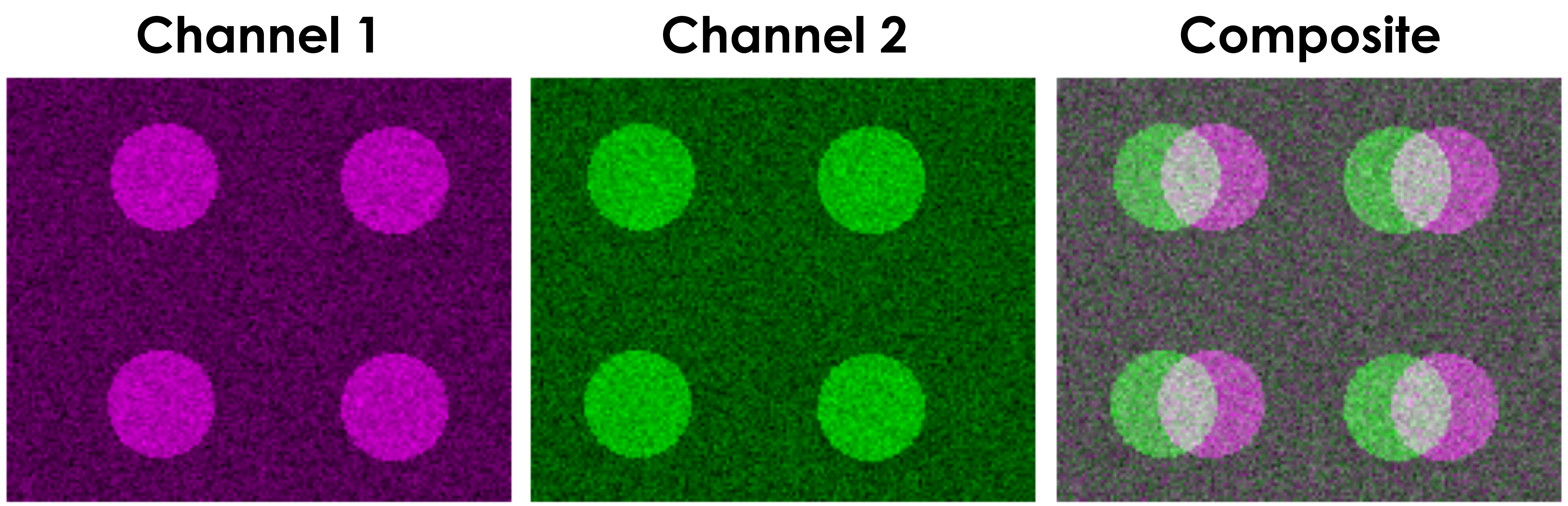
Scatter Plot

all pixels

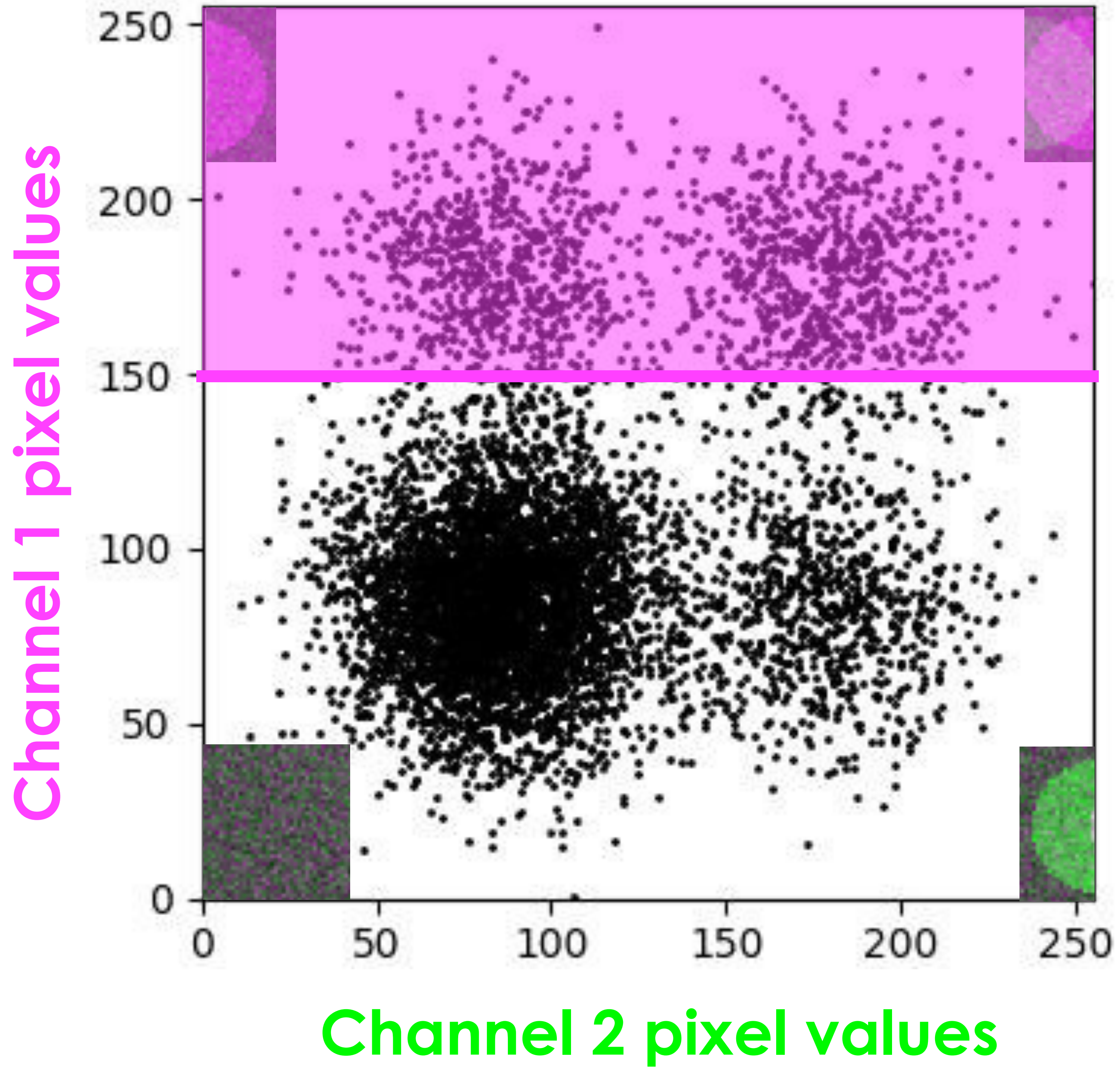


Scatter Plot

visualize thresholds

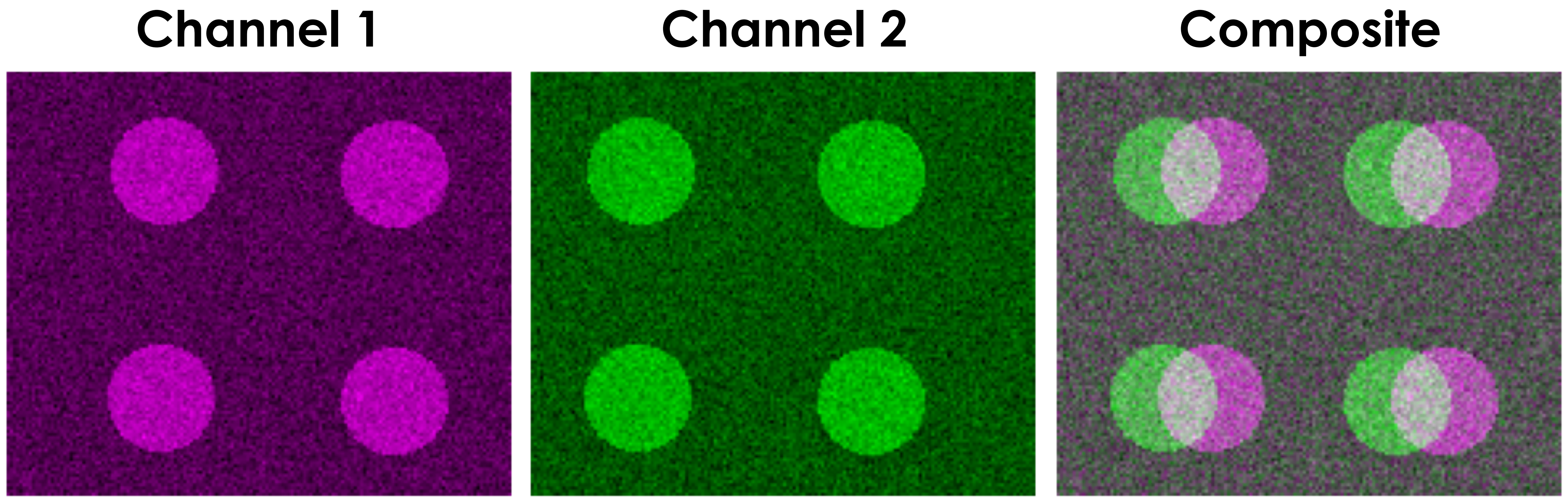


Channel 1 threshold = 150



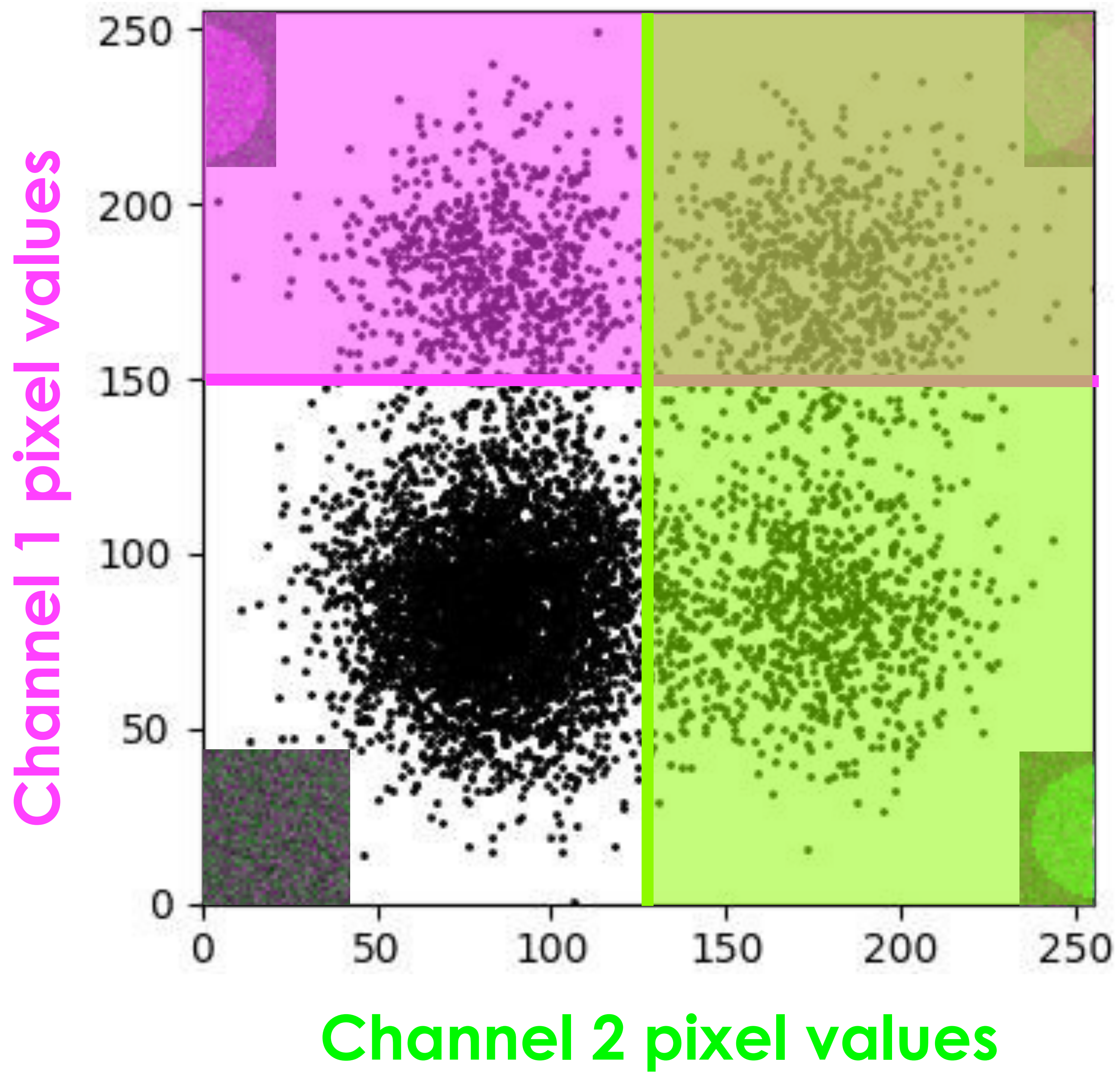
Scatter Plot

visualize thresholds



Channel 1 threshold = 150

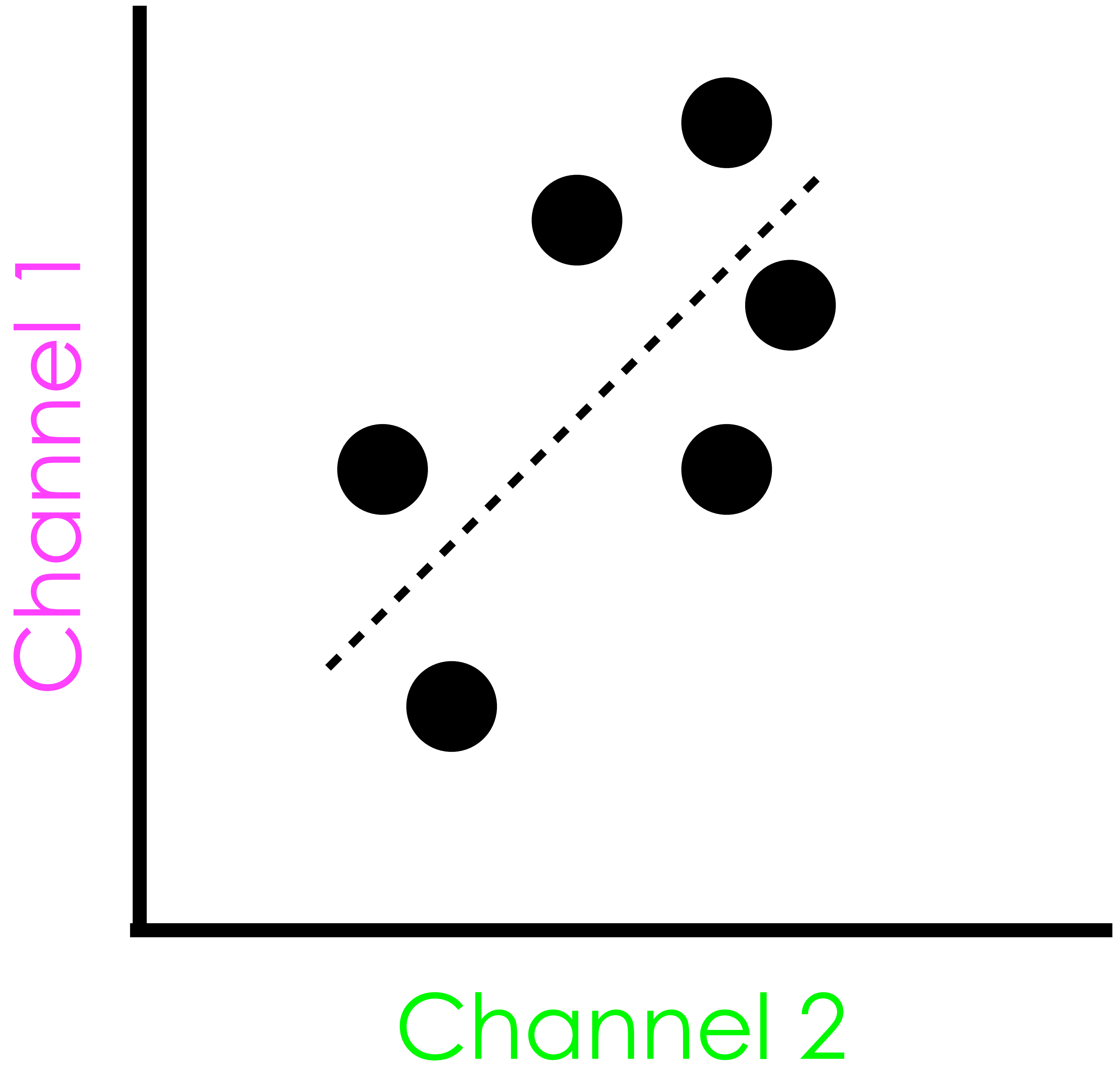
Channel 2 threshold = 140



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 <u>linear</u> 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)?

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

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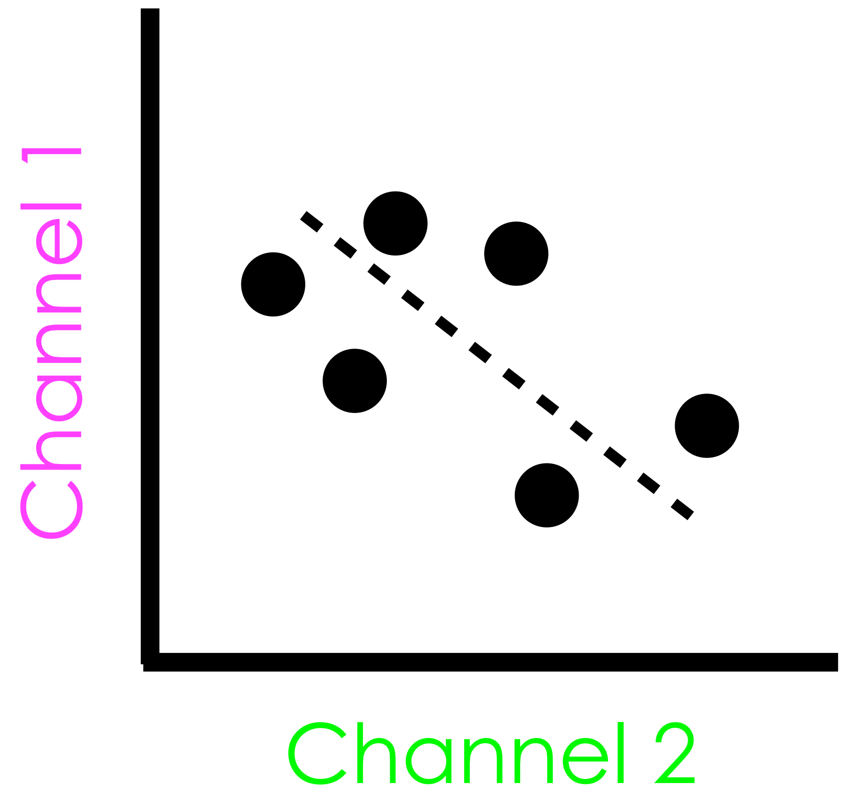
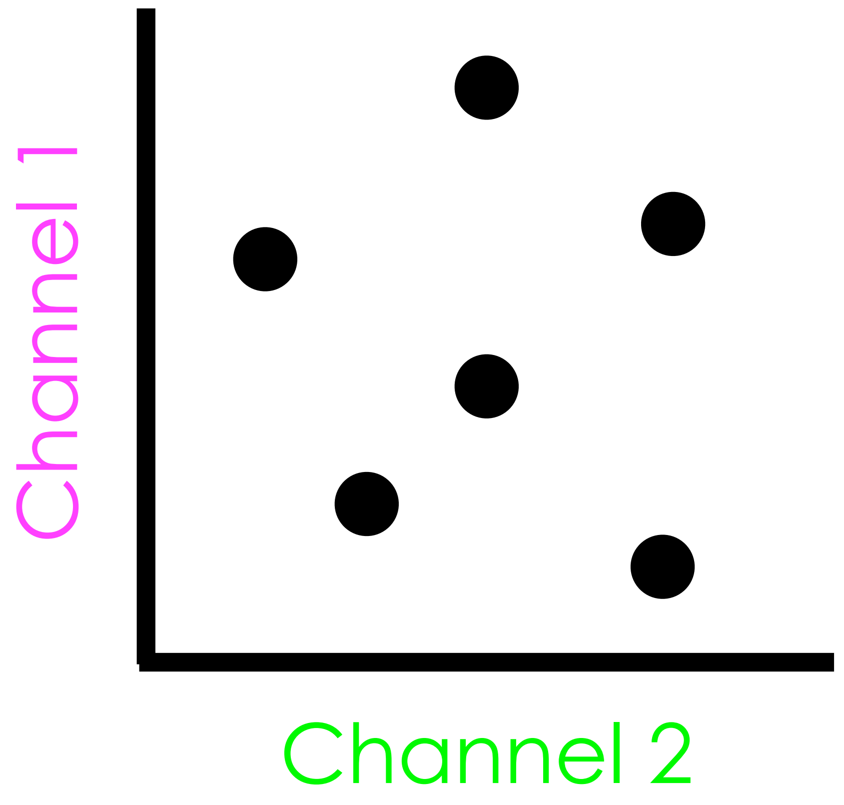
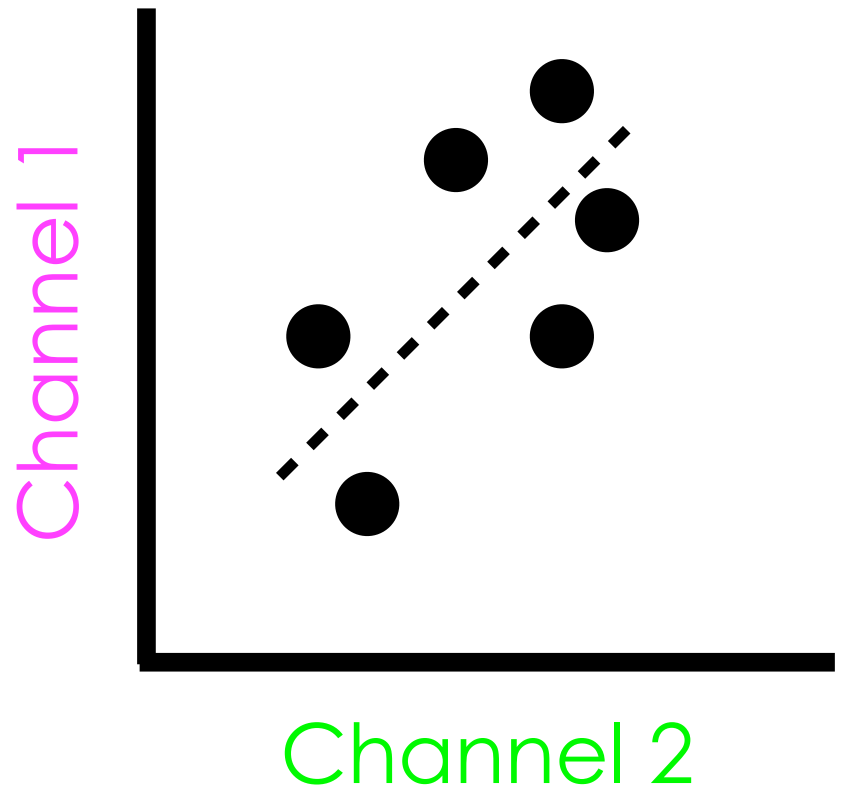


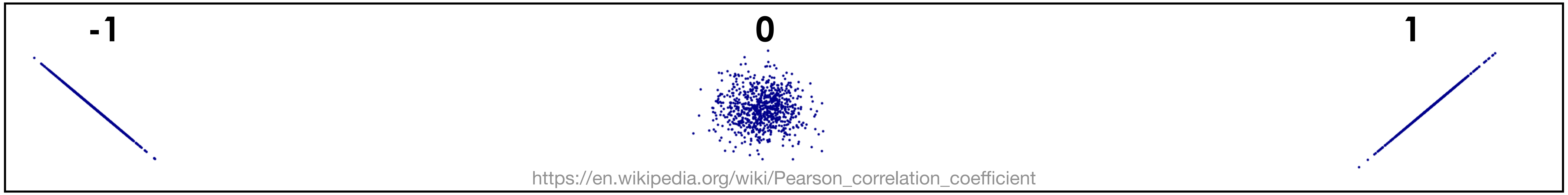
$r_P \sim -1$



$r_P \sim 0$



$r_P \sim 1$



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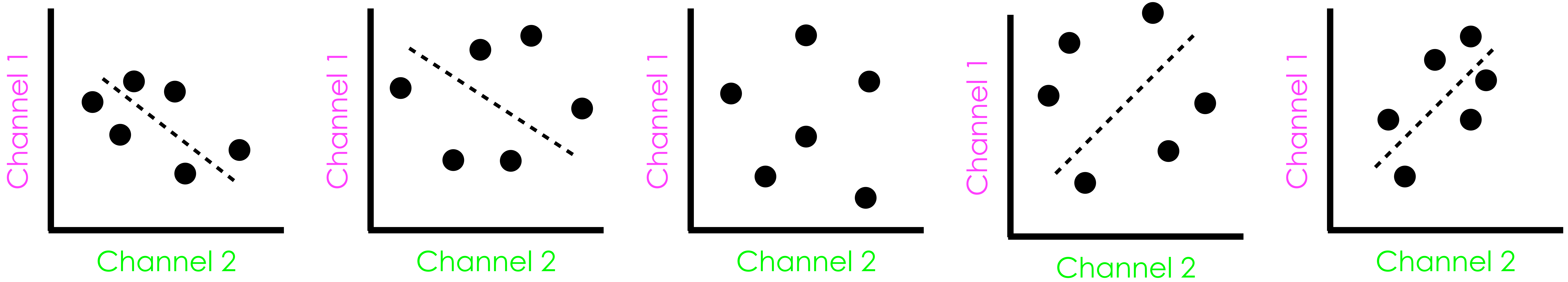
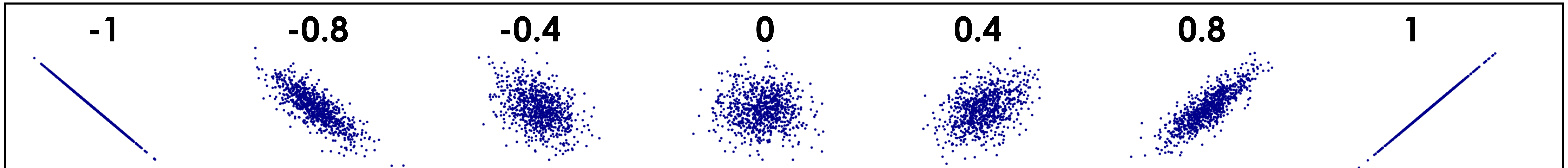
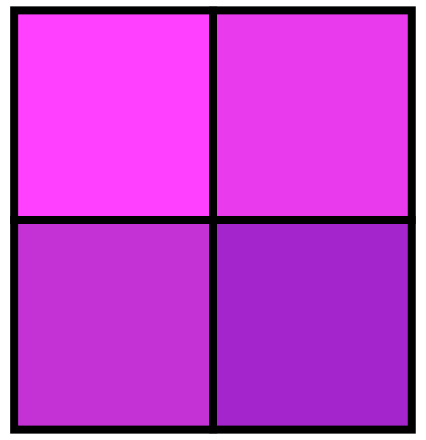


$r_P \sim -1$ | $-1 < r_P < 0$ | $r_P \sim 0$ | $0 < r_P < 1$ | $r_P \sim 1$

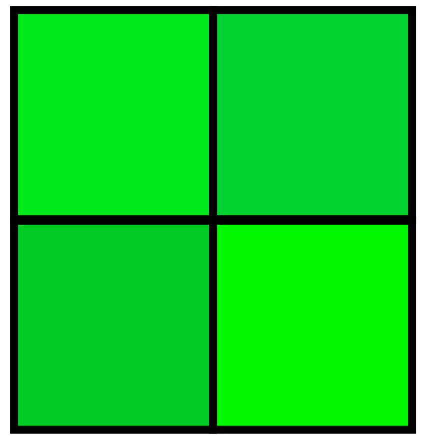


https://en.wikipedia.org/wiki/Pearson_correlation_coefficient

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



200	190
90	80



100	90
70	152

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

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

$(R_i - R_{avg})$ =

200-140	190-140
90-140	80-140

=

60	50
-50	-60

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

$(G_i - G_{avg})$ =

100-103	90-103
70-103	152-103

=

-3	-13
-33	49

$(R_i - R_{avg})(G_i - G_{avg})$ =

60	50
-50	-60

x

-3	-13
-33	49

=

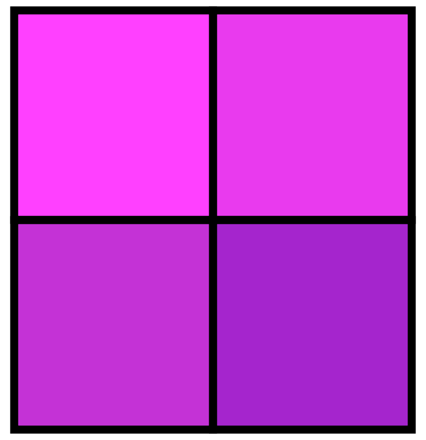
-180	-650
1650	-2940

$\sum_i (R_i - R_{avg})(G_i - G_{avg})$ =

-180	-650
1650	-2940

= **-2120**

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



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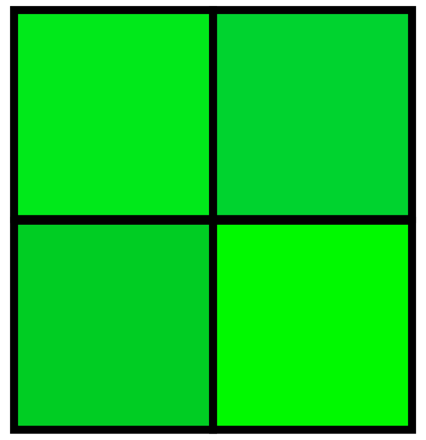


100	90
70	152

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

$(R_i - R_{avg})$ =

60	50
-50	-60

$\sum_i (R_i - R_{avg})^2$ =

60^2	50^2
$(-50)^2$	$(-60)^2$

=

3600	2500
2500	3600

= 12200

$(G_i - G_{avg})$ =

-3	-13
-33	49

$\sum_i (G_i - G_{avg})^2$ =

$(-3)^2$	$(-13)^2$
$(-33)^2$	49^2

=

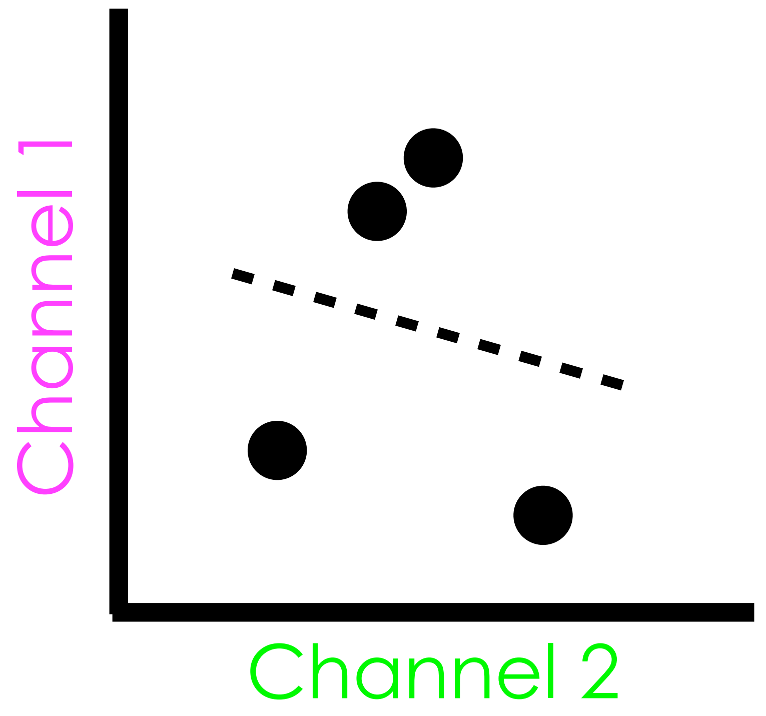
9	169
1089	2401

= 3668

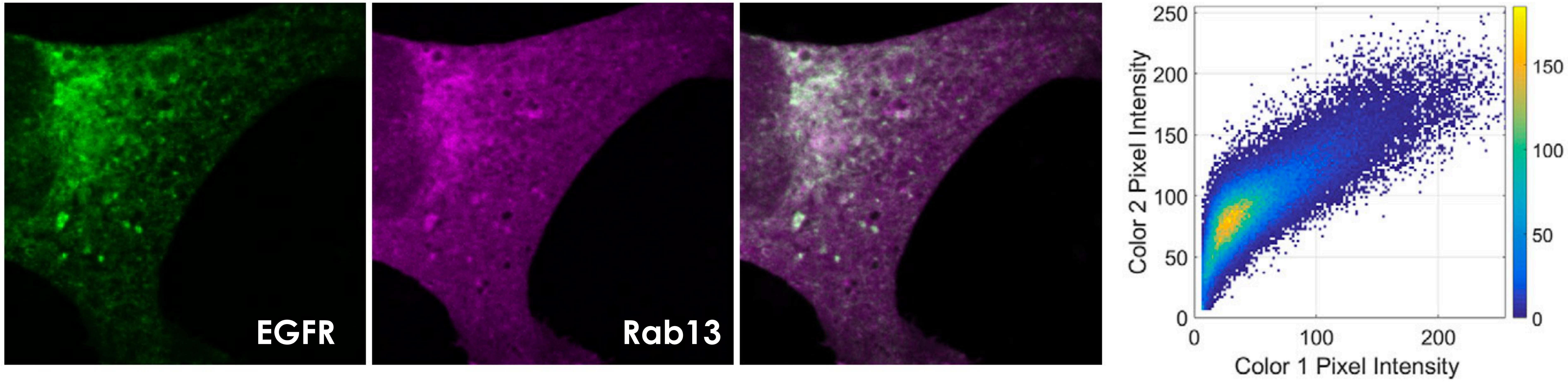
$$\sqrt{\sum_i (R_i - R_{avg})^2 \sum_i (G_i - G_{avg})^2} =$$

$$= \sqrt{12200 \times 3668} = \mathbf{6689.51}$$

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



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

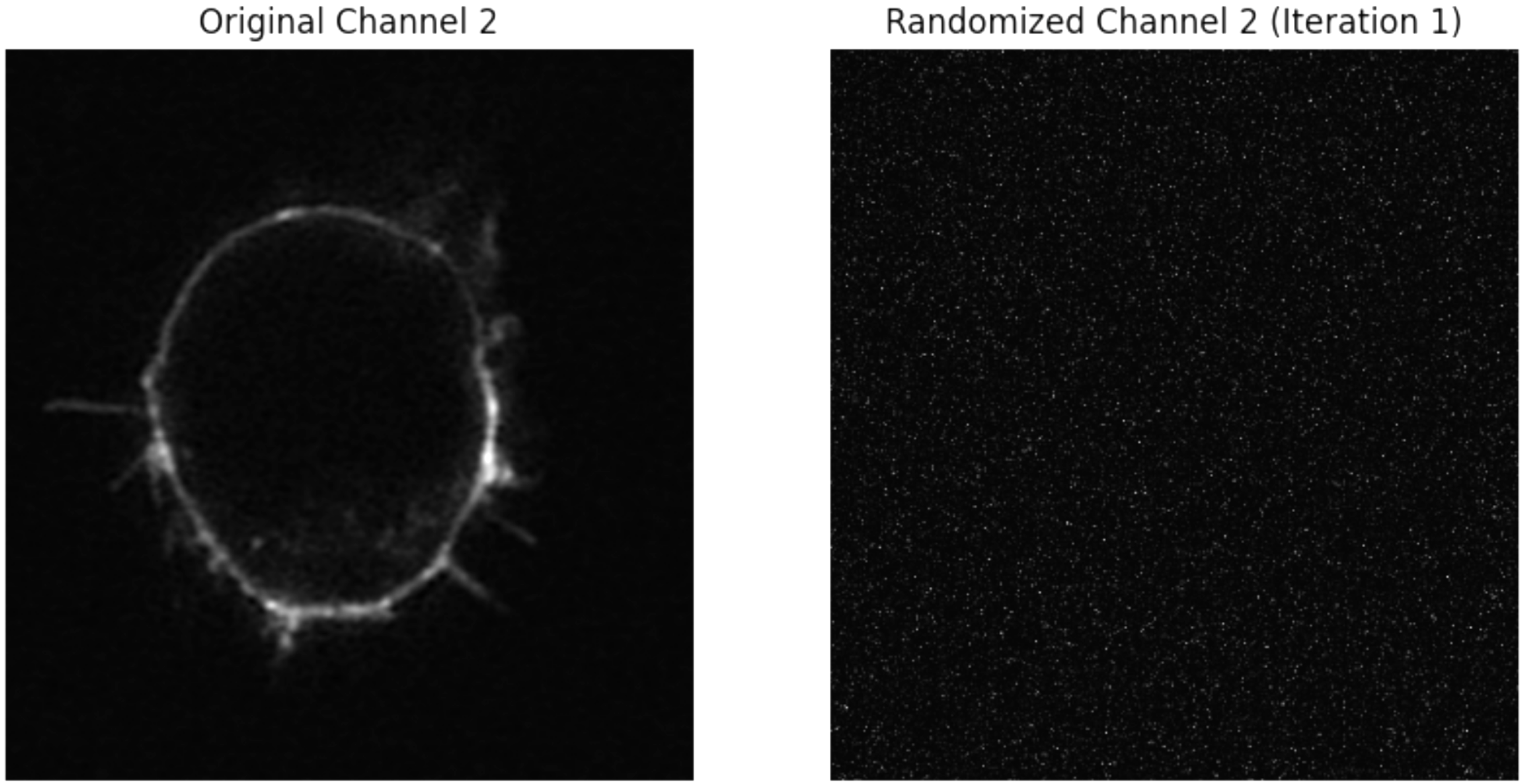


r_P = 0.76 EGFR and Rab13 concentrations predict each other relatively well, indicating a concentration-dependent relationship between these molecules.

Adapted form Aaron et al, 2018

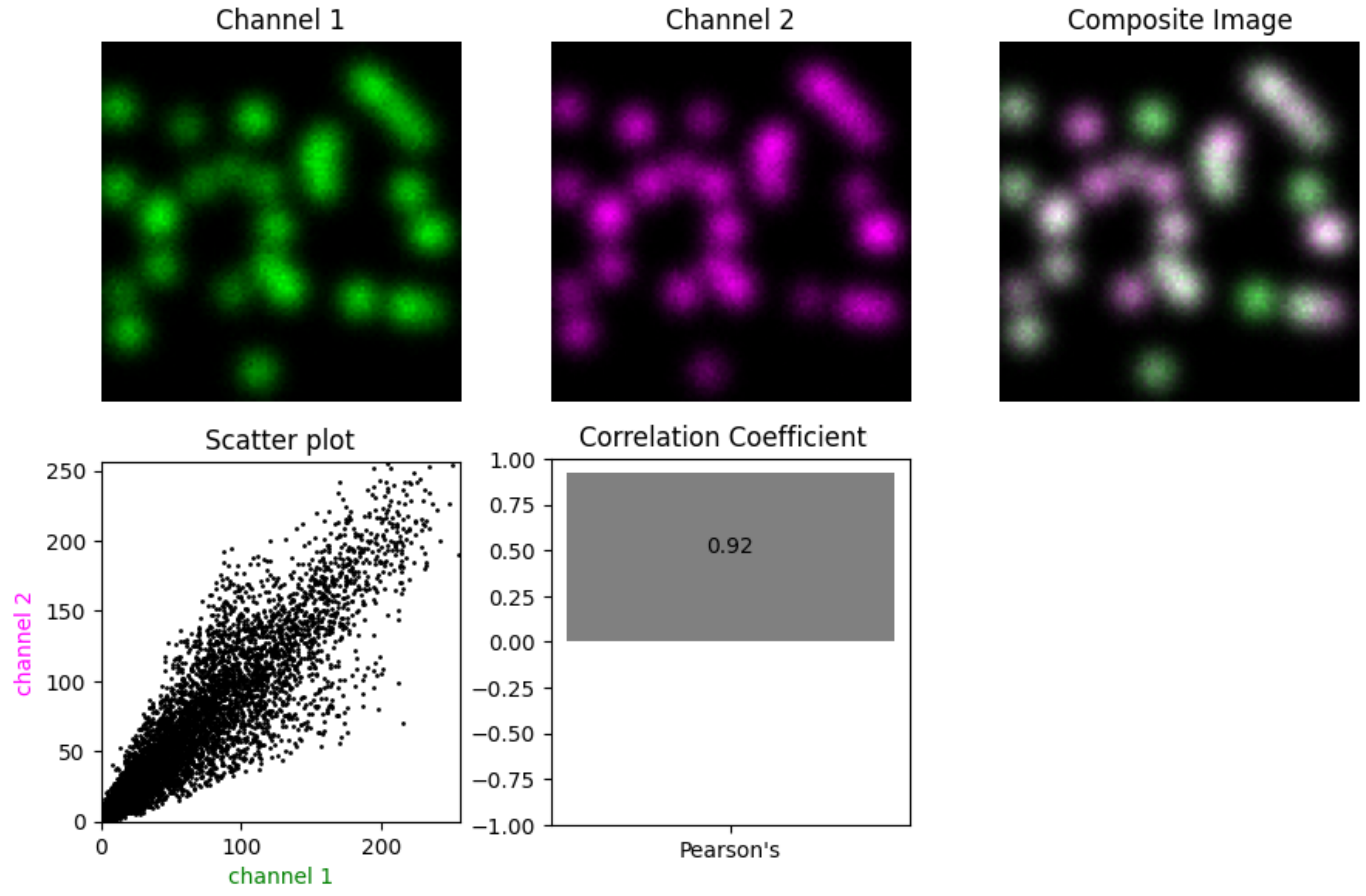
Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

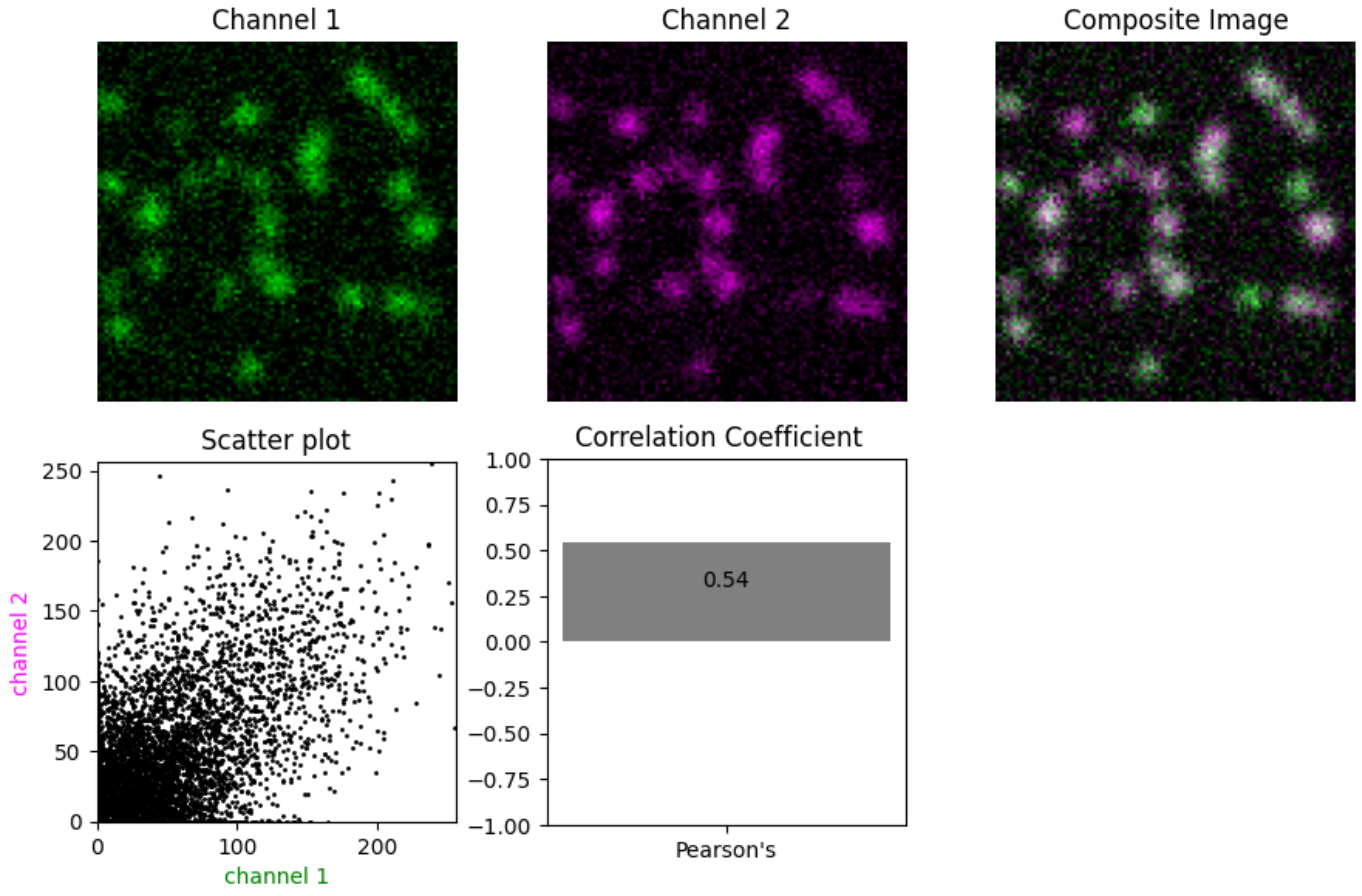
Pixel Randomization



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

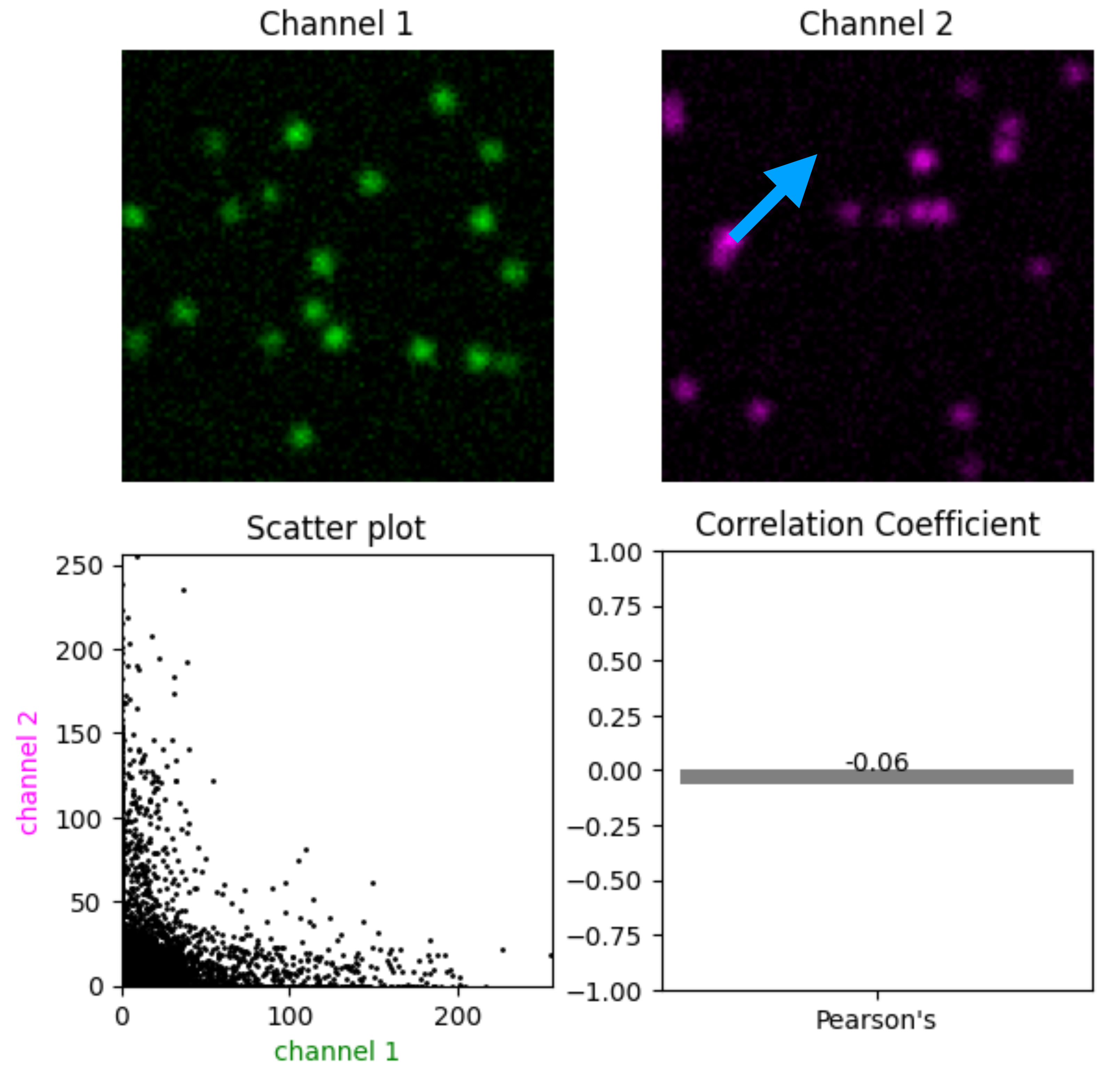
Noise

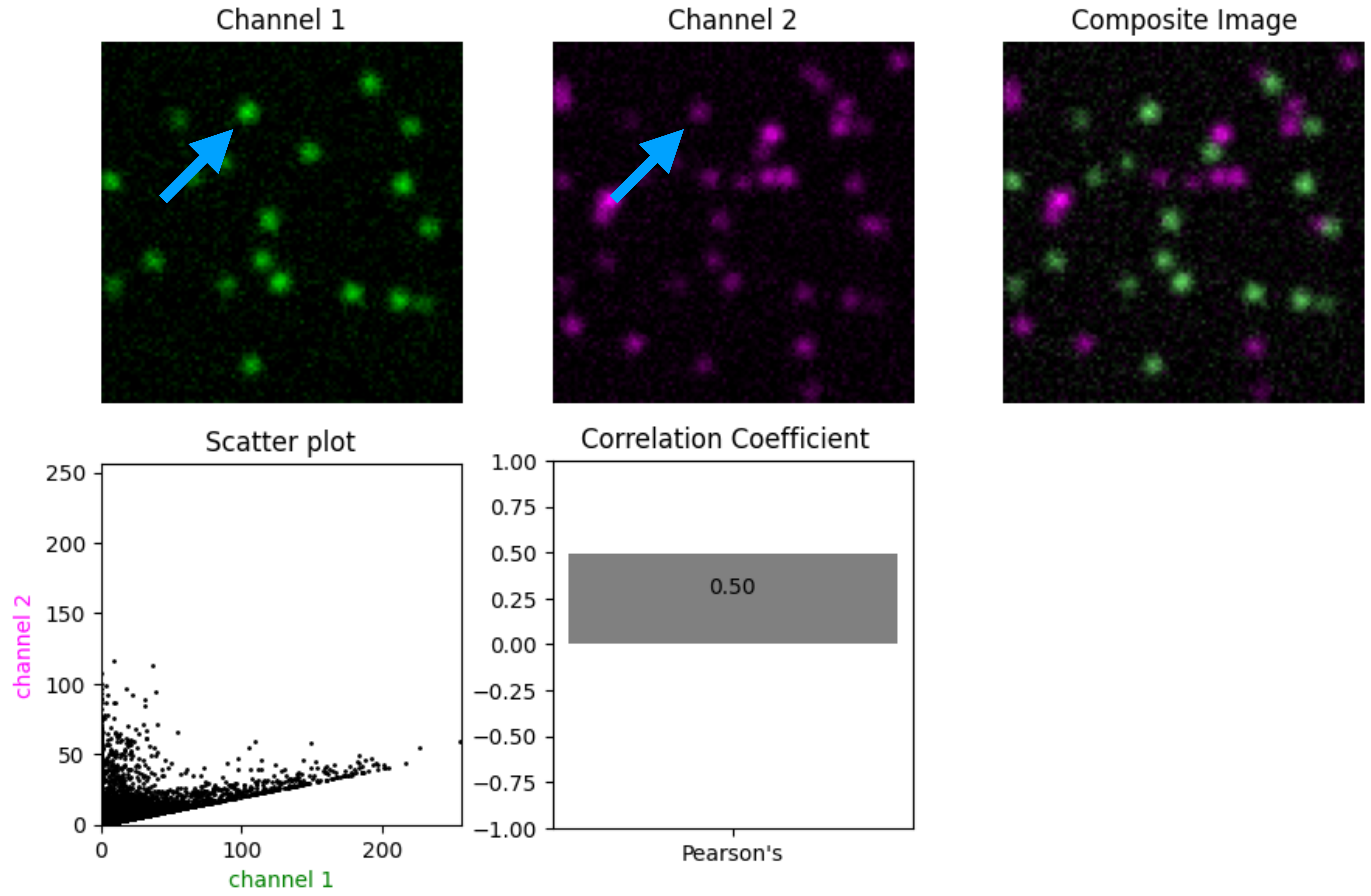




Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

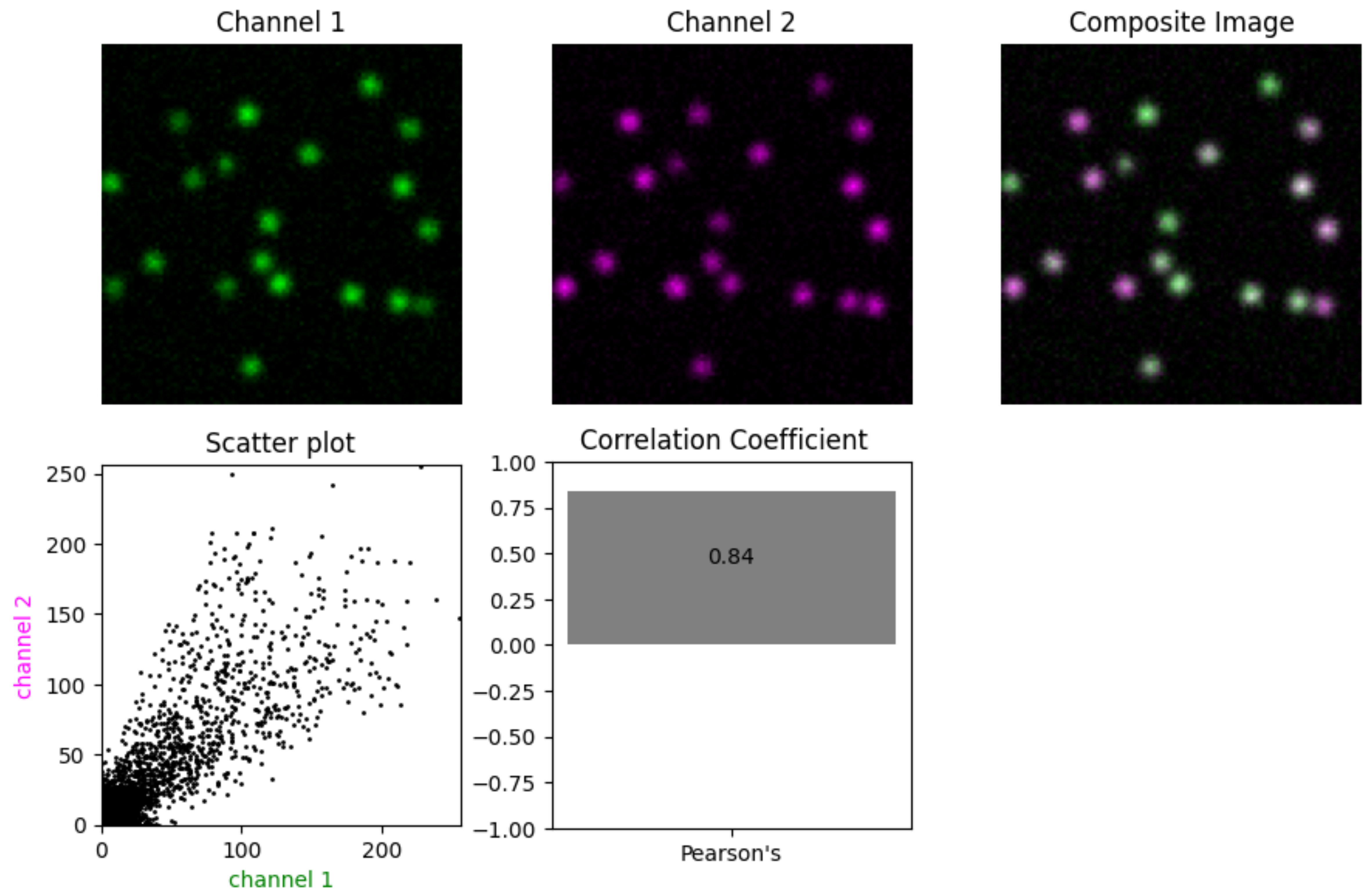
Bleedthrough

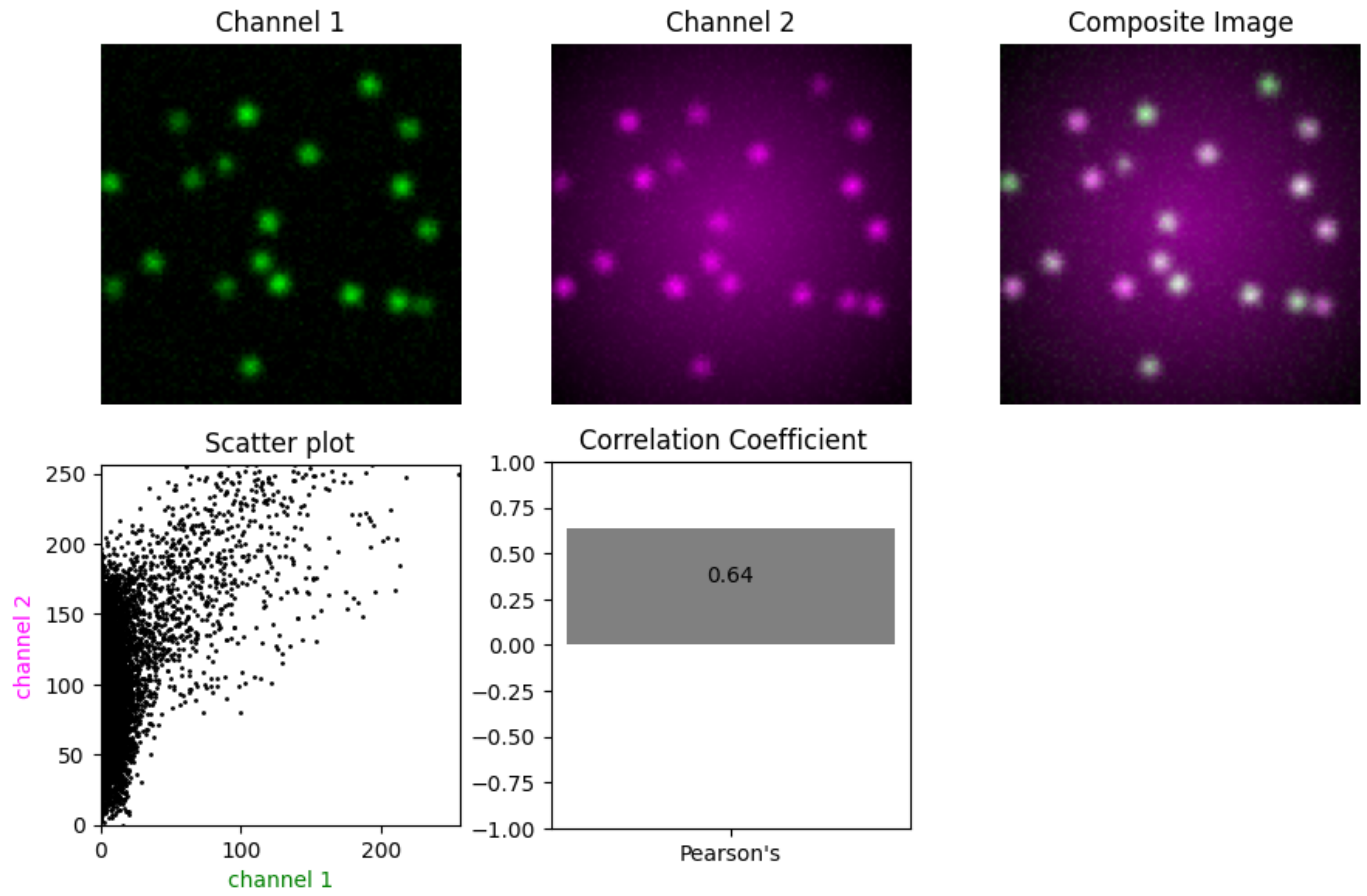




Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

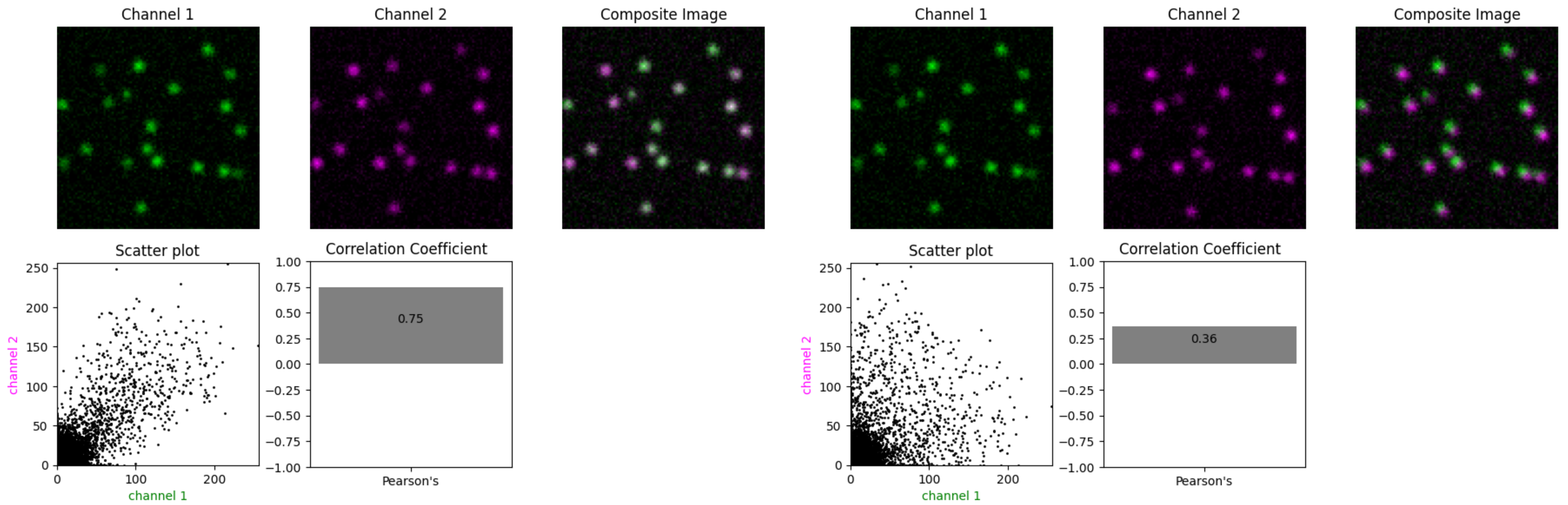
Uneven Illumination





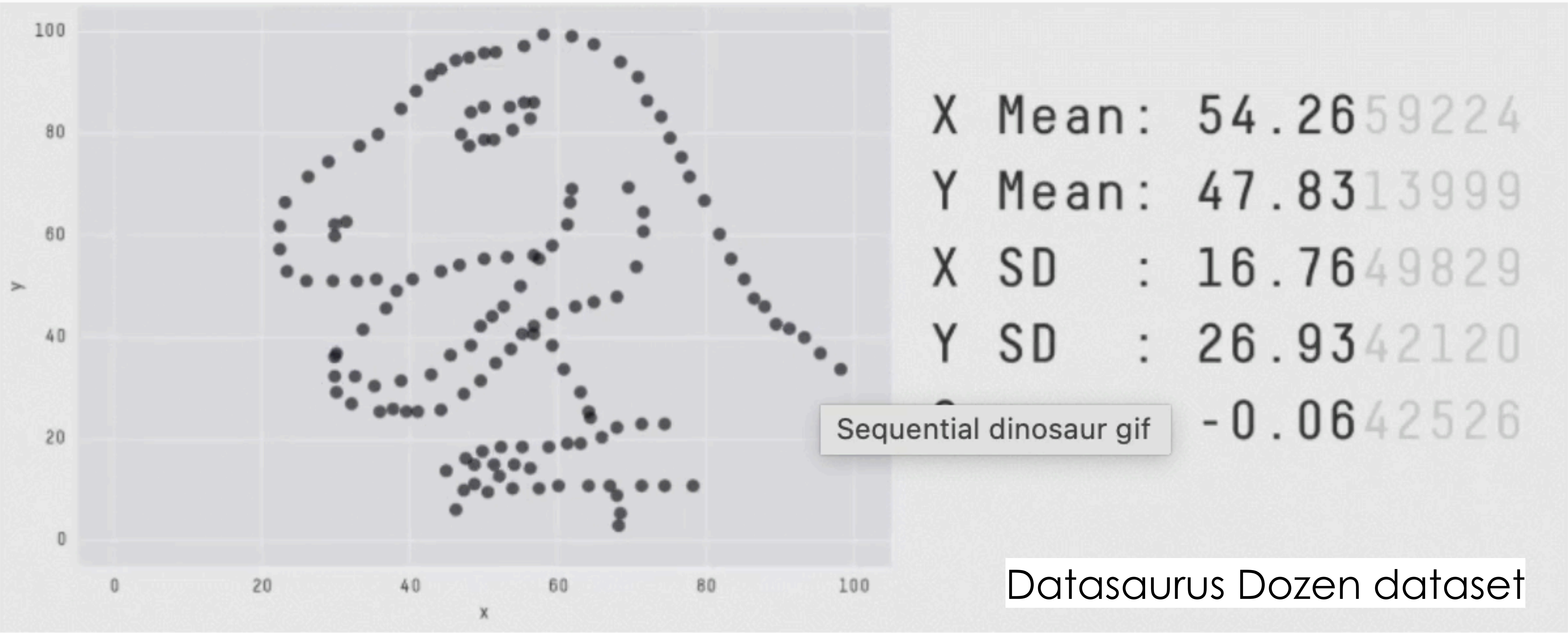
Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

Chromatic Shift



Data Interpretation

plot your data



Datasaurus Dozen dataset

https://www.research.autodesk.com/publications/same-stats-different-graphs/

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

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

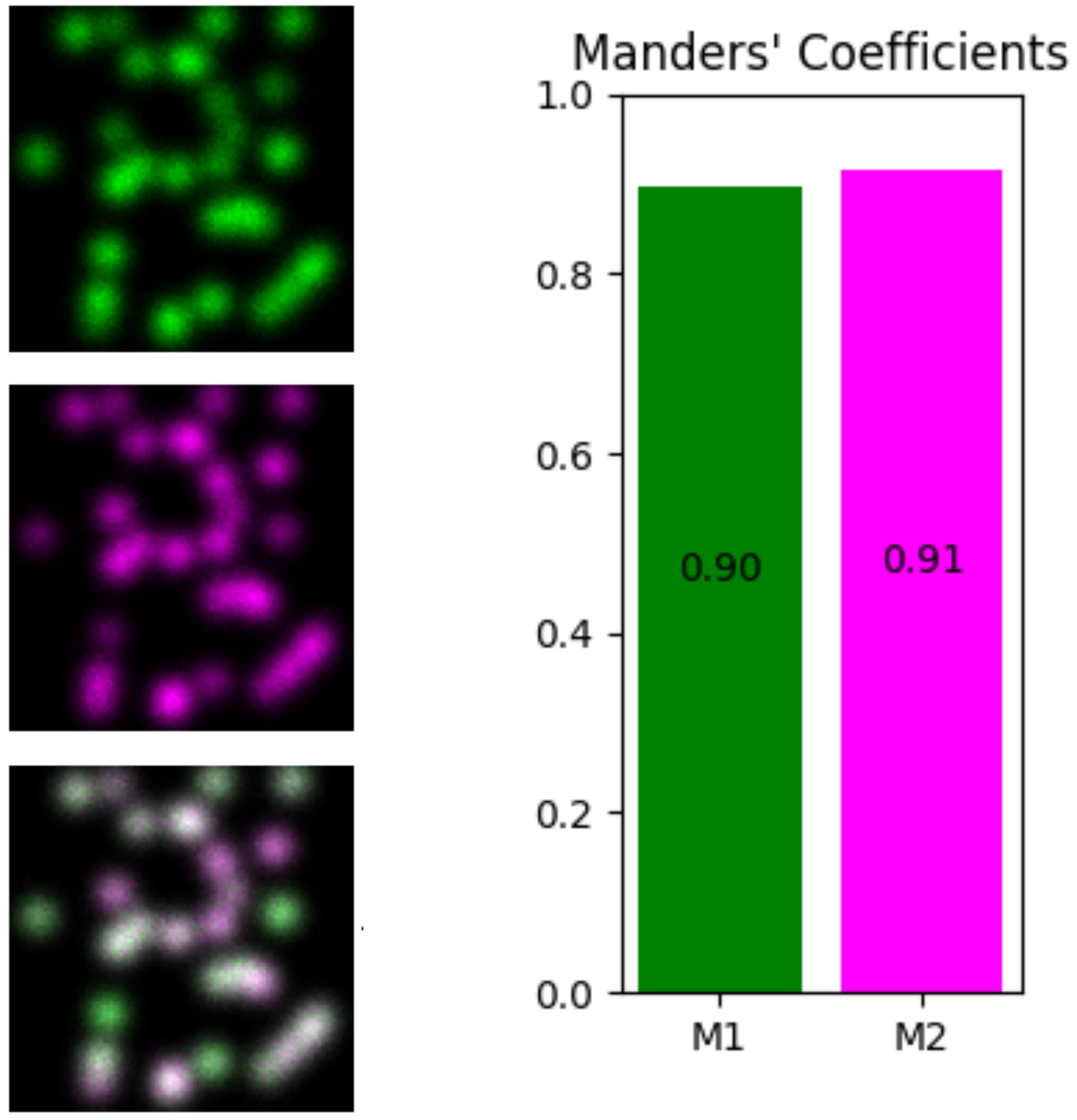
To measure the **proportion of pixel intensity** in one channel **that overlaps with pixel intensity** in the other channel.

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

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

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

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



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

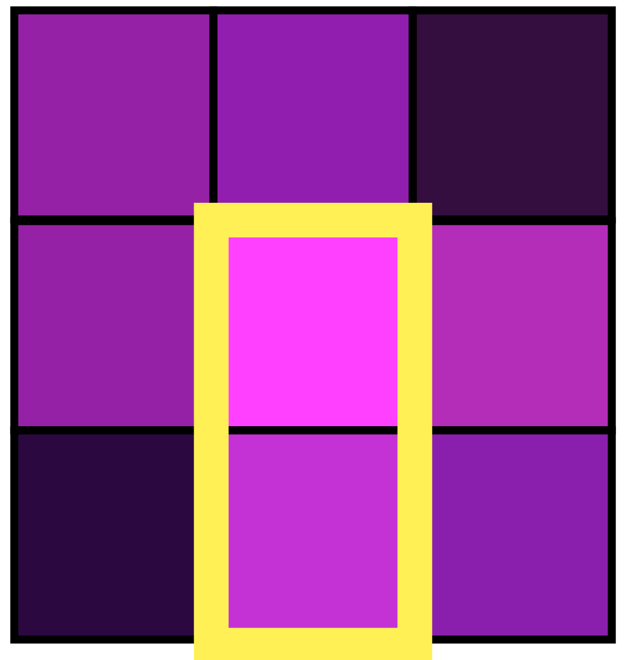
M2 = fraction of channel 2 that co-occurs with channel 1

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

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

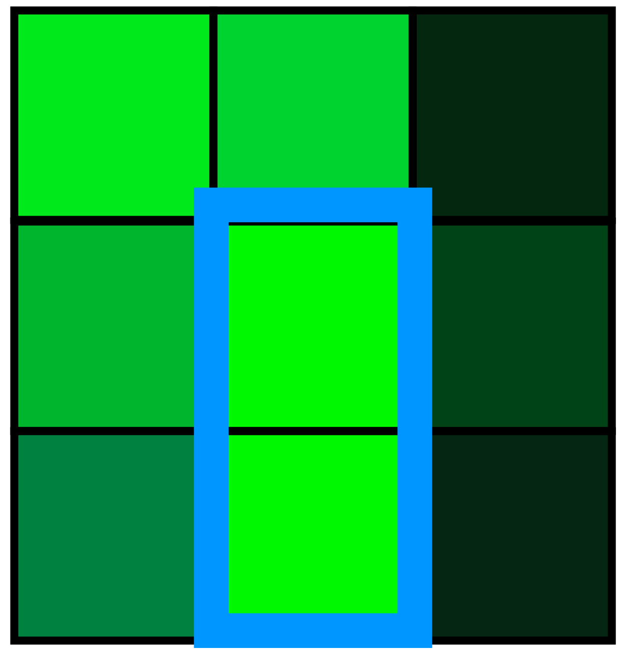
Set a threshold for channel 1:
consider only pixel with a **value > 75**

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



60	65	10
60	200	100
5	90	76

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



100	90	5
60	150	10
50	150	6

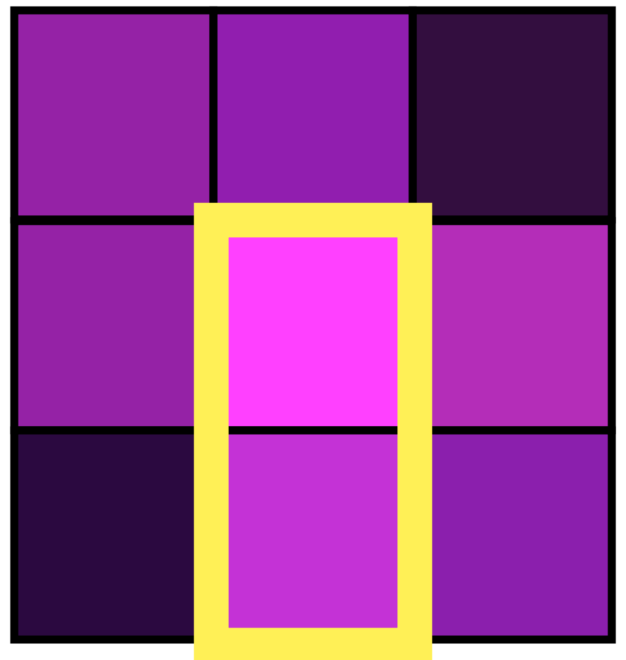
$$\textbf{M2} = \frac{150+150=\mathbf{300}}{100+90+60+150+50+150=\mathbf{600}} = \mathbf{0.5}$$

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

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

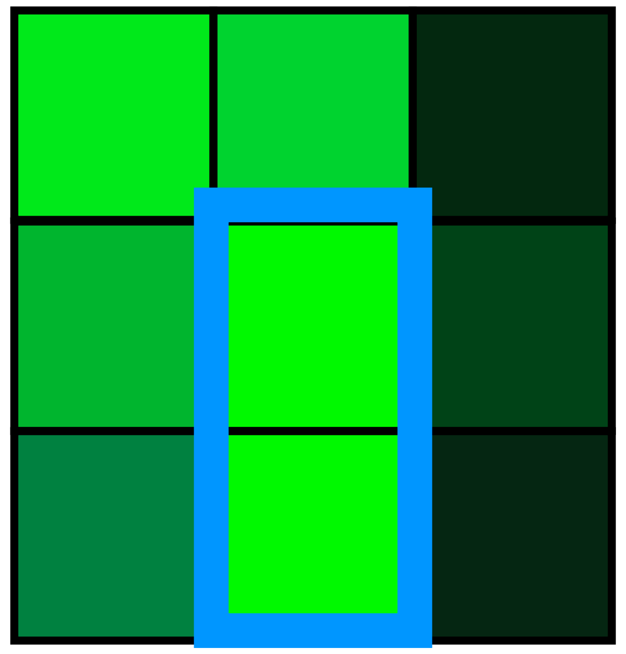
Set a threshold for channel 1:
consider only pixel with a value > 75

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



60	65	10
60	200	100
5	90	76

M1 = 0.62



100	90	5
60	150	10
50	150	6

M2 = 0.5

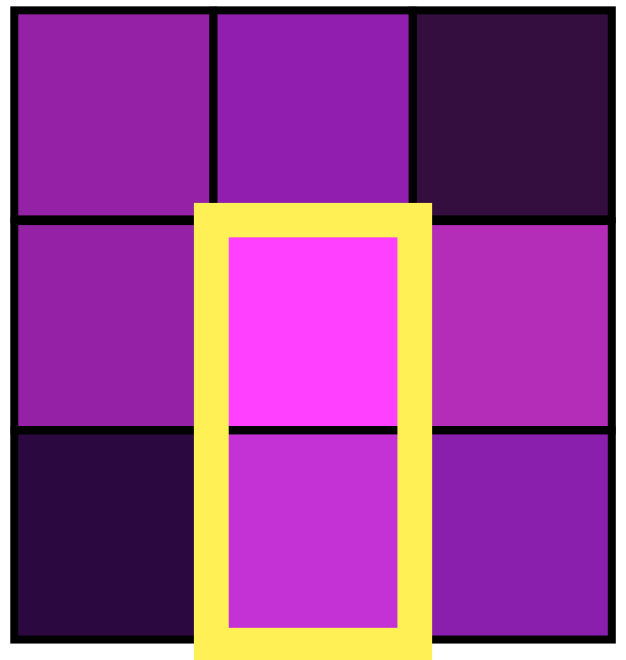
- Mander's **M1** and **M2 can be different** from each other

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

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

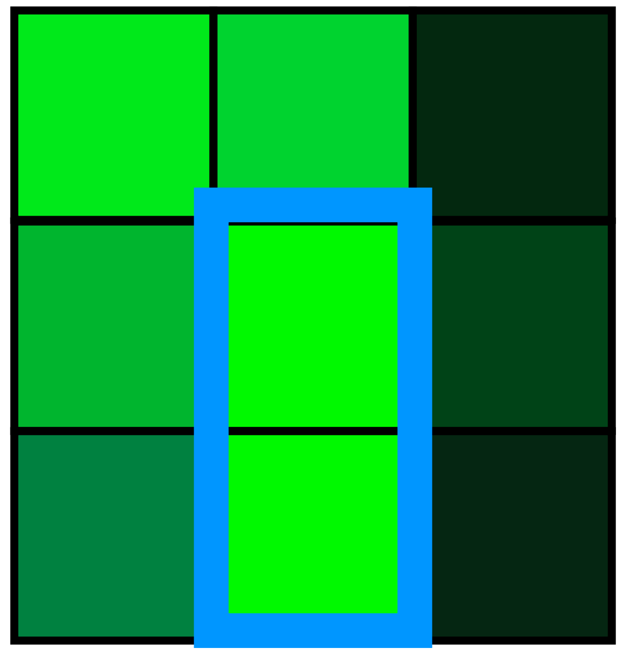
Set a threshold for channel 1:
consider only pixel with a value > 75

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



60	65	10
60	200	100
5	90	76

M1 = 0.62



100	90	5
60	150	10
50	150	6

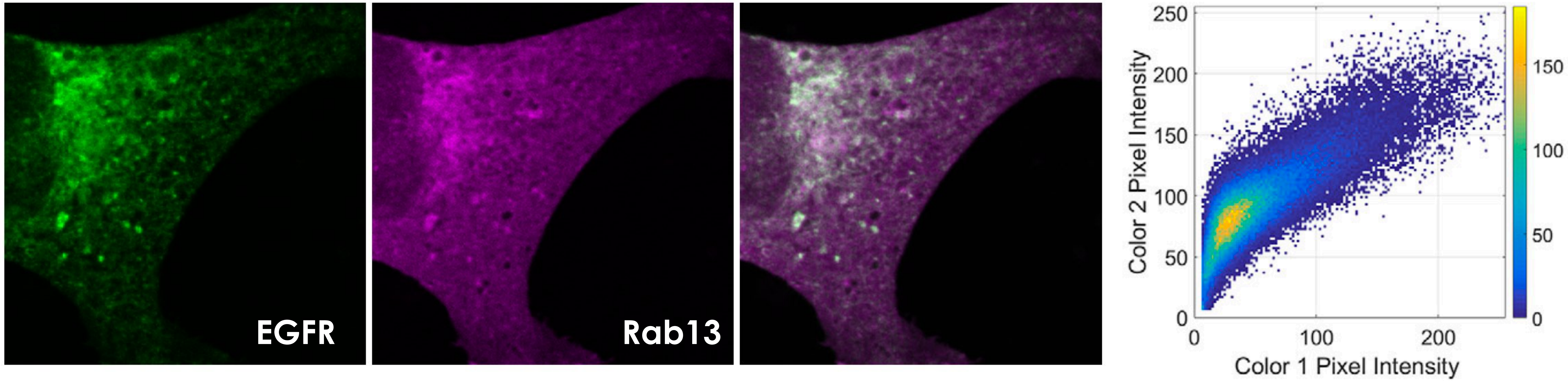
M2 = 0.5

- 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.

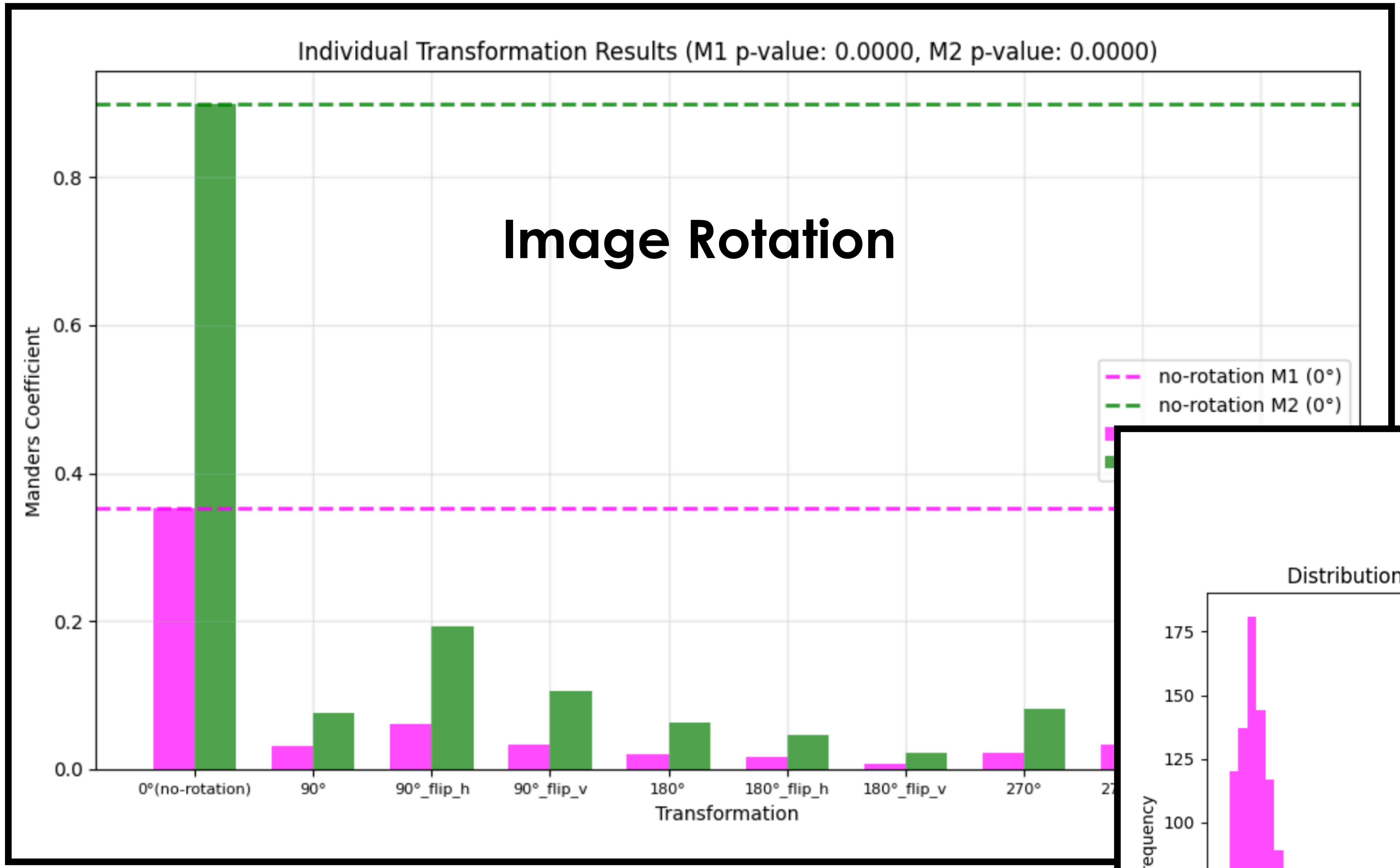
M1 = 0.99

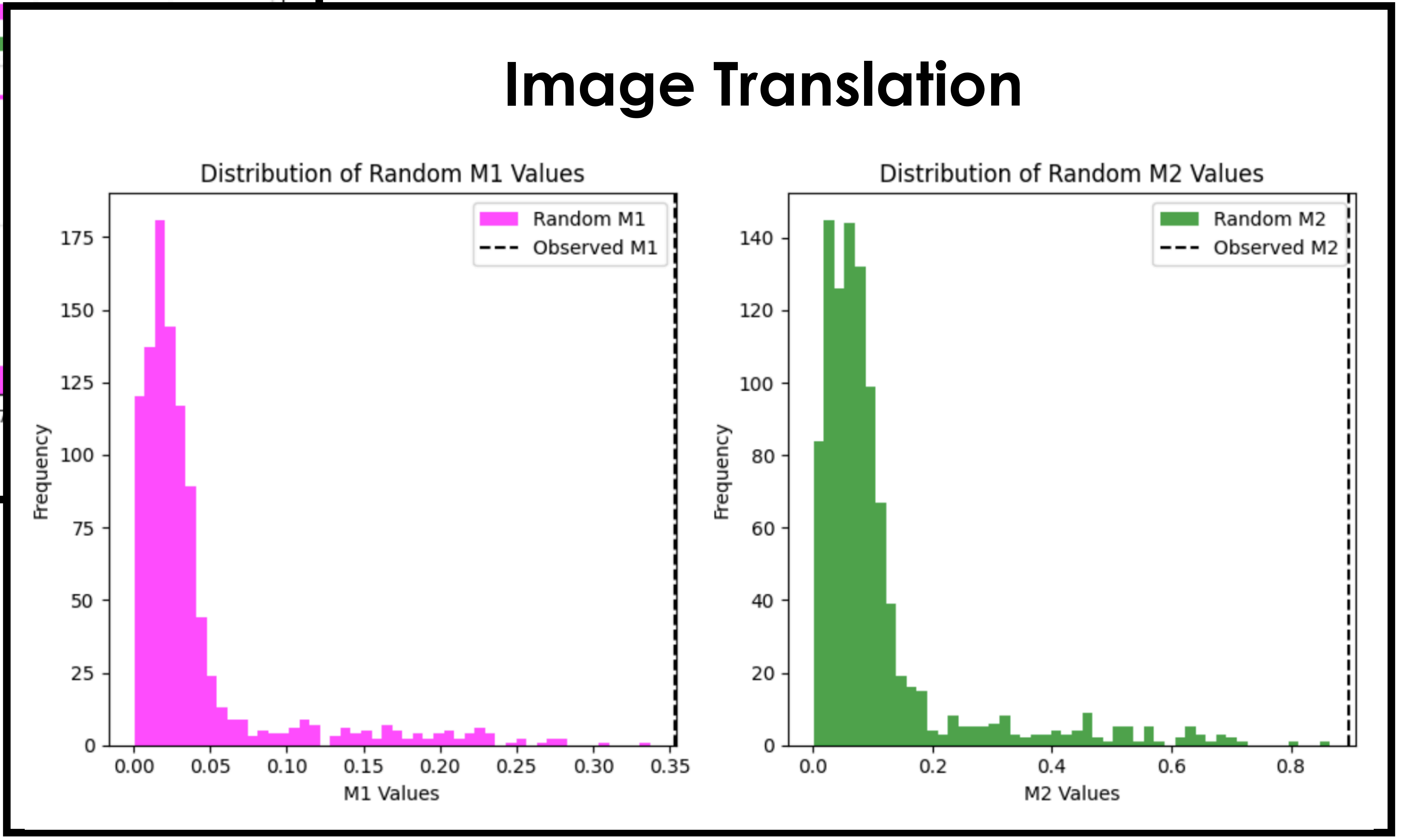
M2 = 0.44

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

Adapted form Aaron et al, 2018

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



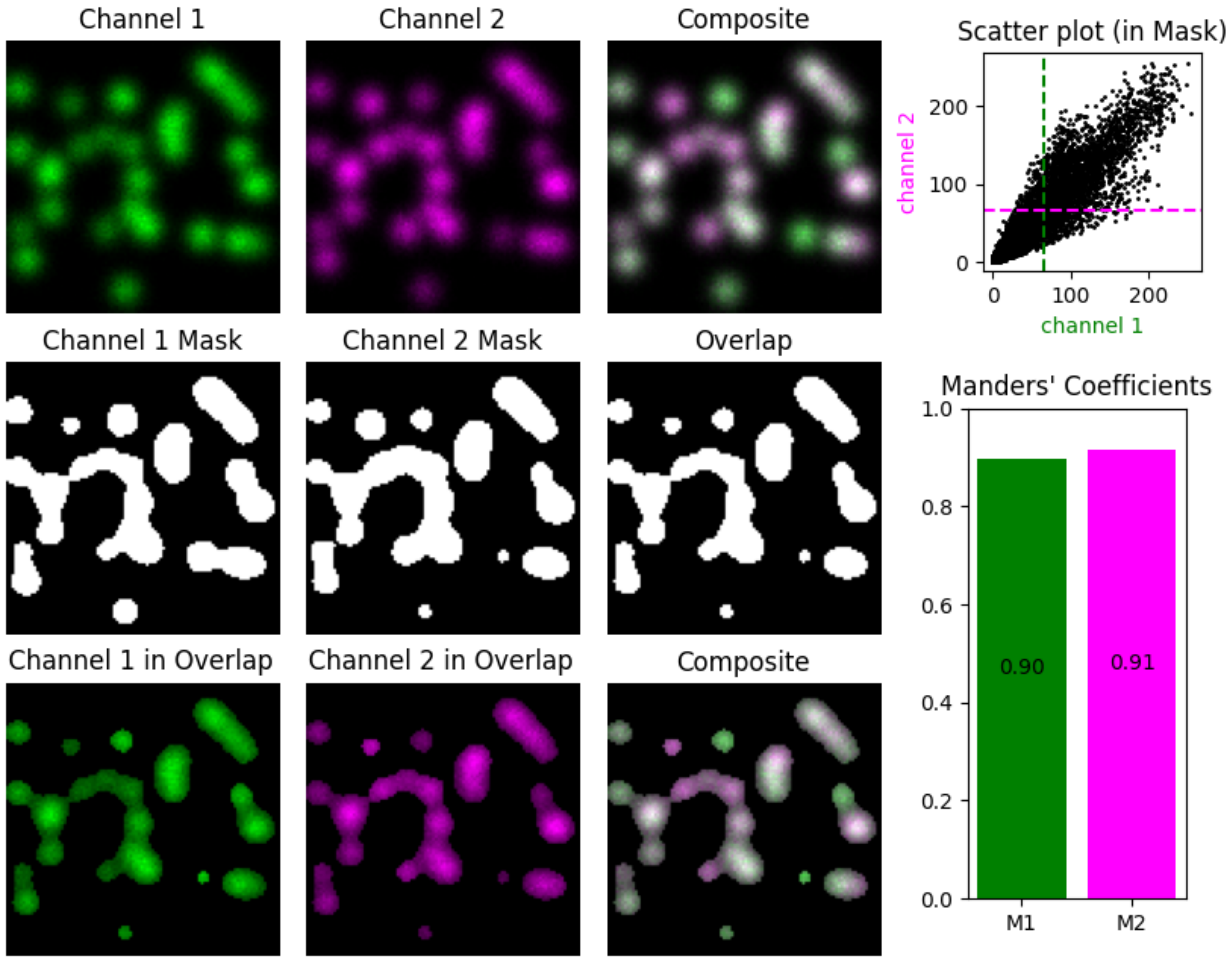


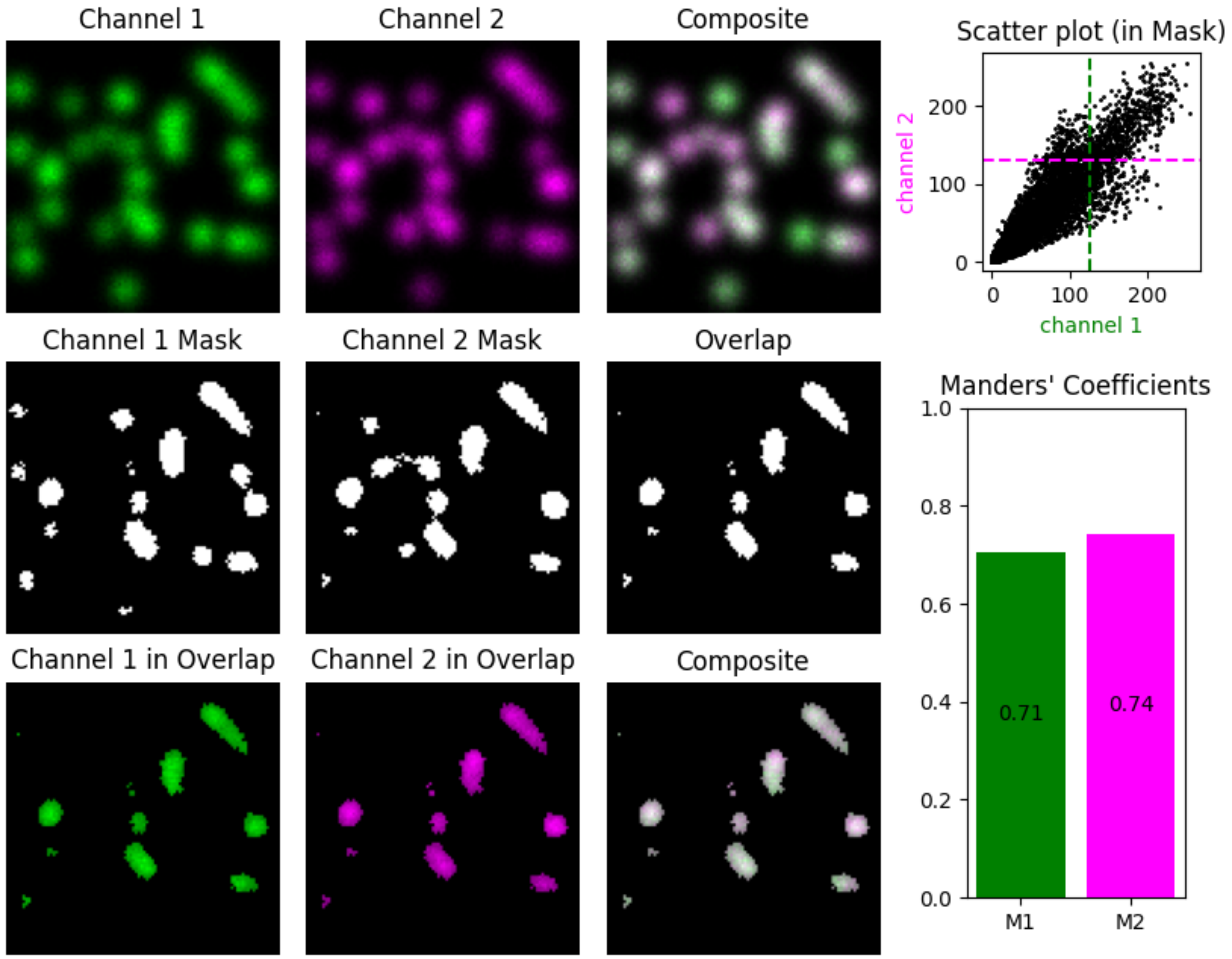
Block Scrambling

…

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

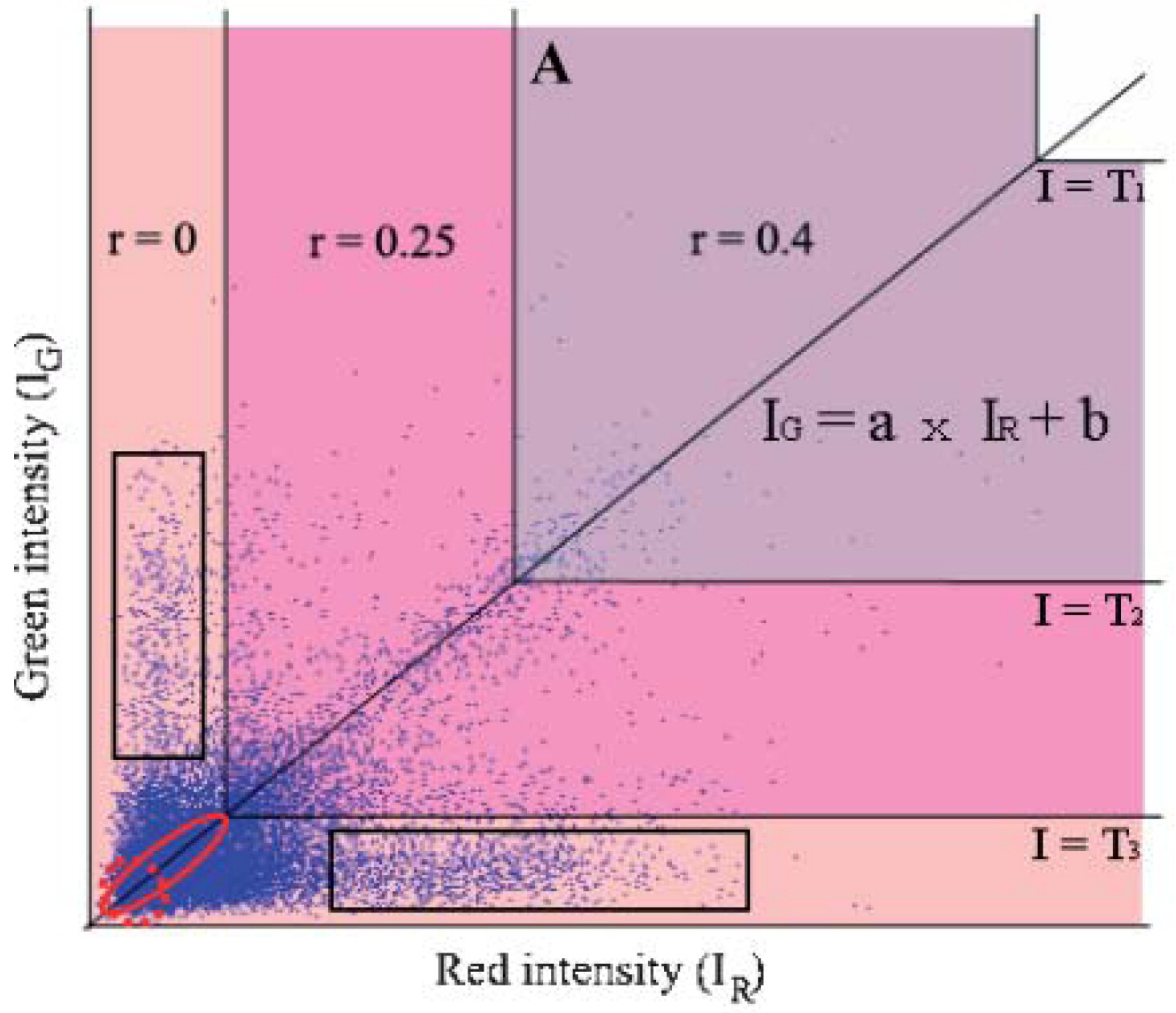
Highly **depends** on **threshold**





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

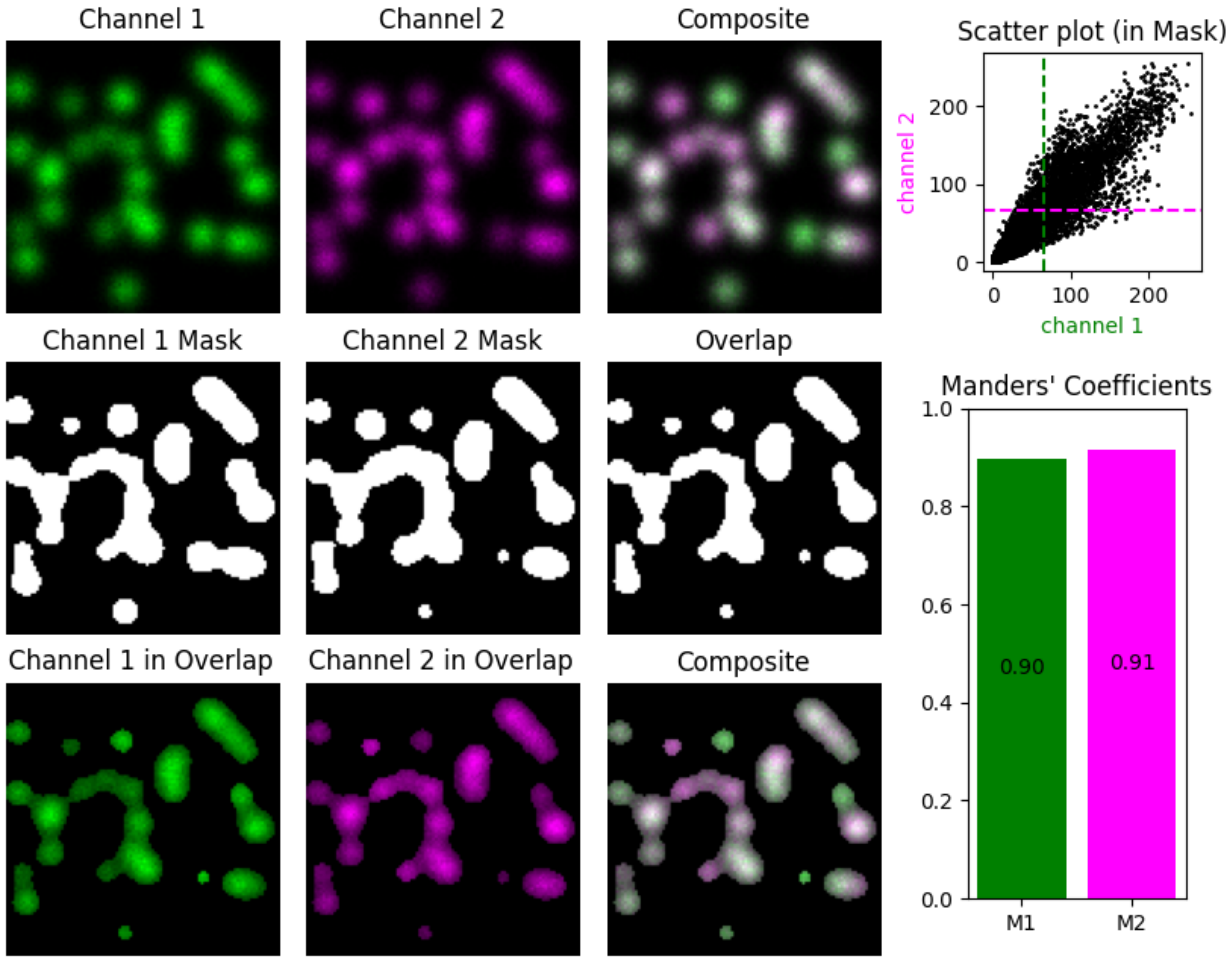
Costes Auto-Threshold

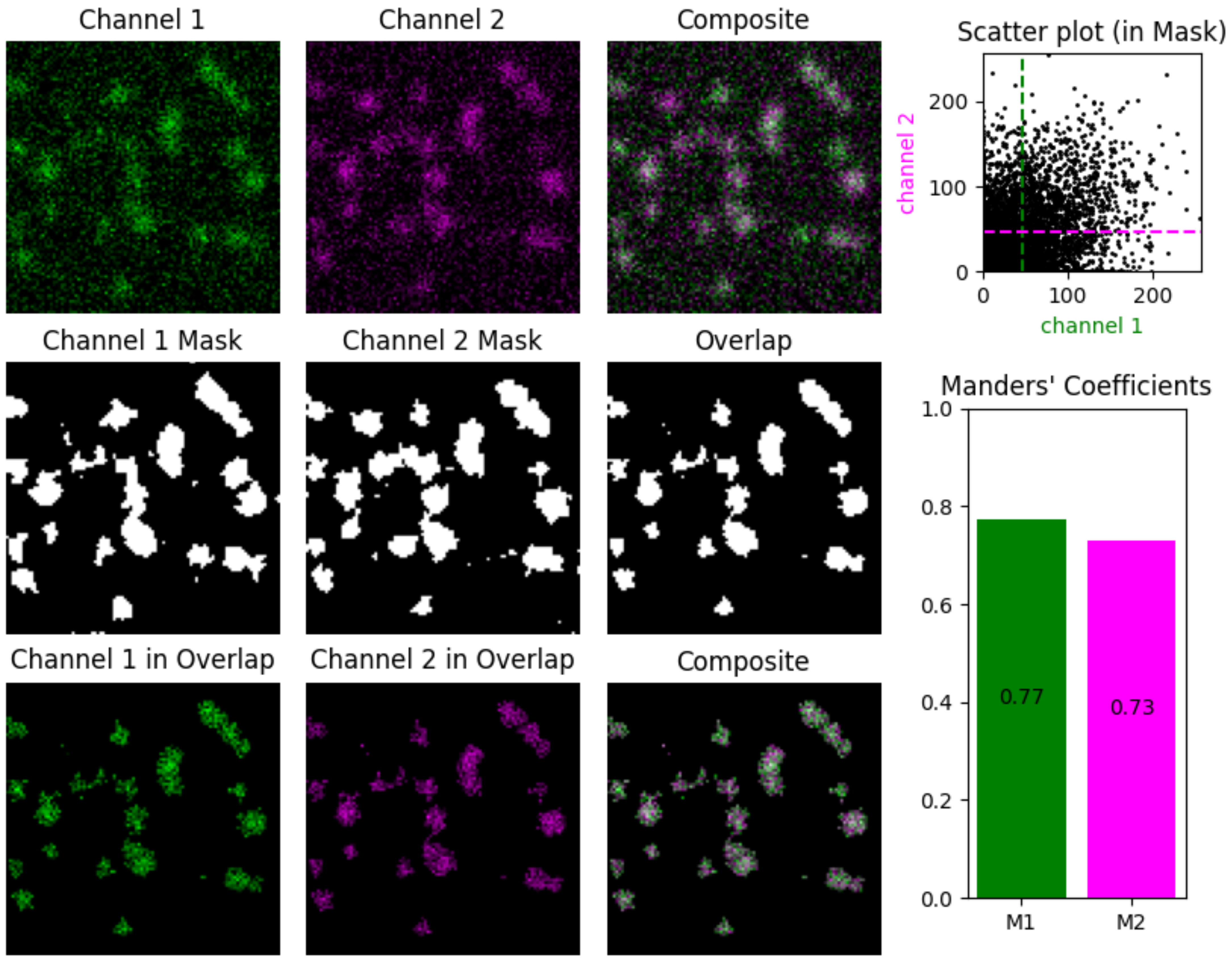


Costes et all., Automatic and Quantitative Measurement of Protein-Protein Colocalization in Live Cells

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

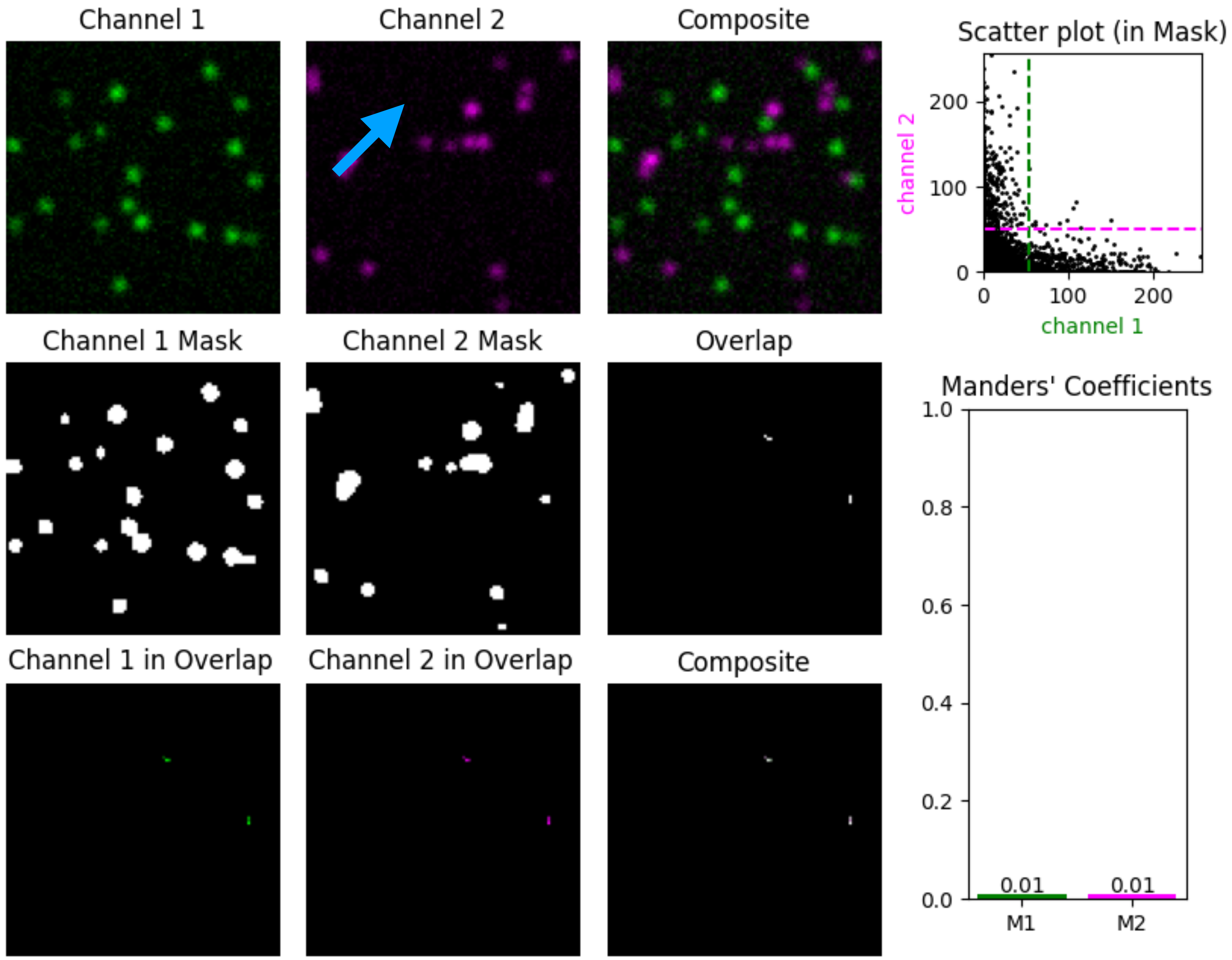
Noise

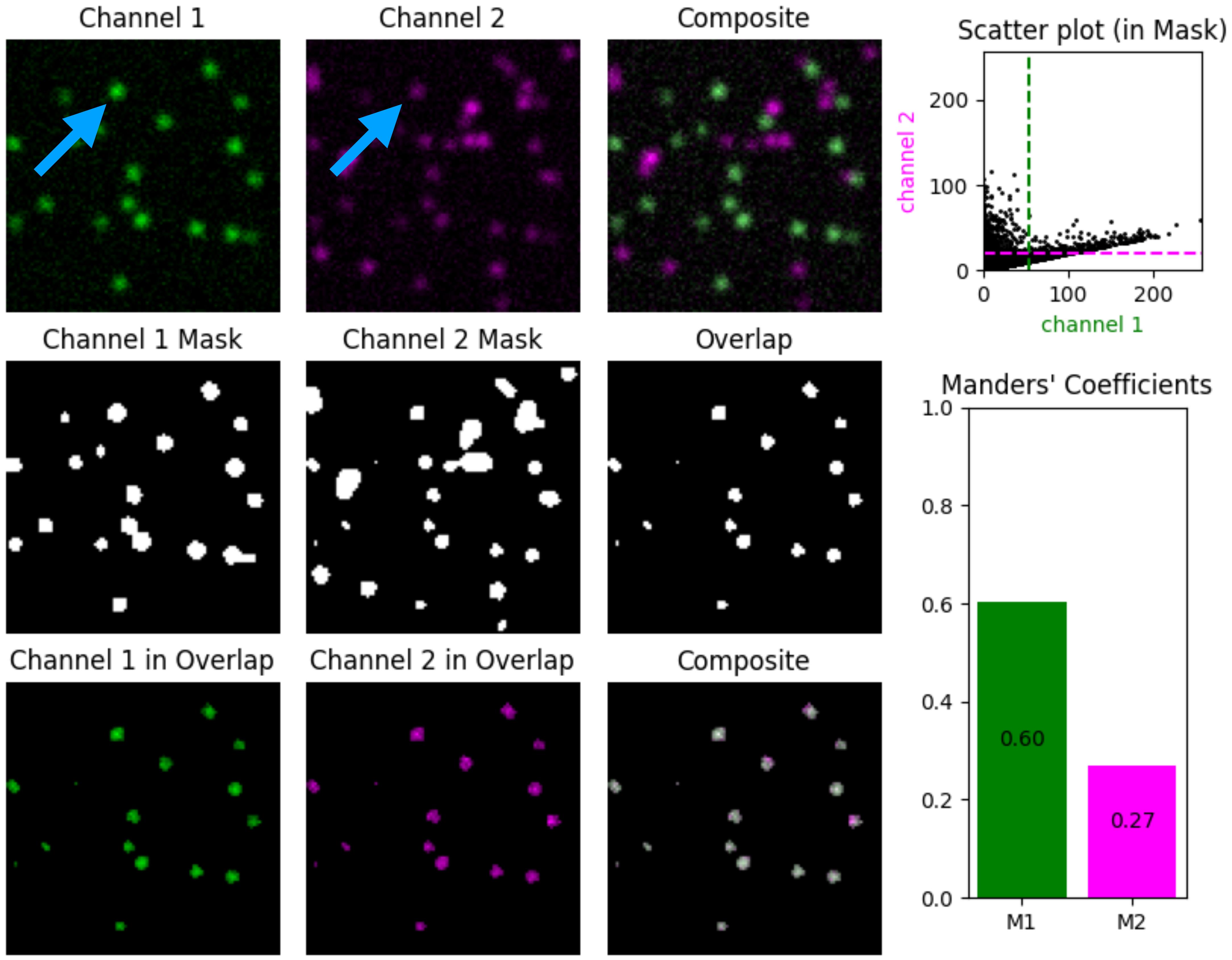




Intensity/Pixel-based: Mander's correlation coefficients (co-occurrence)

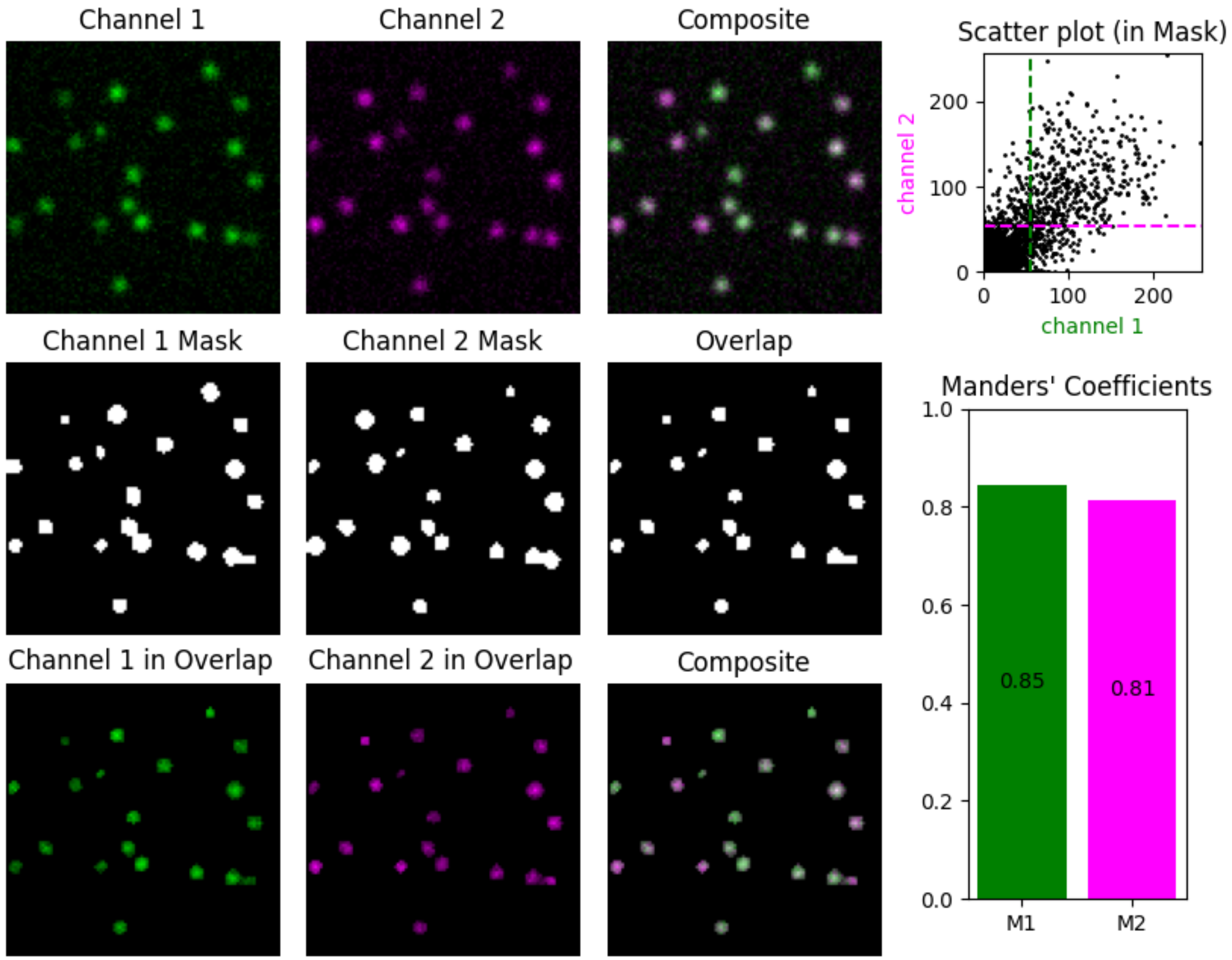
Bleedthrough

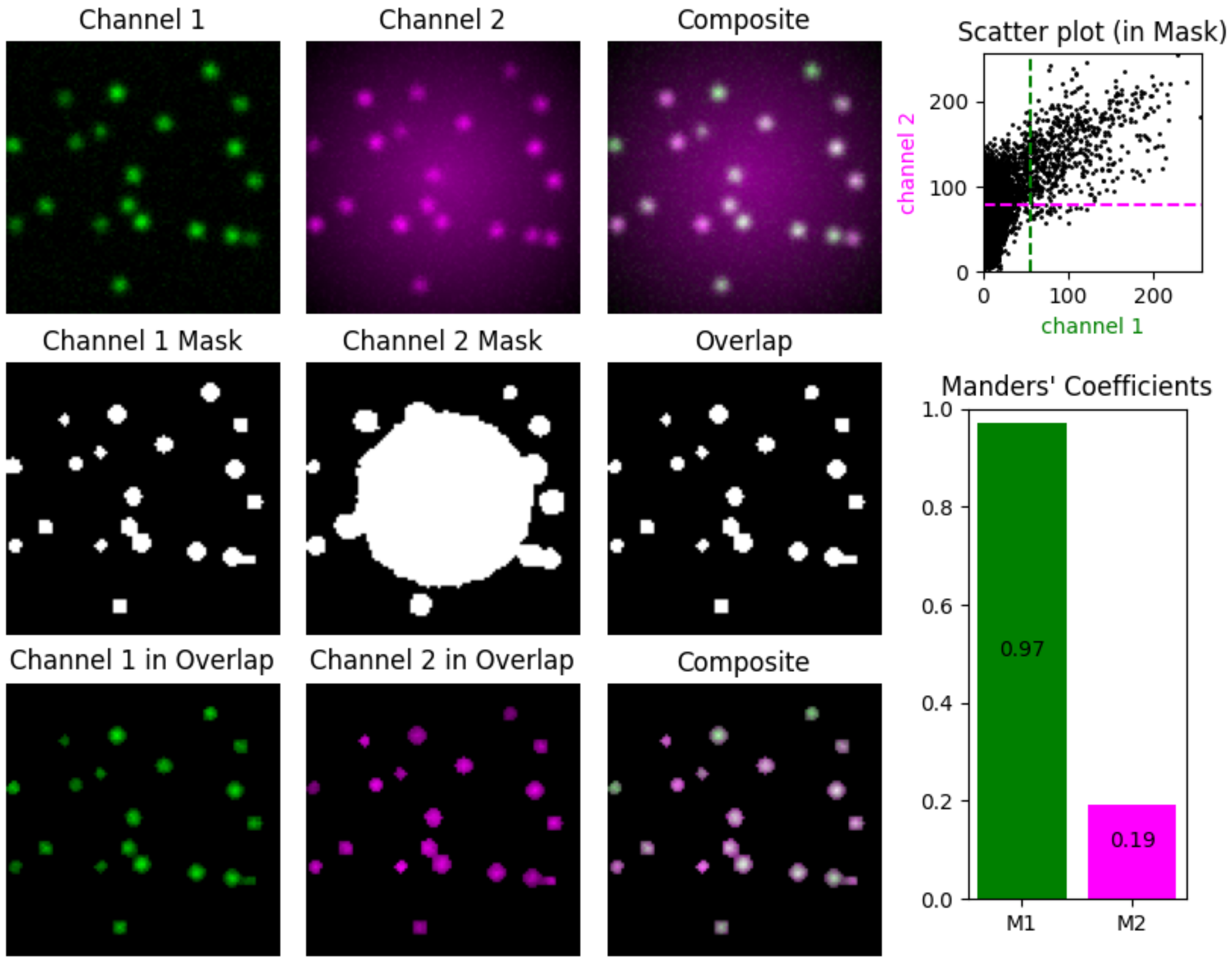




Intensity/Pixel-based: Mander’s correlation coefficients (co-occurrence)

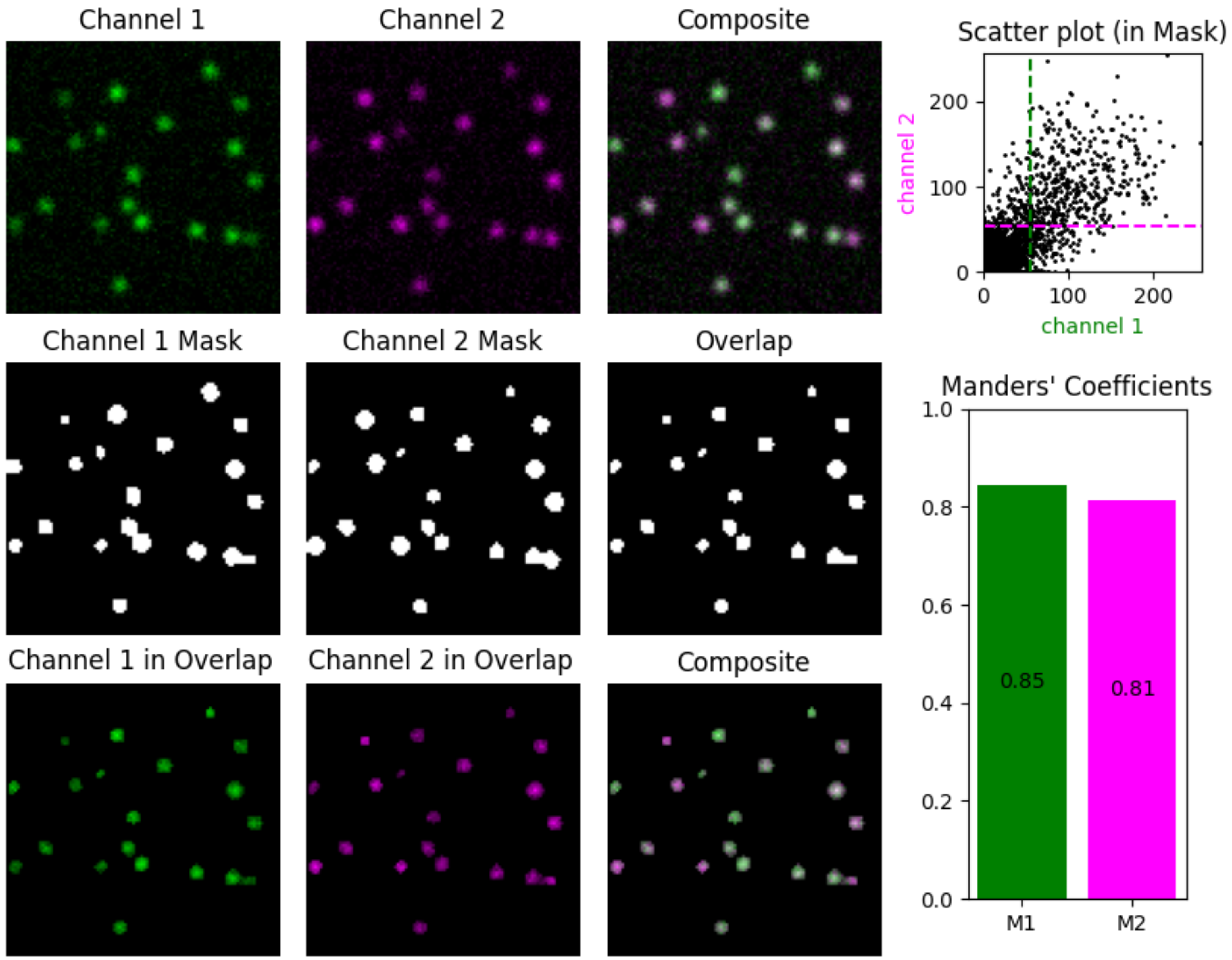
Uneven Illumination

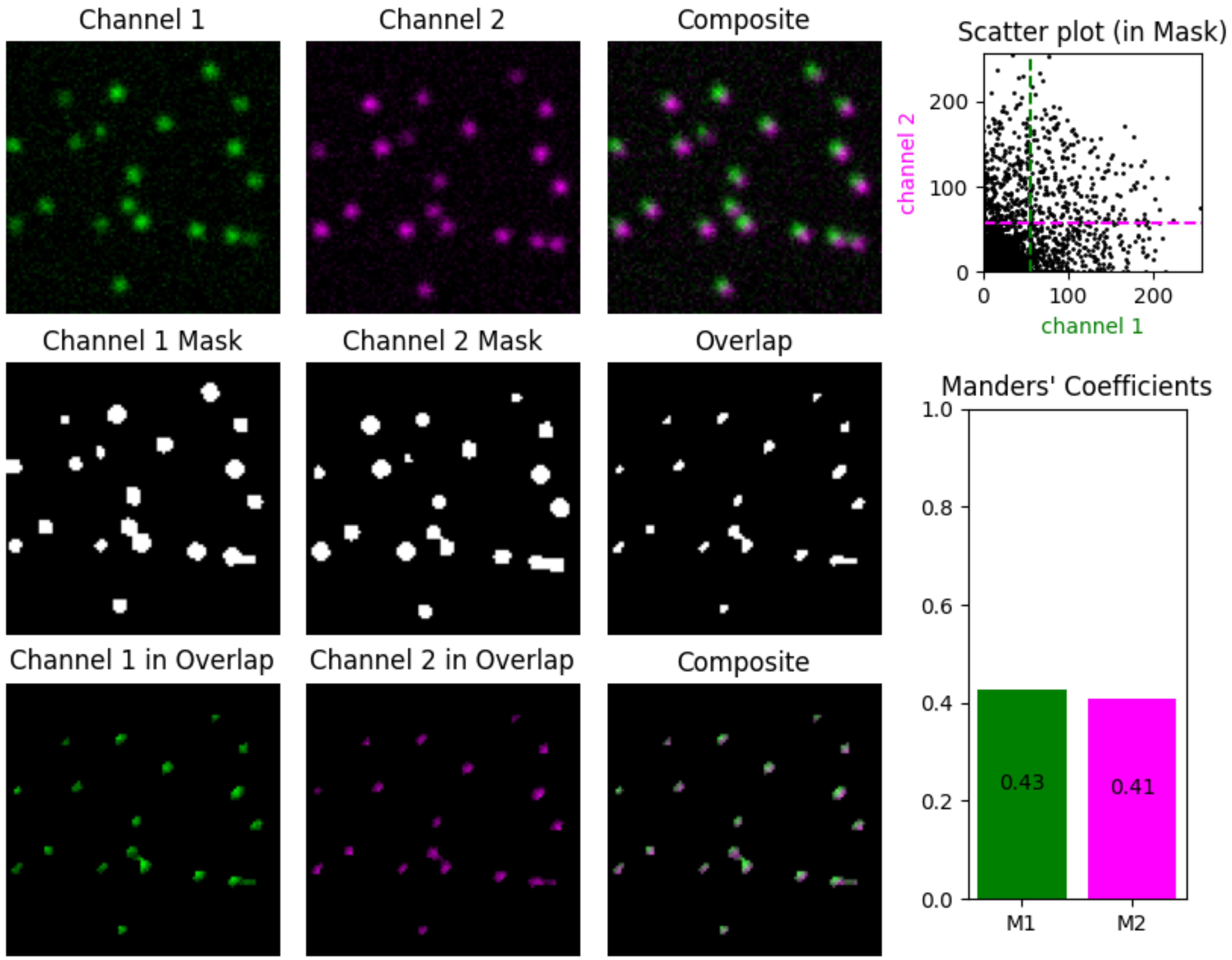




Intensity/Pixel-based: Mander’s correlation coefficients (co-occurrence)

Chromatic Shift





Summary

Coloclization 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.

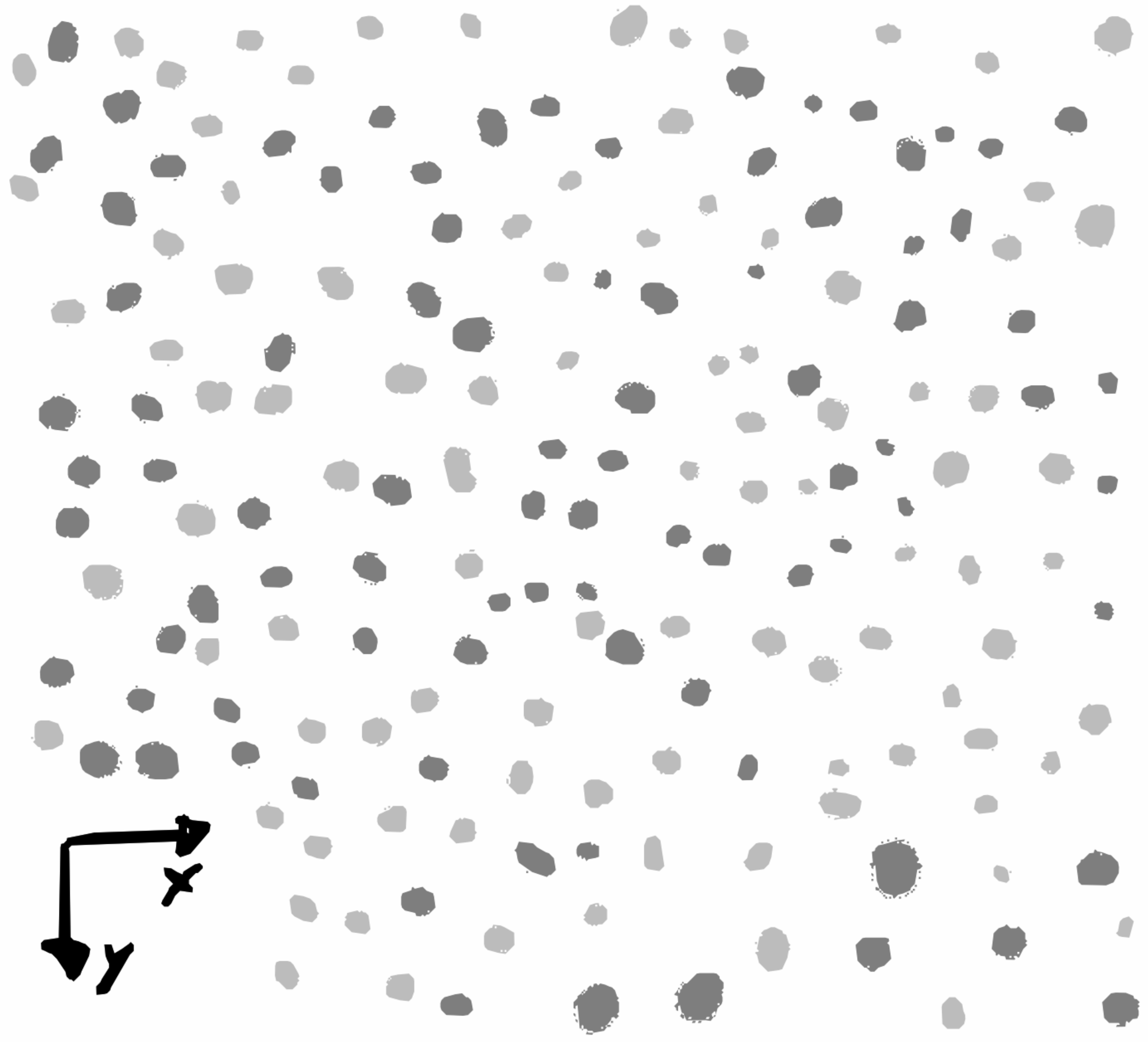
Report how you did the analysis ("Analysis was performed with ImageJ." is not a good way to report what you did)

Spatial statistics: Object-based colocalization

What are (2D) objects?

Points

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

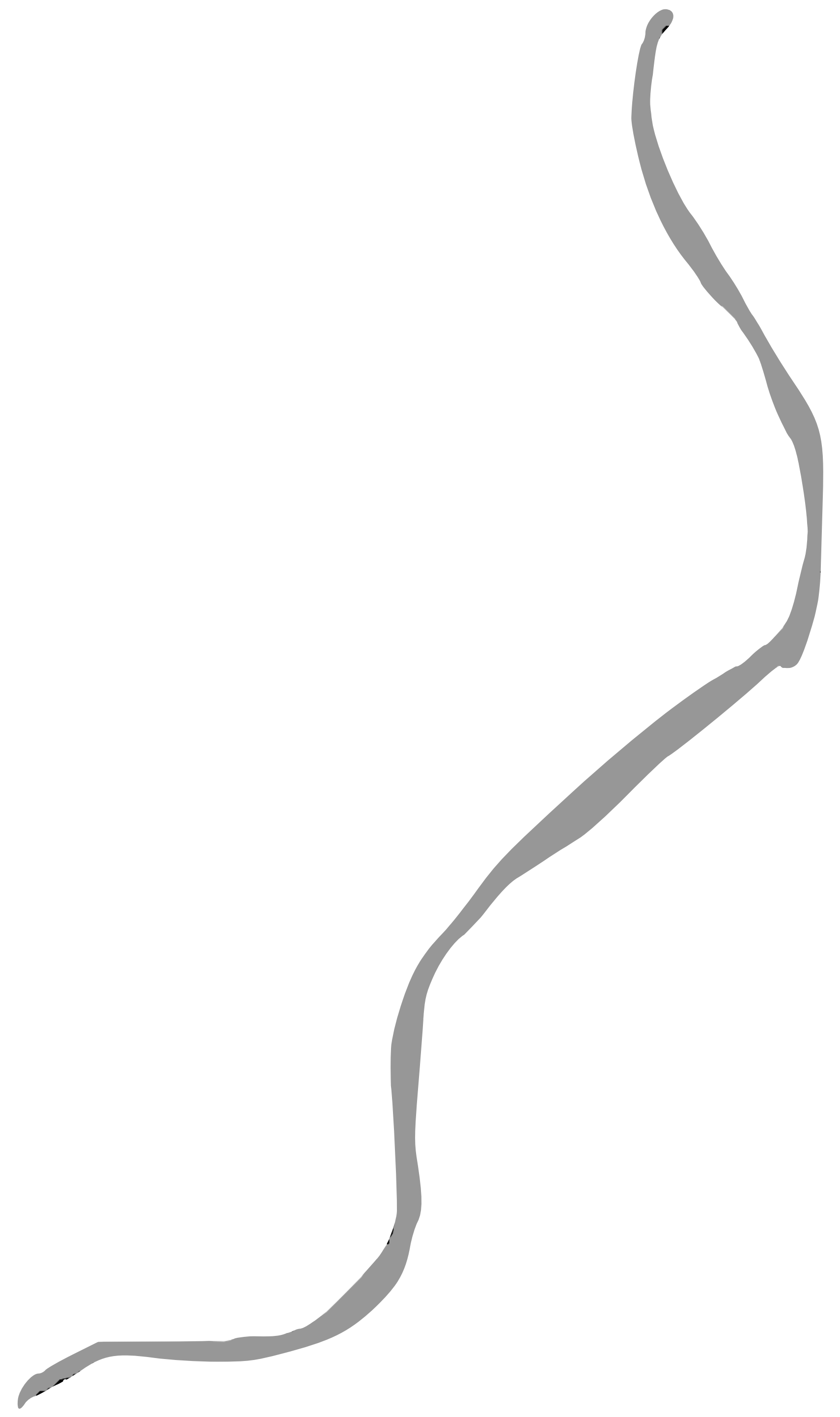


Extracting coordinates — examples:
Spotiflow: Dominguez Mantes, A., Herrera, A., Khven, I. *et al.* Spotiflow: accurate and efficient spot detection for fluorescence microscopy with deep stereographic flow regression. *Nat Methods* **22**, 1495–1504 (2025). https://doi.org/10.1038/s41592-025-02662-x
SMLM fitting in Python: E.g. https://github.com/ZhuangLab/storm-analysis

What are (2D) objects?

Lines

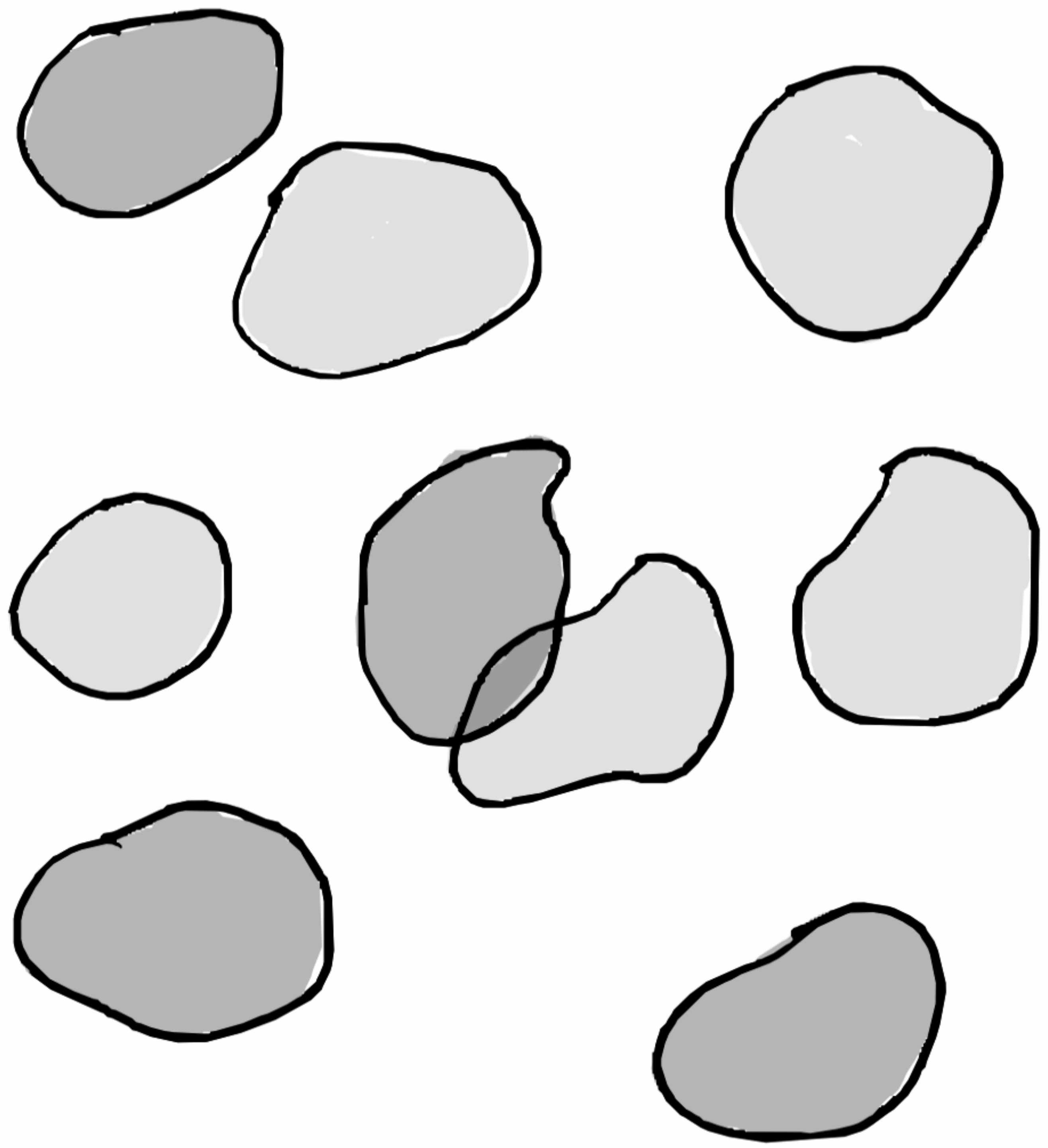
- Geometrically: length, but no width
- Obtained by segmentation
- Example: A tissue boundary



What are (2D) objects?

Bounded regions

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

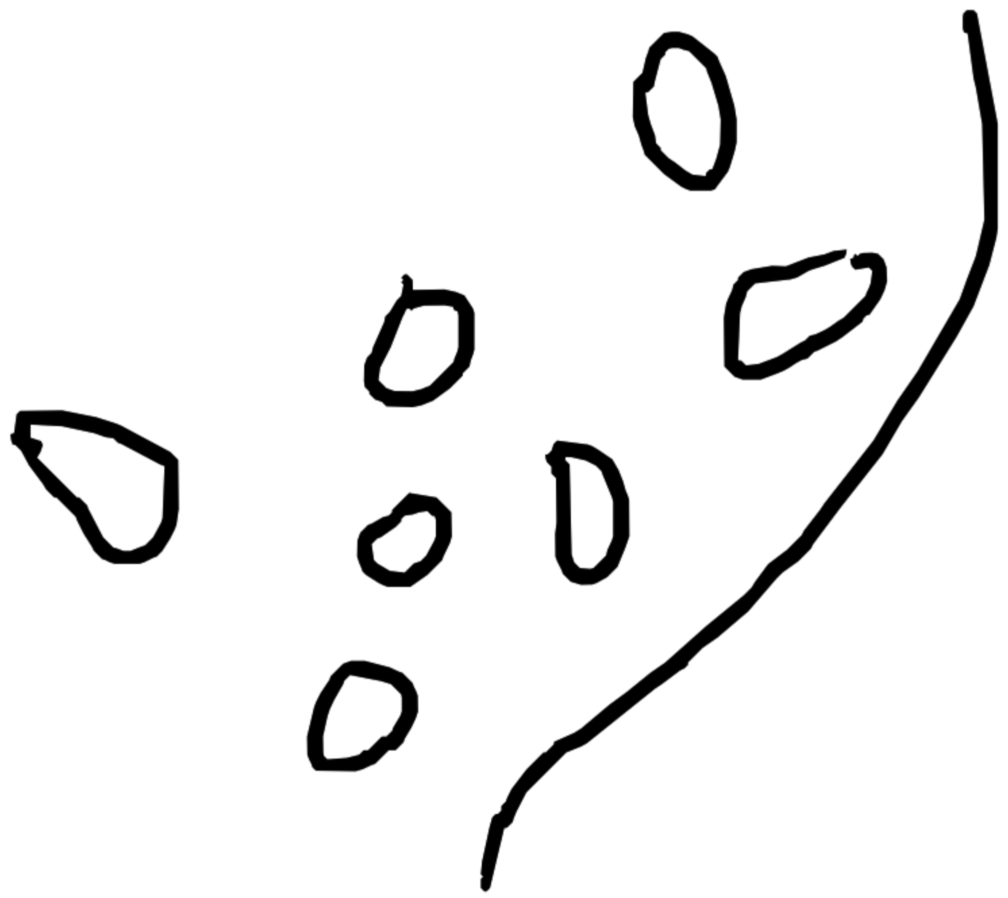


Cellpose-SAM: superhuman generalization for cellular segmentation
Marius Pachitariu, Michael Rariden, Carsen Stringer
bioRxiv 2025.04.28.651001; doi: https://doi.org/10.1101/2025.04.28.651001

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},

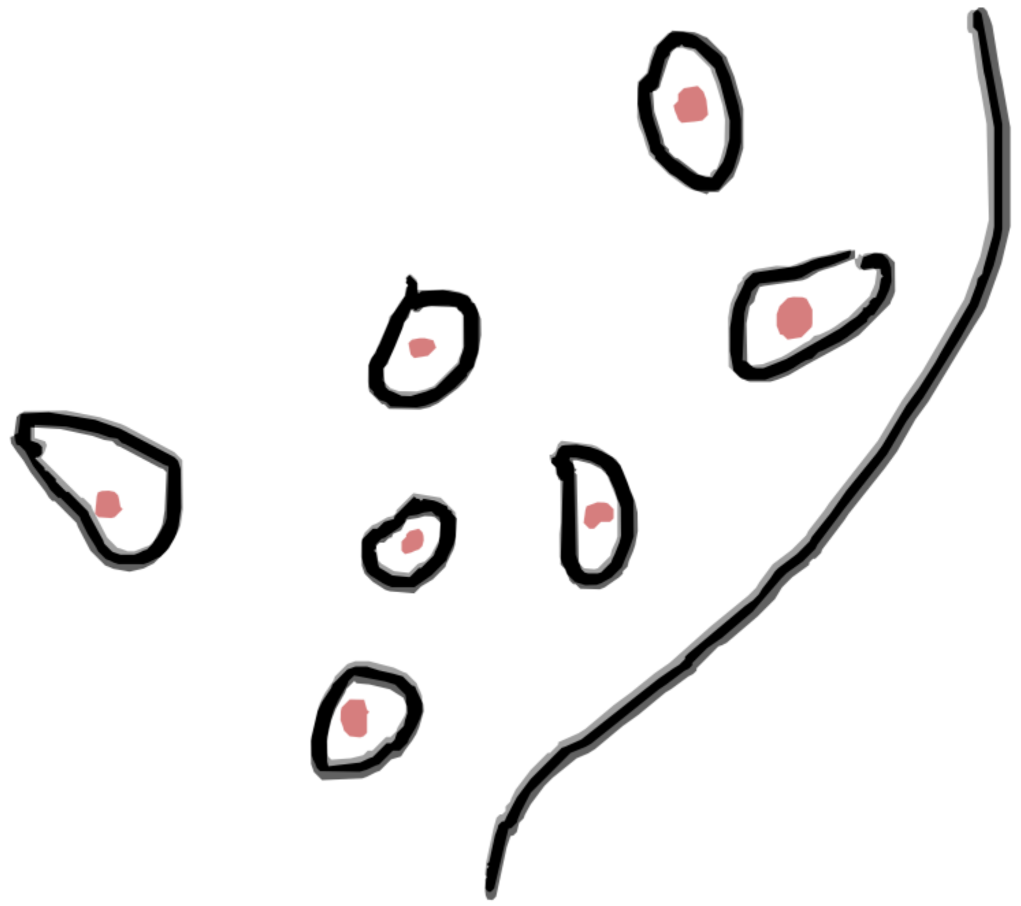
What are (2D) objects?

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



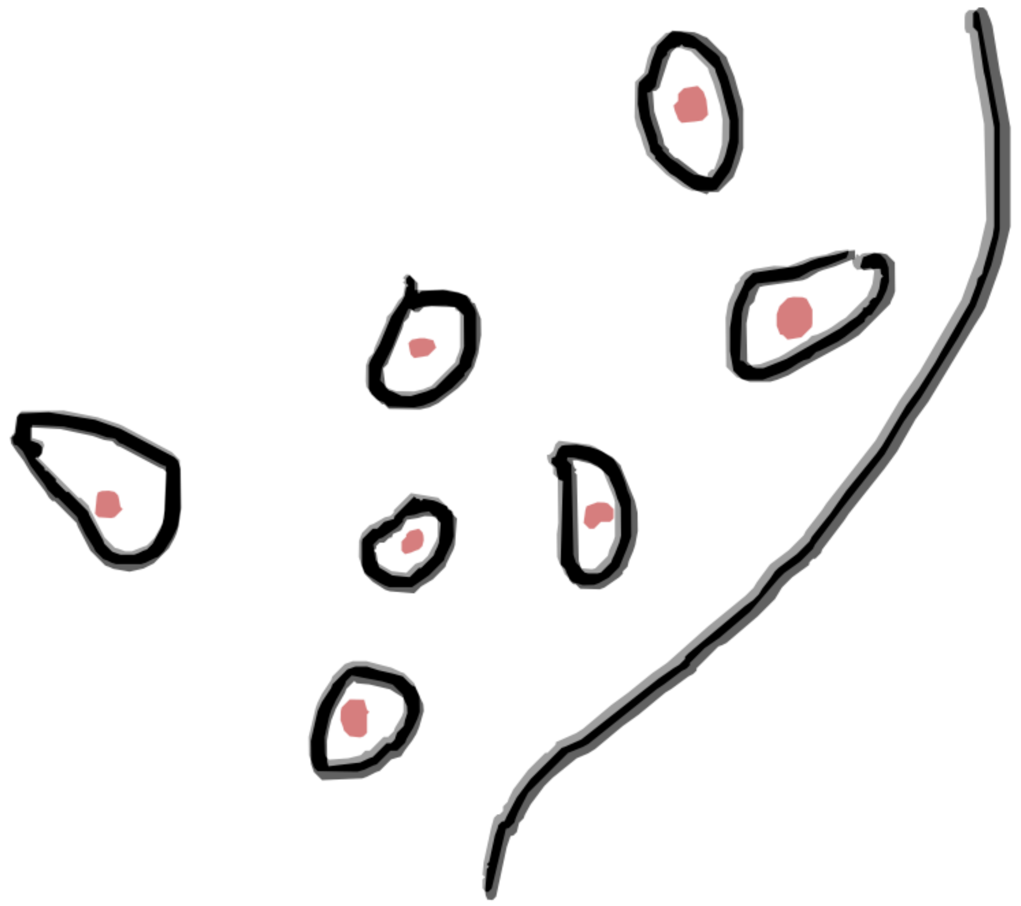
What are (2D) objects?

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

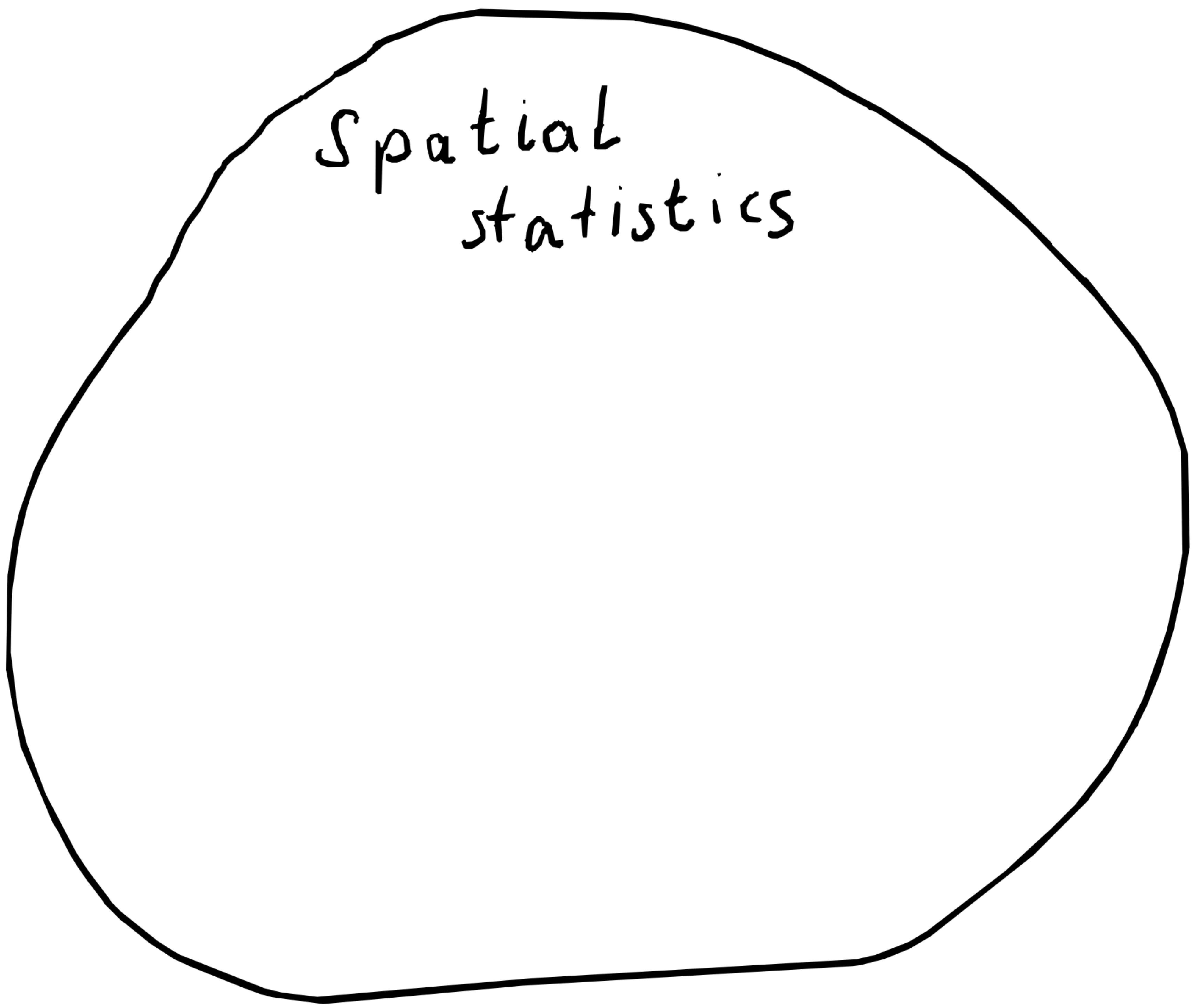


What are (2D) objects?

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

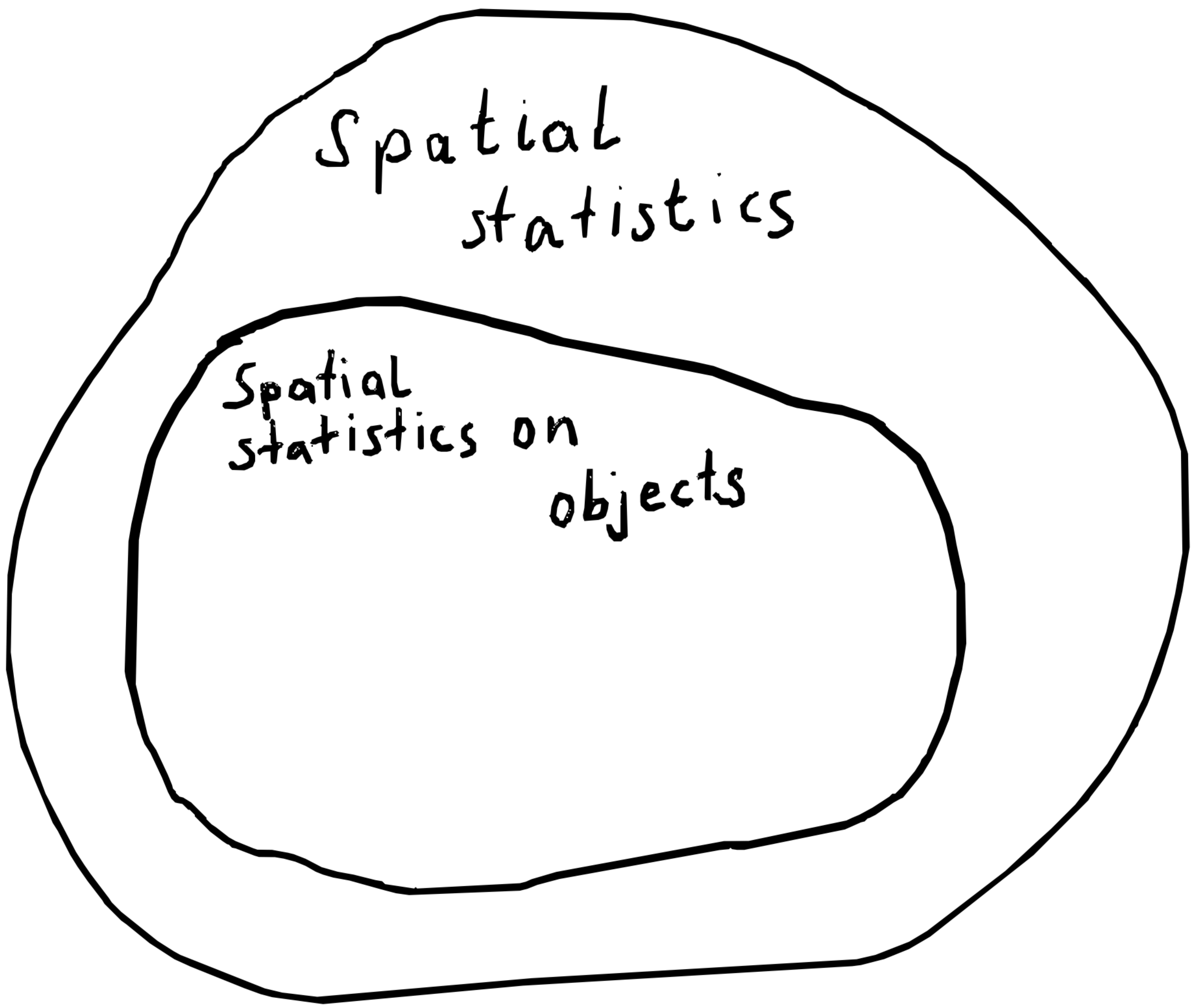


What is “object-based” colocalization

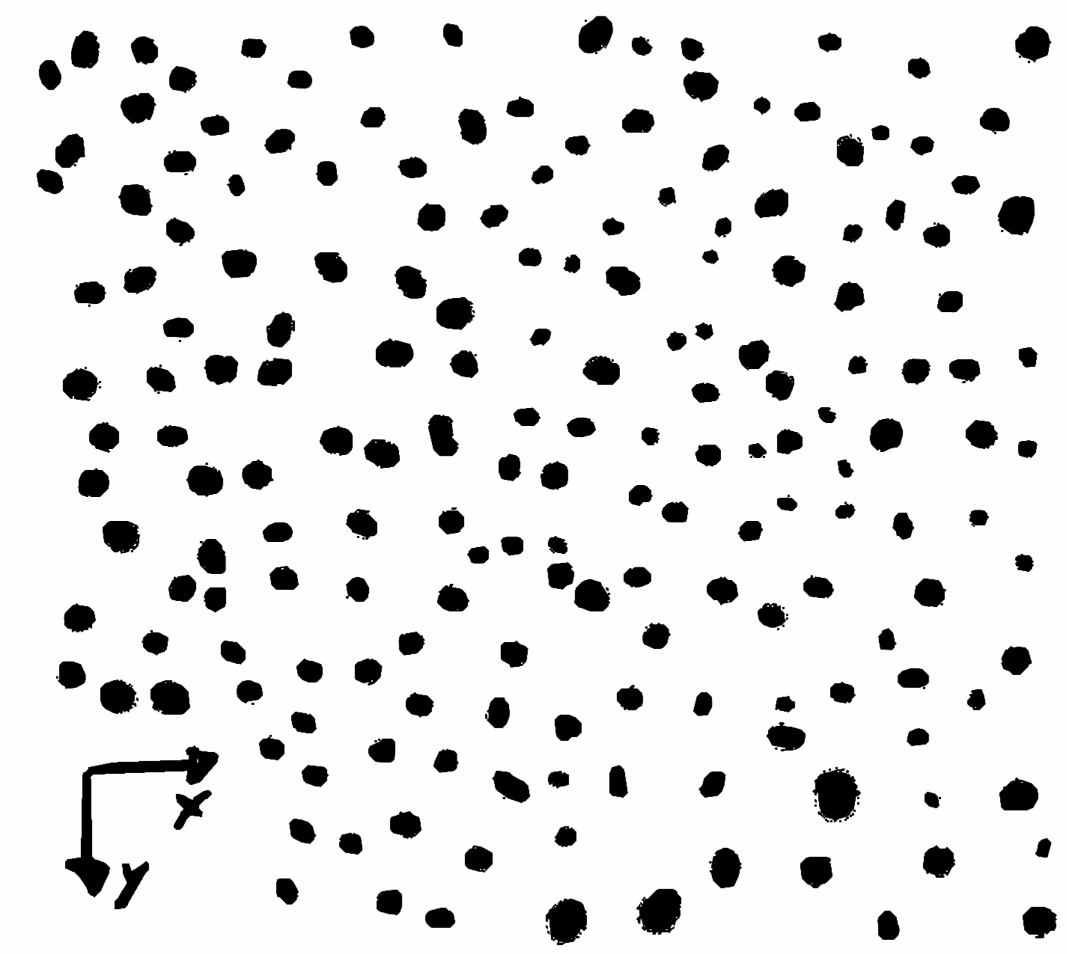


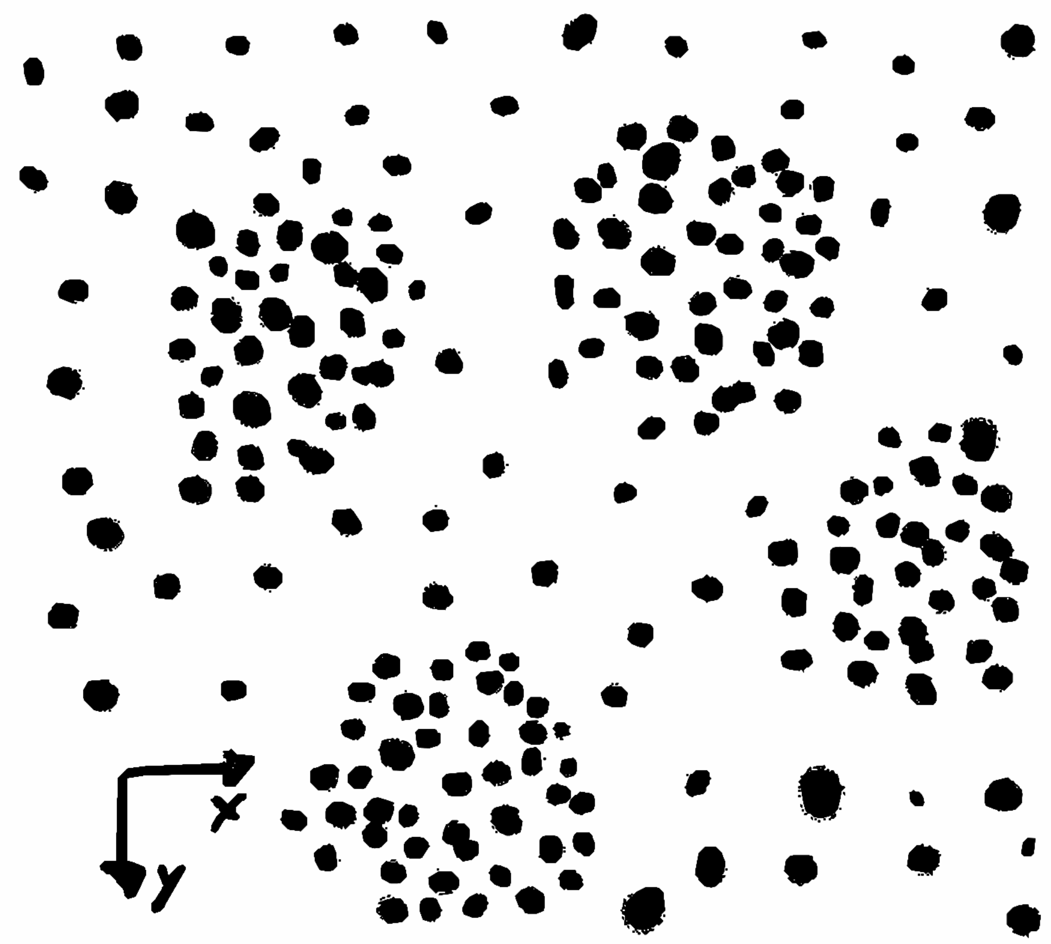
- Spatial statistics deals with spatial data

What is “object-based” colocalization

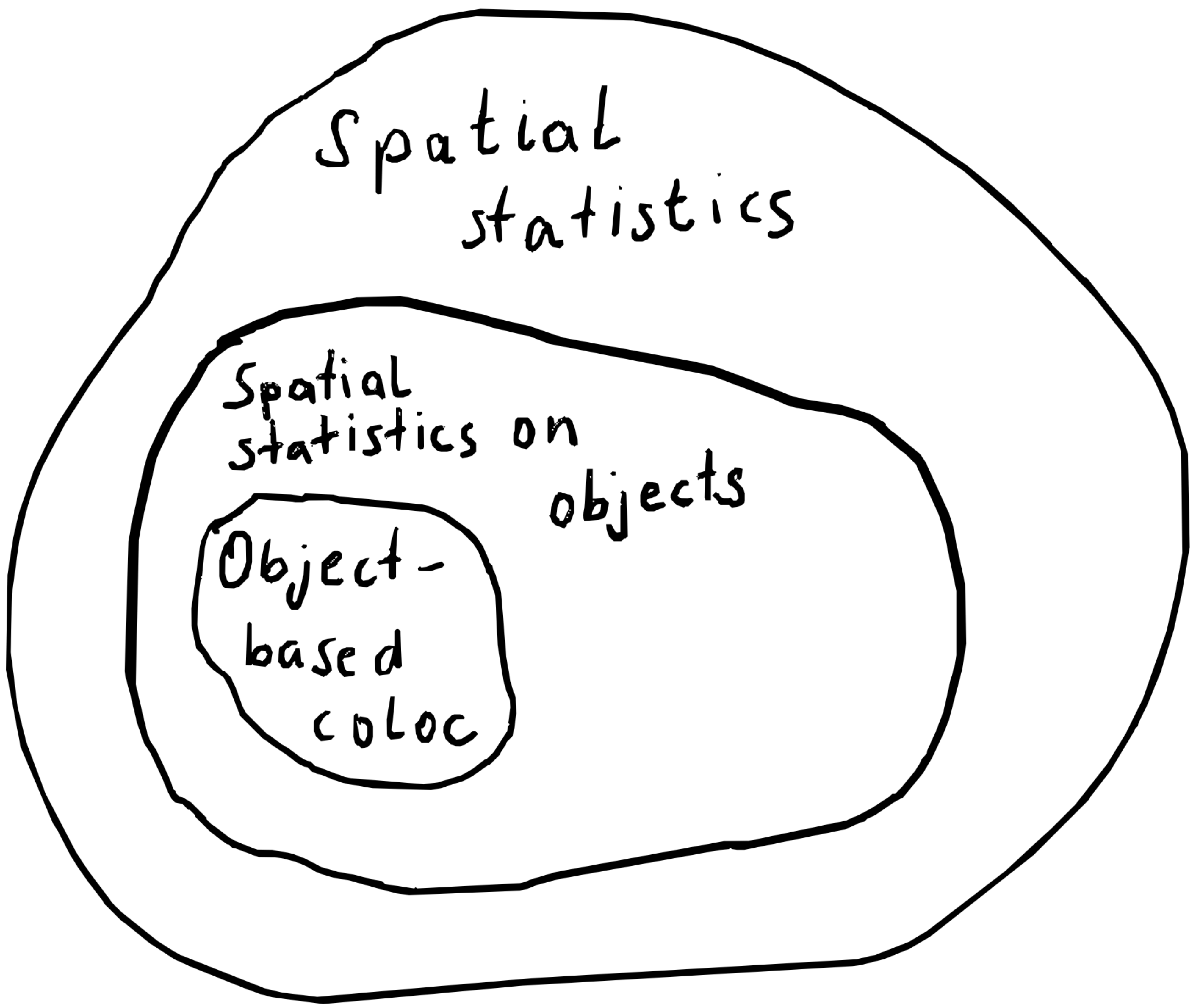


- Spatial statistics deals with spatial data
- These data can be objects

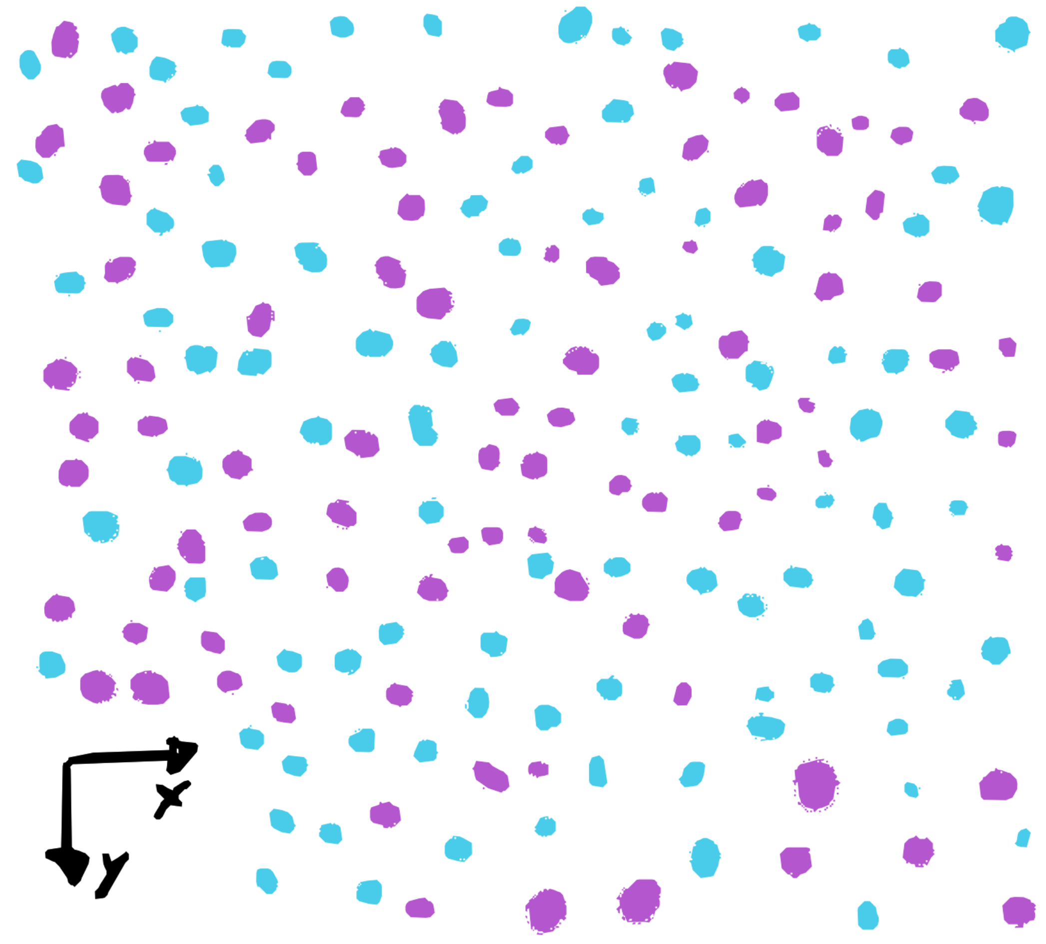


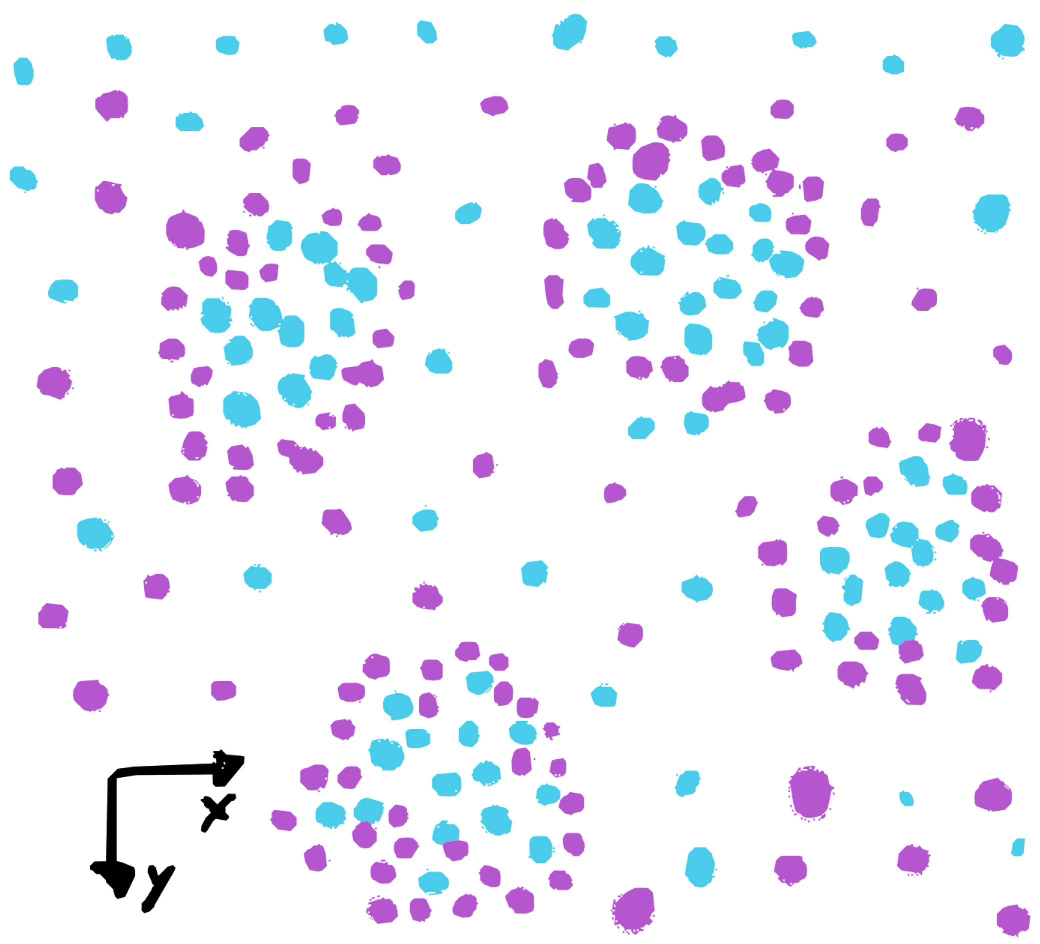


What is “object-based” colocalization

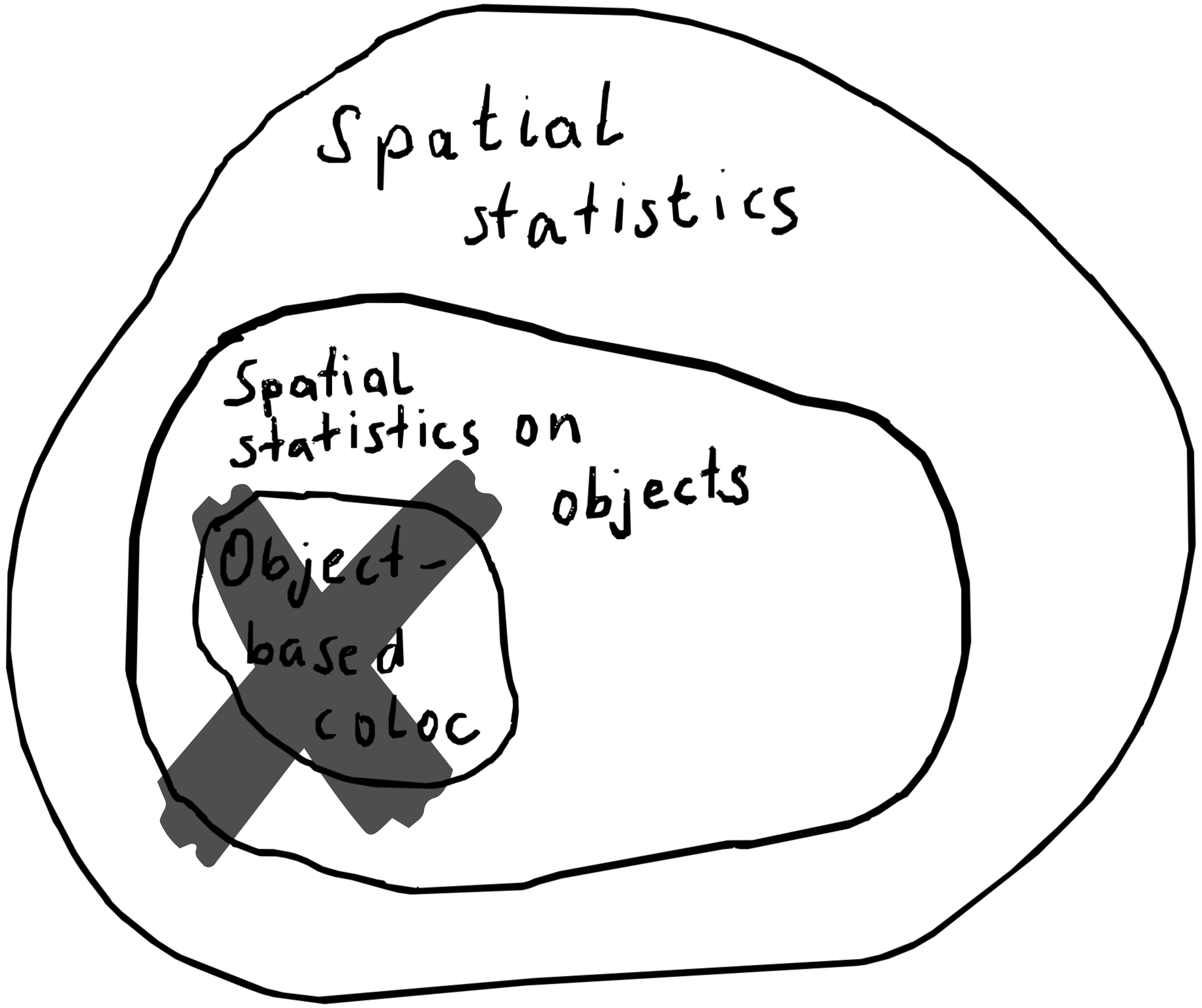


- Spatial statistics deals with spatial data
- These data can be objects
- Objects can be of more than one class

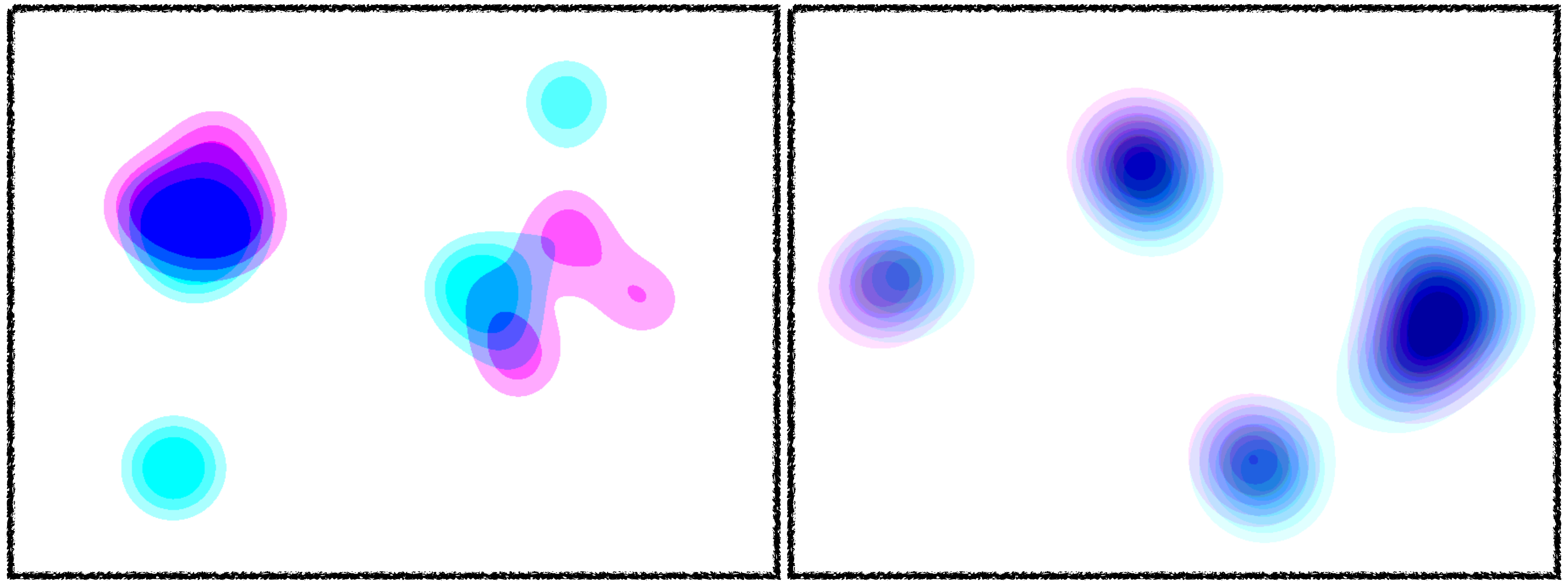




What is “object-based” colocalization

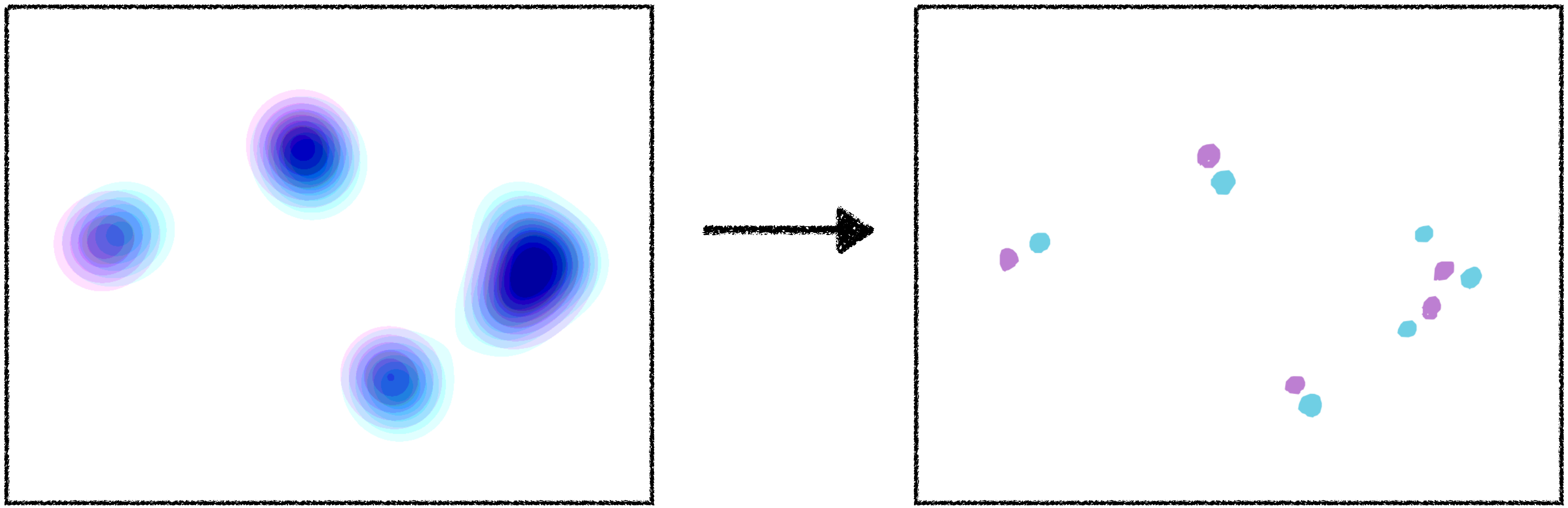


- Colocalization is an illusion of the diffraction limit of microscopy
- Colocalization does not exist!



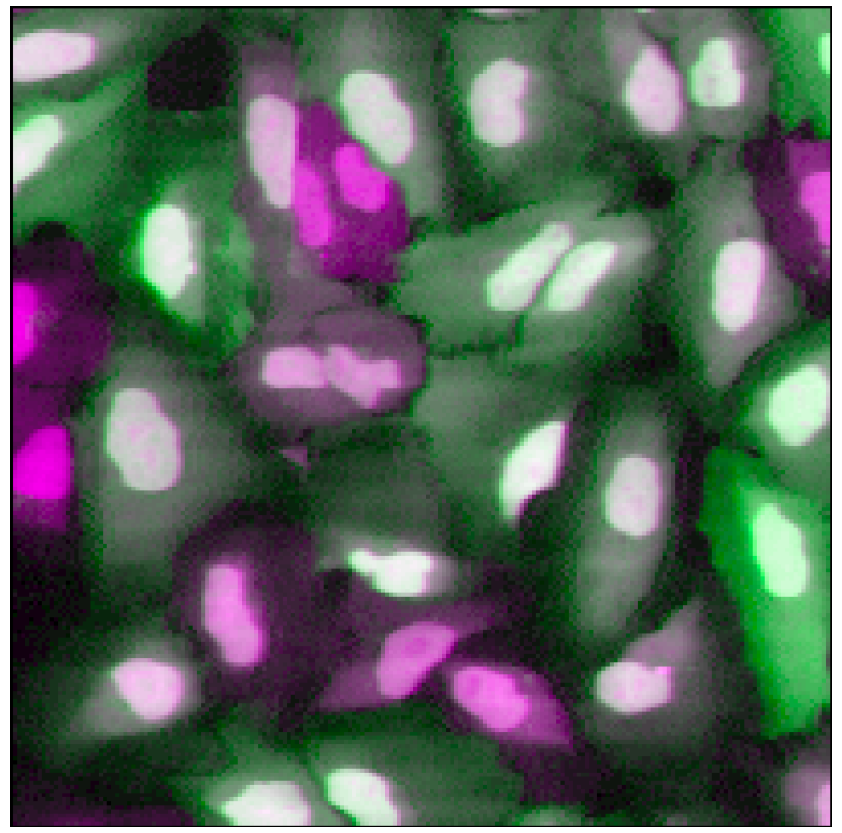
What is “object-based” colocalization

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



Recap: Intensity-based coloc

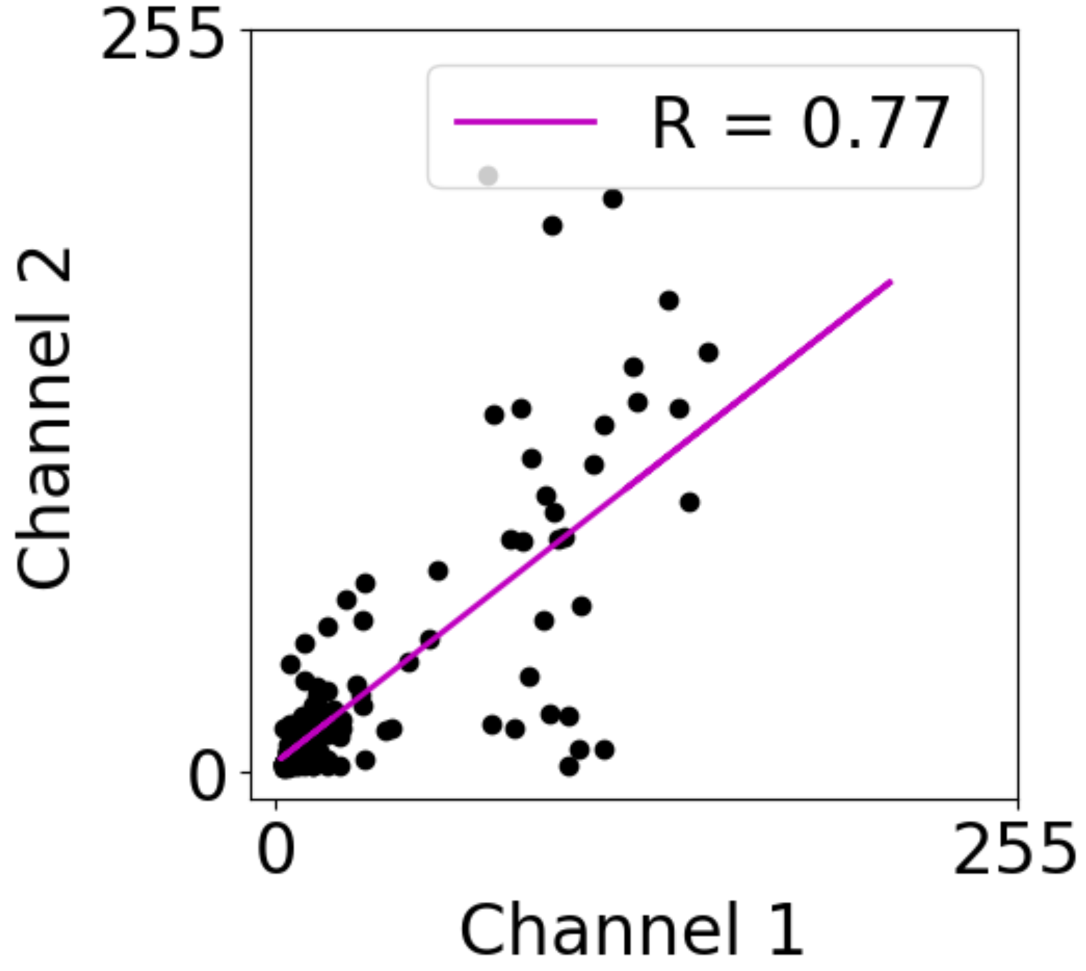
Bleedthrough
Chromatic aberration
Controls
Resolution
SNR
...



Segmentation

Thresholding

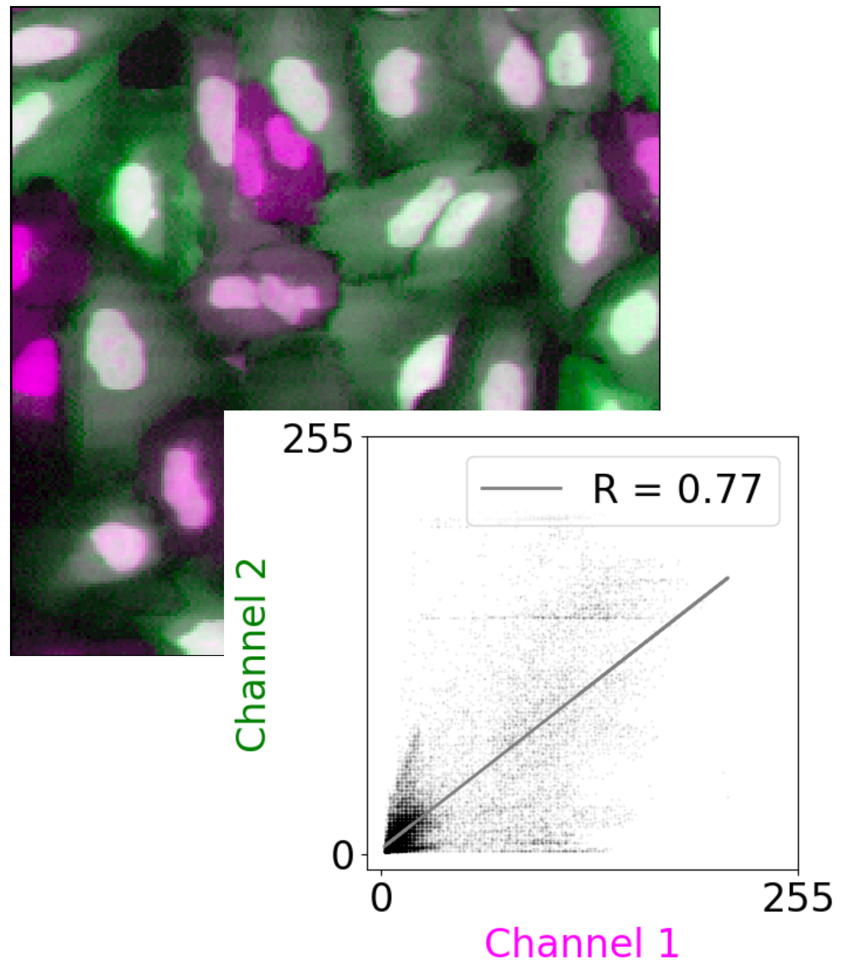
Intensity-based coloc



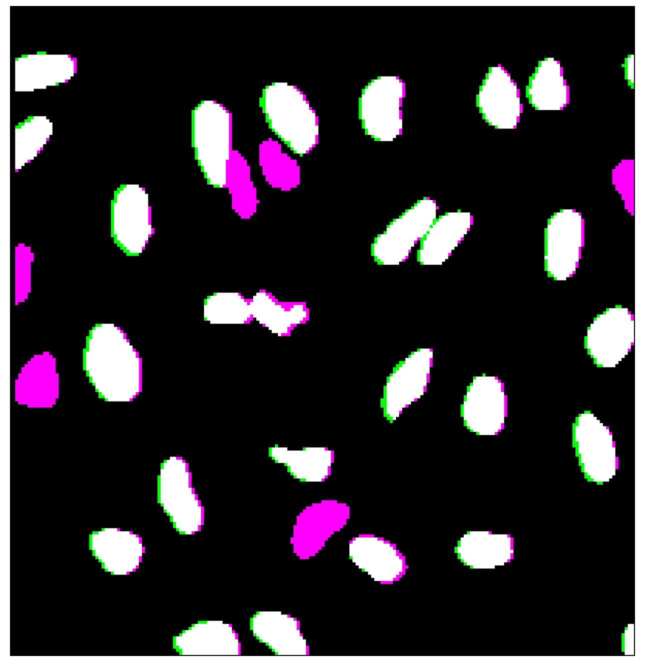
Data: Image set BBBC014v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [dx.doi.org/10.1038/nmeth.2083].

Intensity-based vs. Object-based coloc

Purely intensity-based

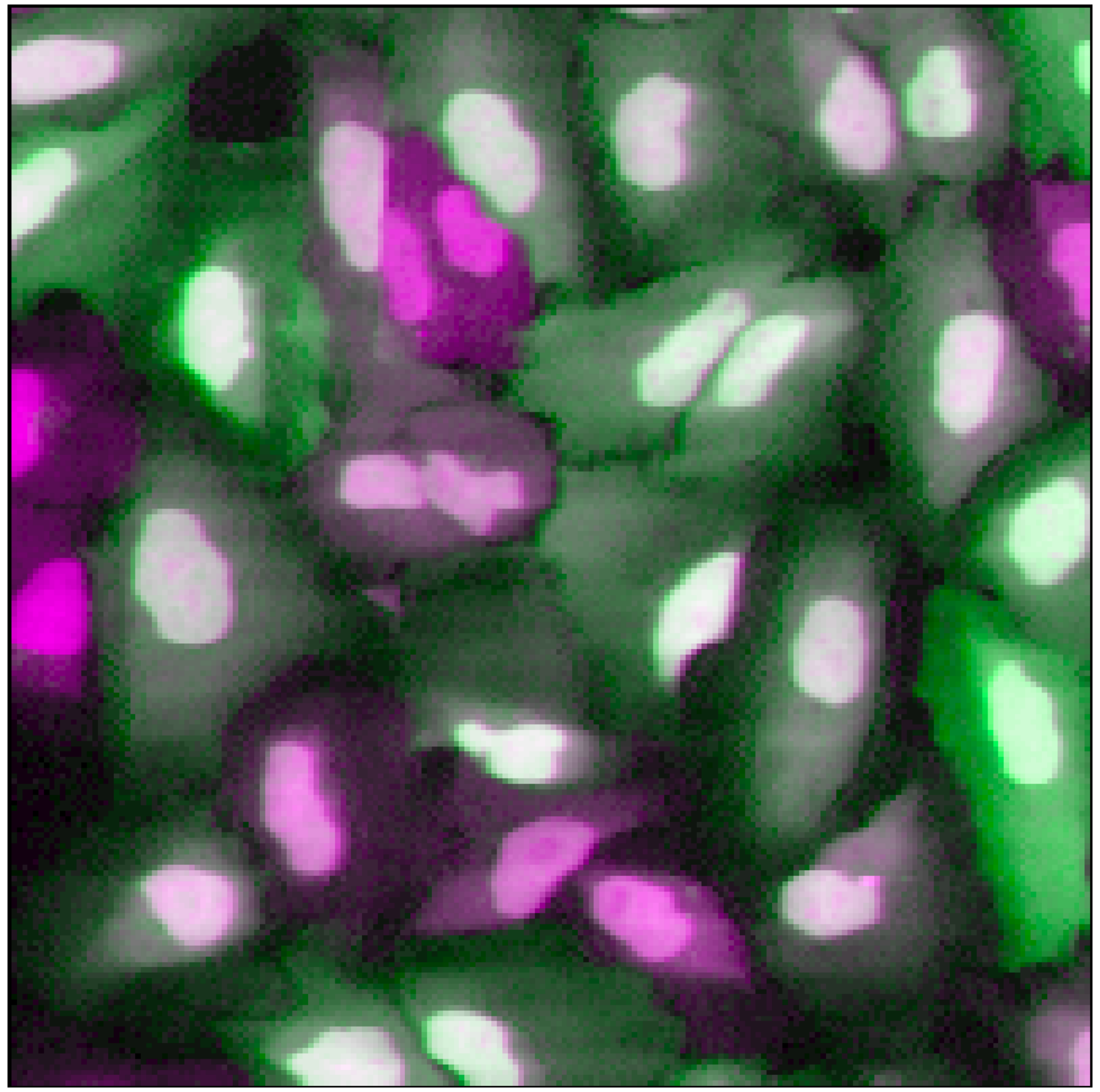


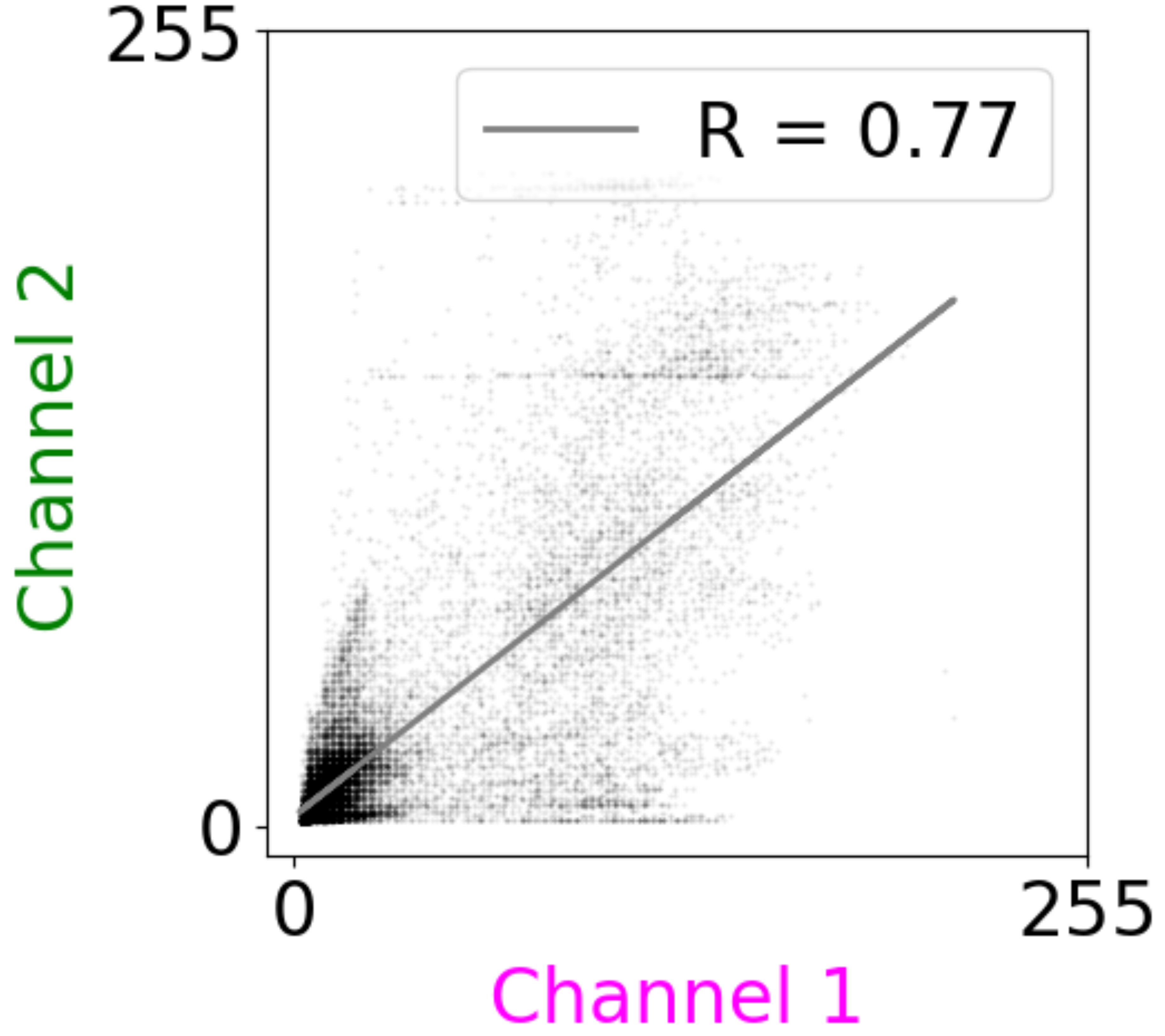
Object-based



Data: Image set BBBC014v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [dx.doi.org/10.1038/nmeth.2083].

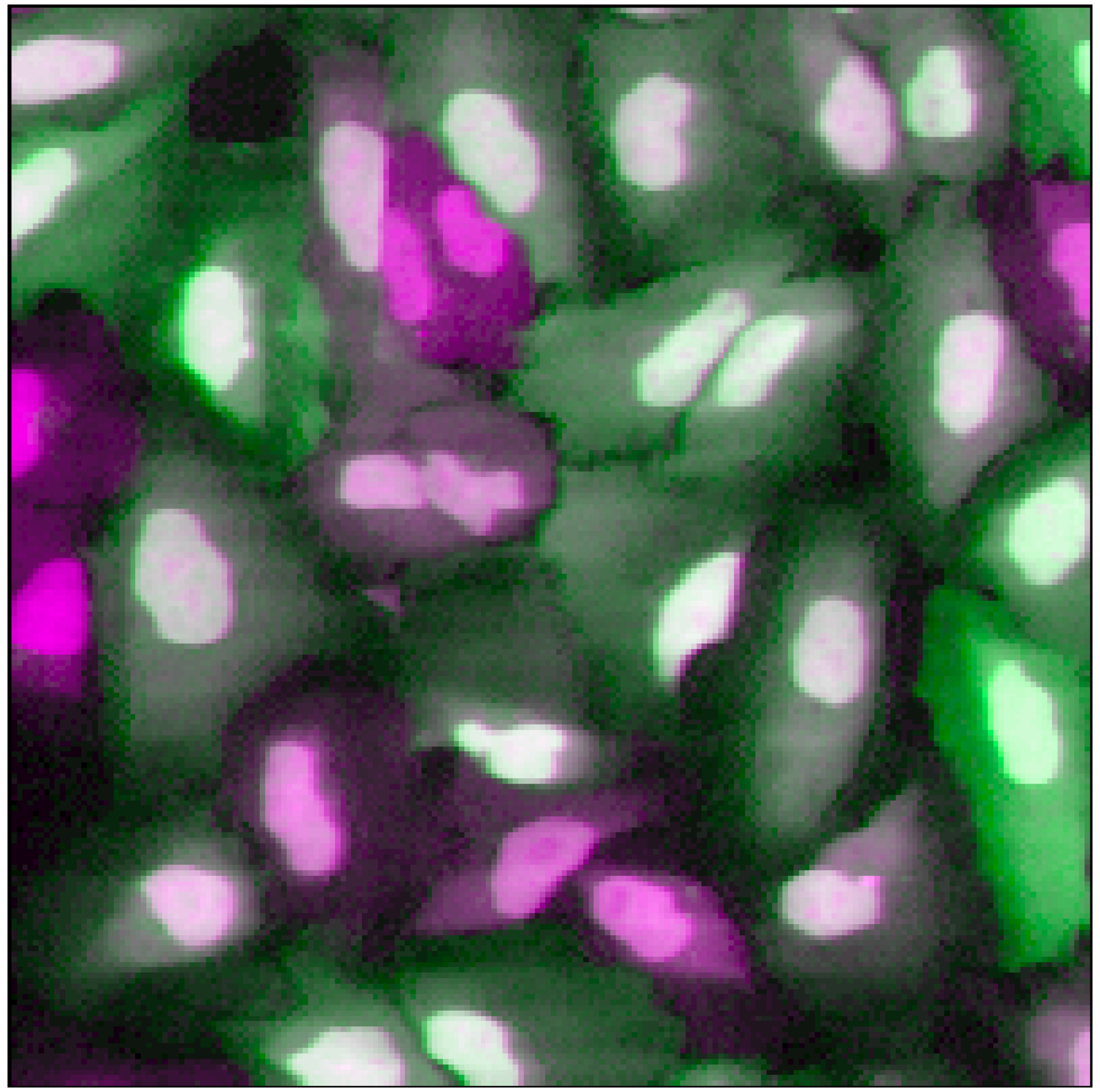
Intensity-based *could* discard spatial information





Data: Image set BBBC014v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [dx.doi.org/10.1038/nmeth.2083].

Object based analysis discards intensity-values

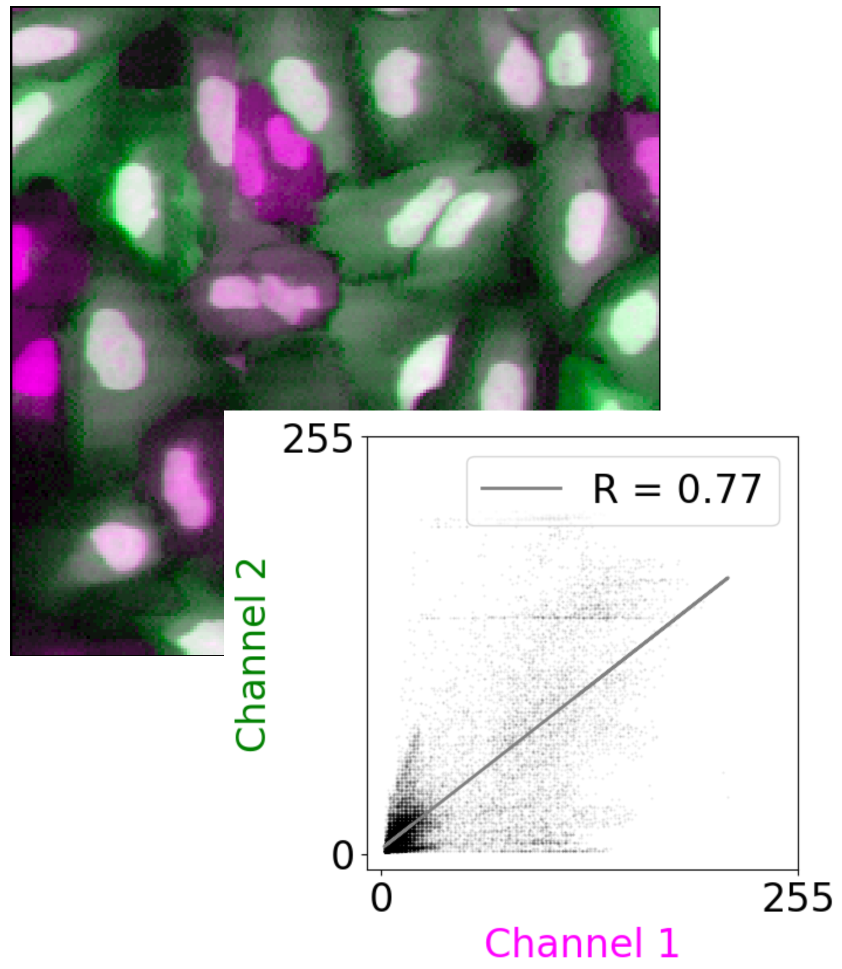


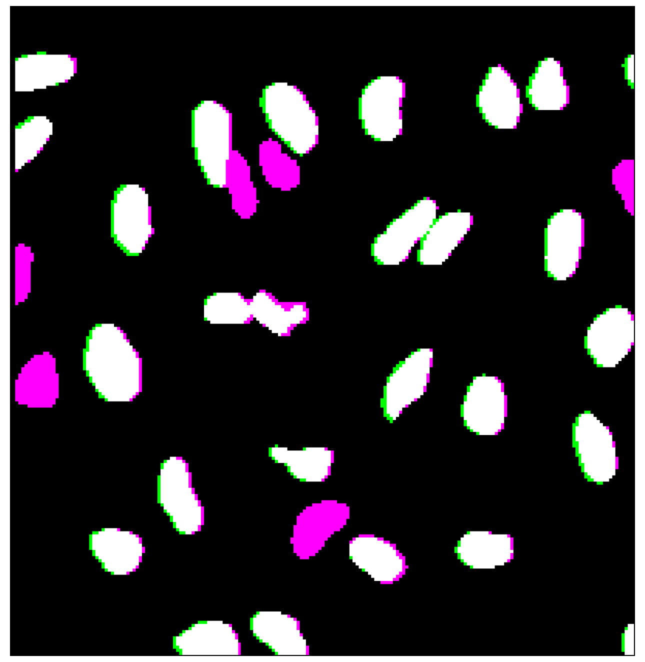
Data: Image set BBBC014v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [dx.doi.org/10.1038/nmeth.2083].

Intensity-based vs. Object-based coloc

Purely intensity-based

Object-based





▲ Only pixel intensity values

▲ Thresholding

▲ Segmentation

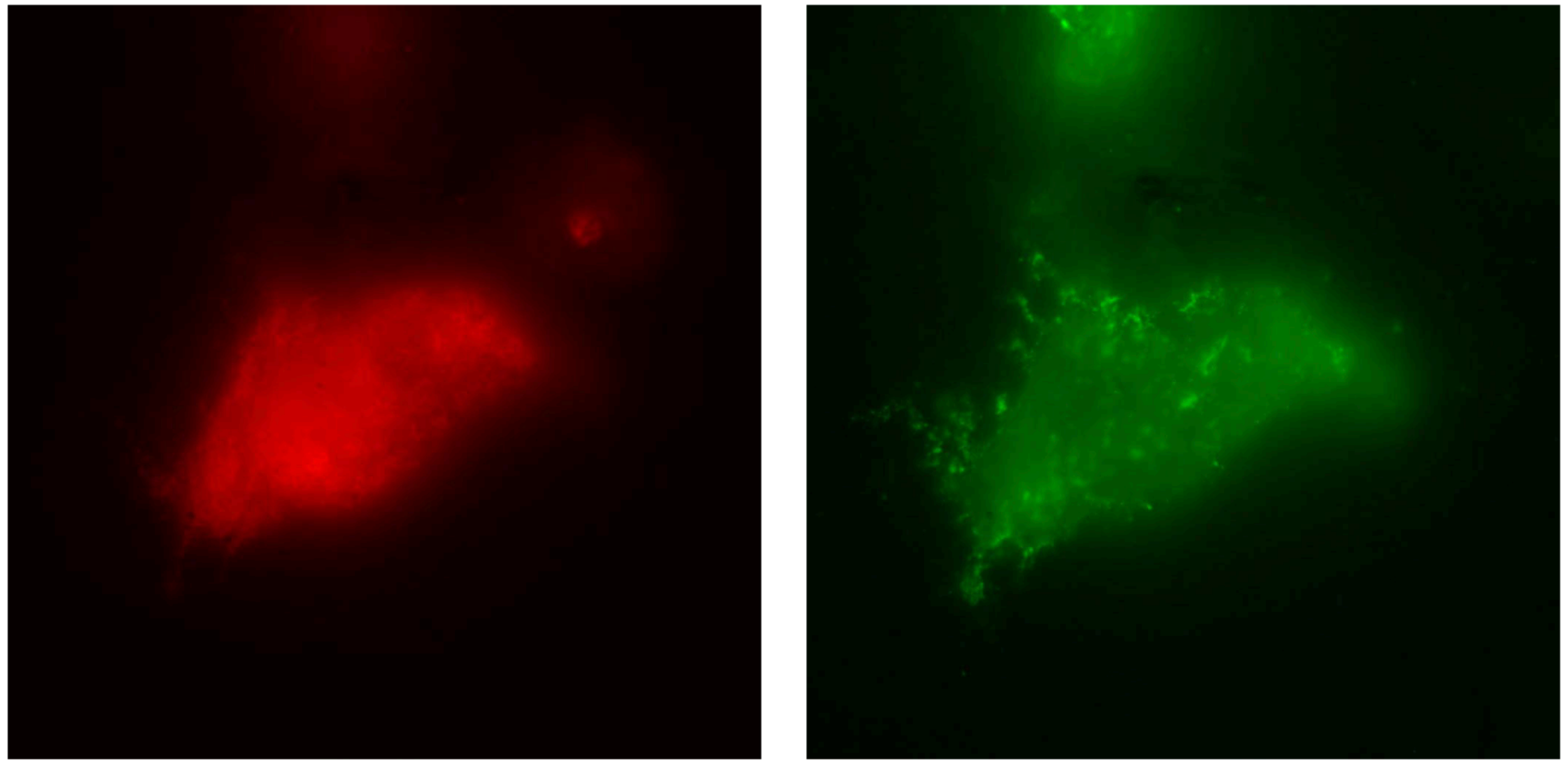
▲ Discards pixel intensity values

Data: Image set BBBC014v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [dx.doi.org/10.1038/nmeth.2083].

When to use object-based methods

Intensity-based

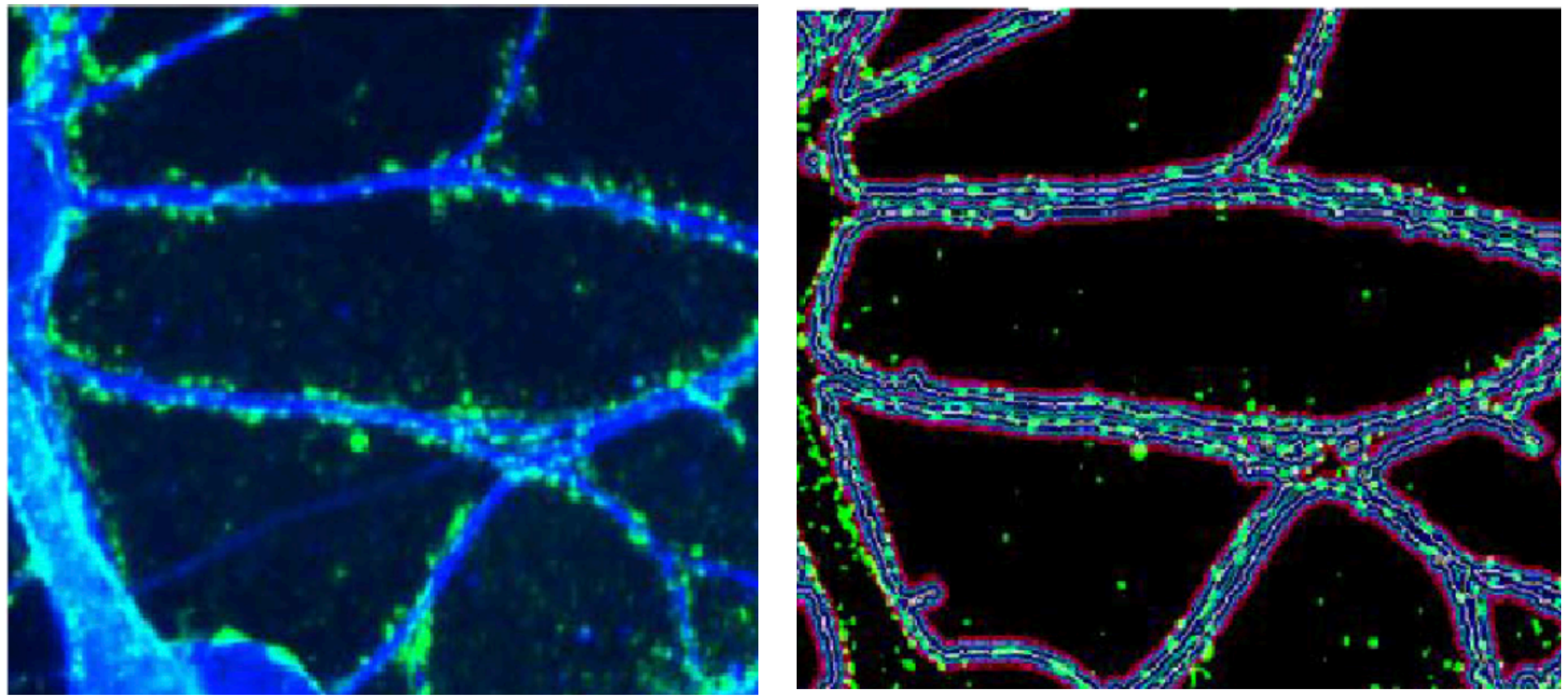
- Segmentation difficult



Two HIV components in HeLa cells

Object-based

- Objects are discrete & segmentable



Neuronal dendrites (blue)
and synaptic spots (green)

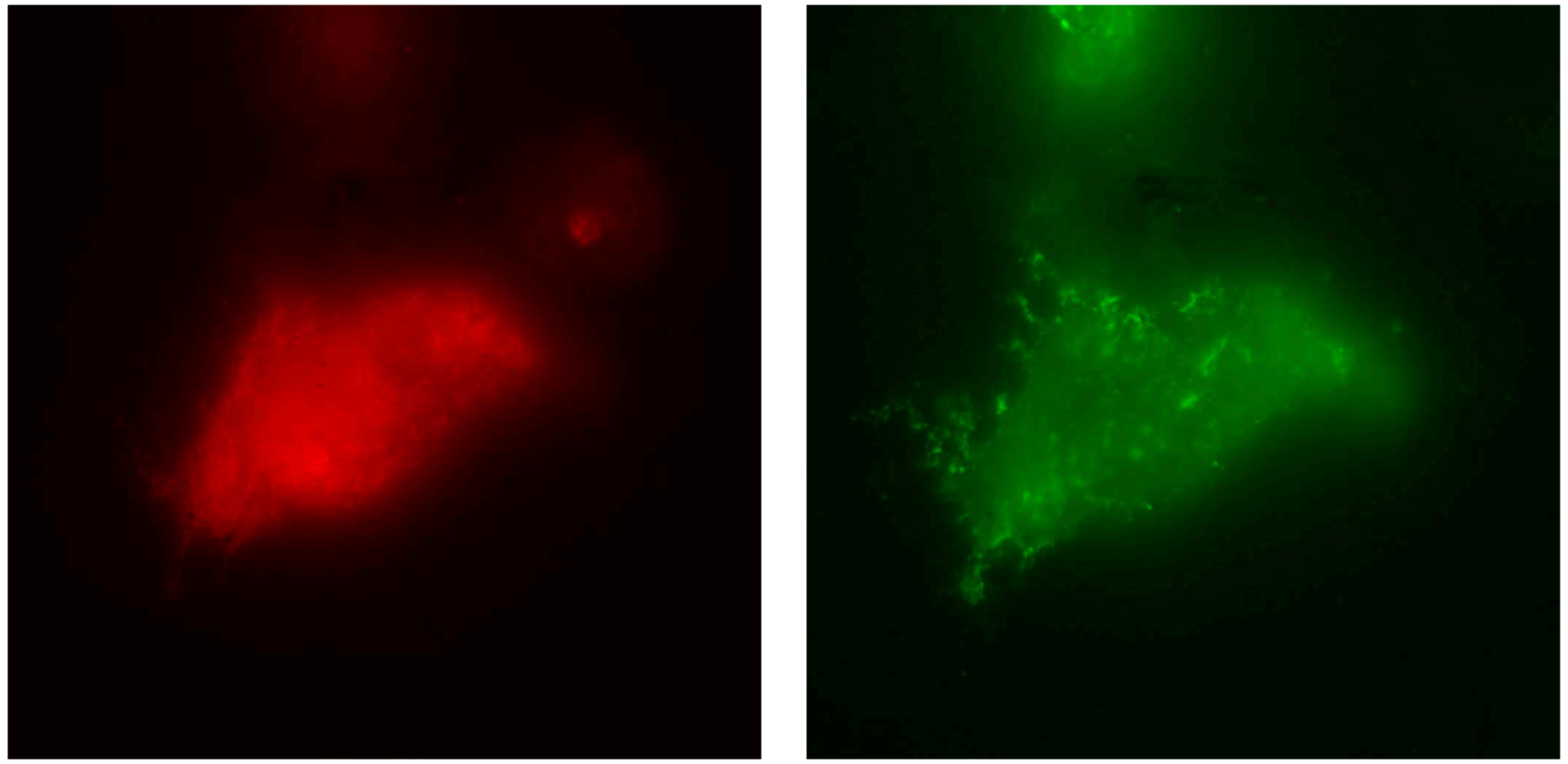
Wang S, Arena ET, Becker JT, Bement WM, Sherer NM, Eliceiri KW, Yuan M. Spatially Adaptive Colocalization Analysis in Dual-Color Fluorescence Microscopy. IEEE Trans Image Process. 2019 Apr 4. doi: 10.1109/TIP.2019.2909194. Epub ahead of print. PMID: 30951467.

Modified from: S. Mukherjee, C. Gonzalez-Gomez, L. Danglot, T. Lagache and J. -C. Olivo-Marin, "Generalizing the Statistical Analysis of Objects' Spatial Coupling in Bioimaging," in *IEEE Signal Processing Letters*, vol. 27, pp. 1085-1089, 2020, doi: 10.1109/LSP.2020.3003821.

When to use object-based methods

Intensity-based

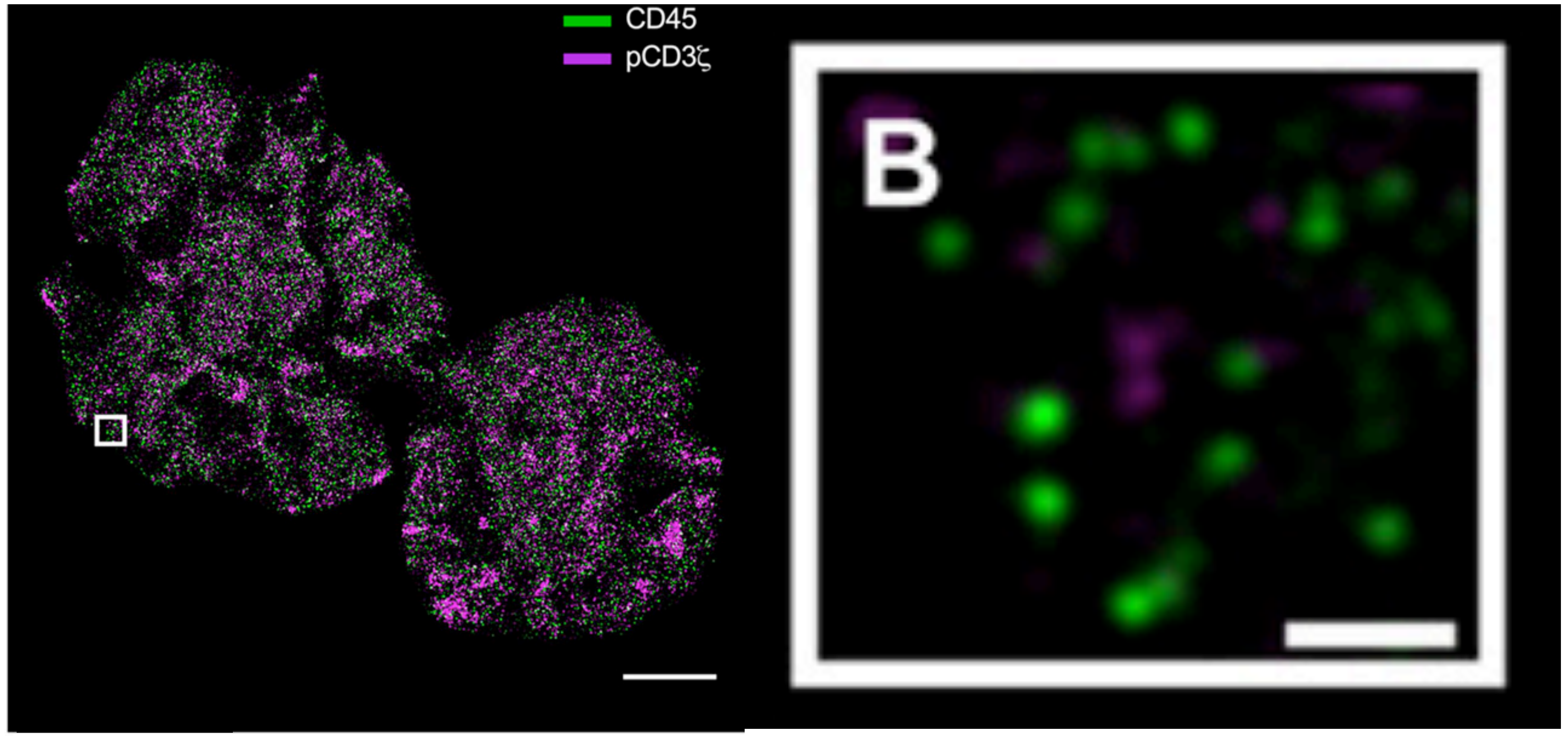
- Often in diffraction limited settings



Two HIV components in HeLa cells

Object-based

- Often in super-resolution settings



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

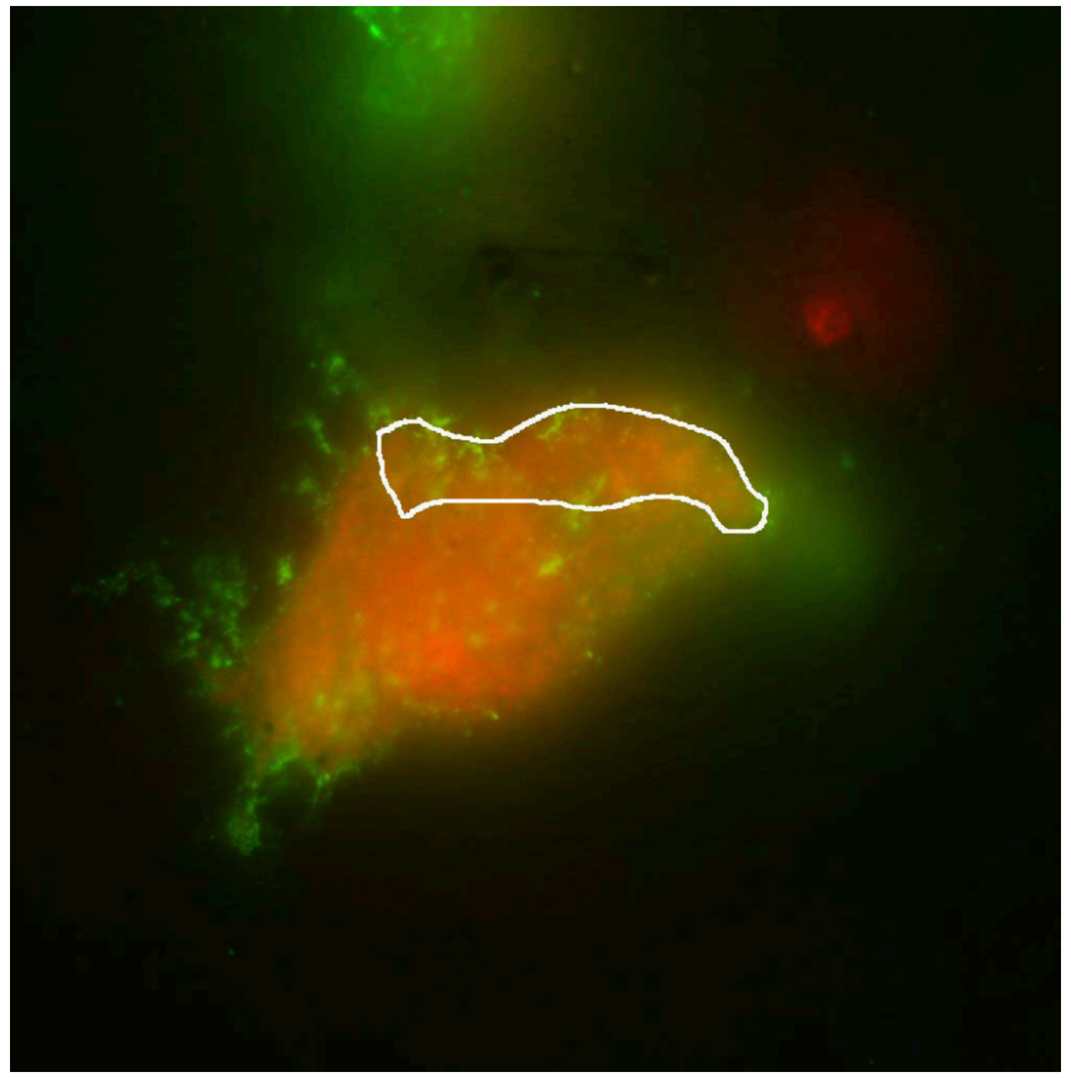
Wang S, Arena ET, Becker JT, Bement WM, Sherer NM, Eliceiri KW, Yuan M. Spatially Adaptive Colocalization Analysis in Dual-Color Fluorescence Microscopy. IEEE Trans Image Process. 2019 Apr 4. doi: 10.1109/TIP.2019.2909194. Epub ahead of print. PMID: 30951467.

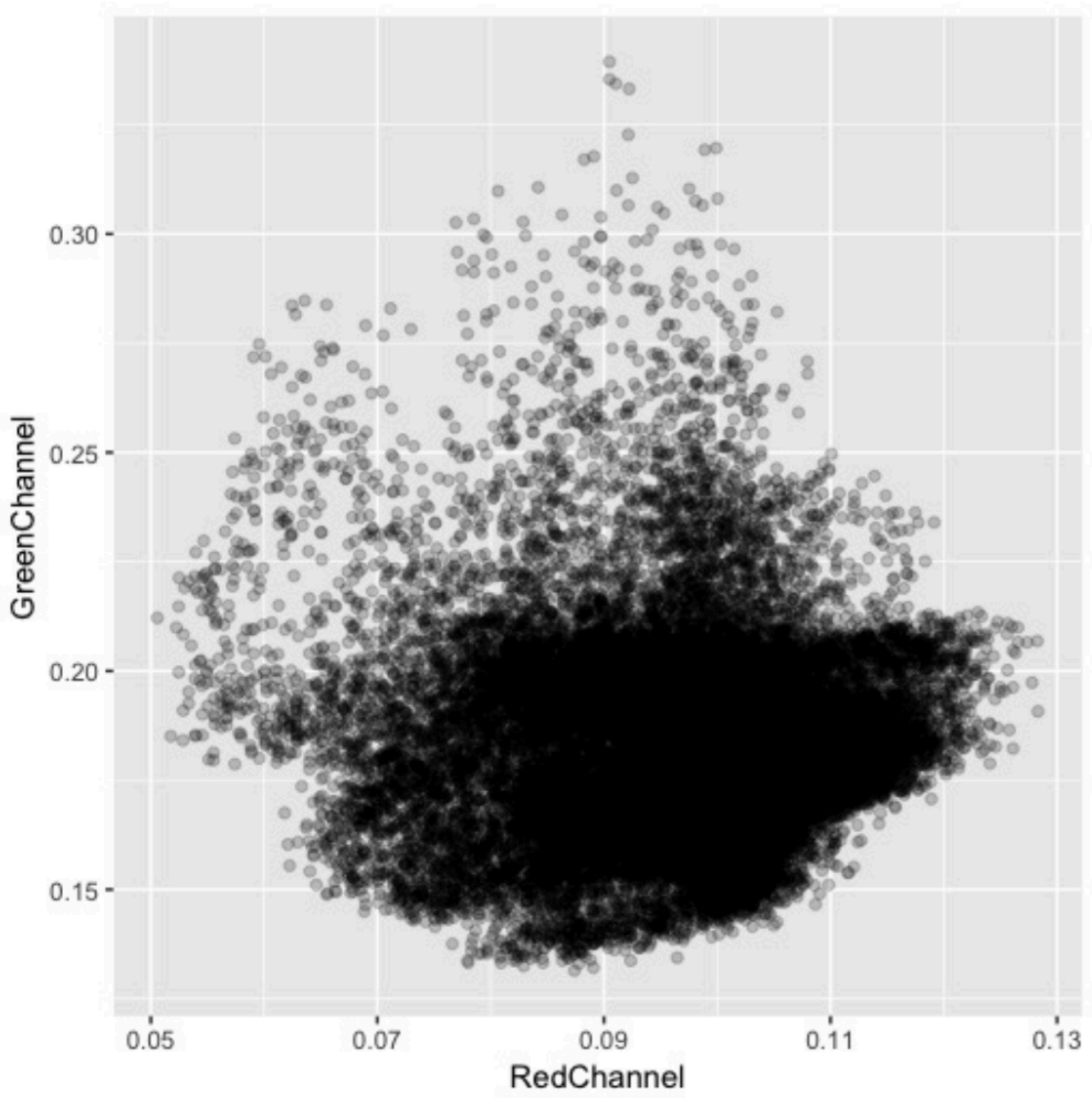
Modified from: Simao Coelho *et al*., Ultraprecise single-molecule localization microscopy enables in situ distance measurements in intact cells. *Sci. Adv.* **6**, eaay8271 (2020). DOI: 10.1126/sciadv.aay8271

When to use object-based methods

Intensity-based

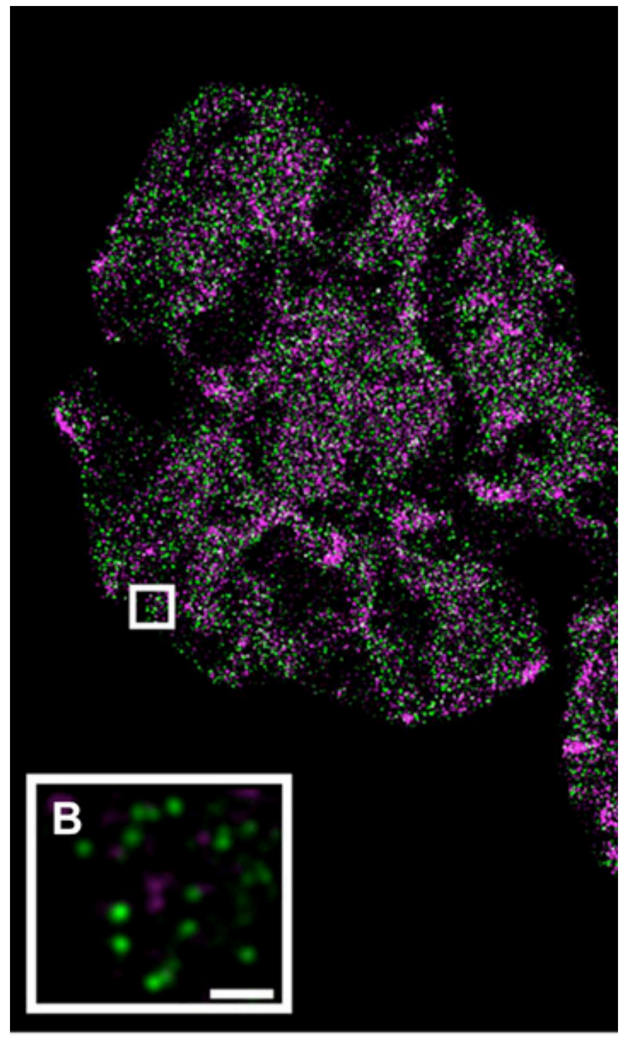
- Statistical quantities
  - “What correlation”

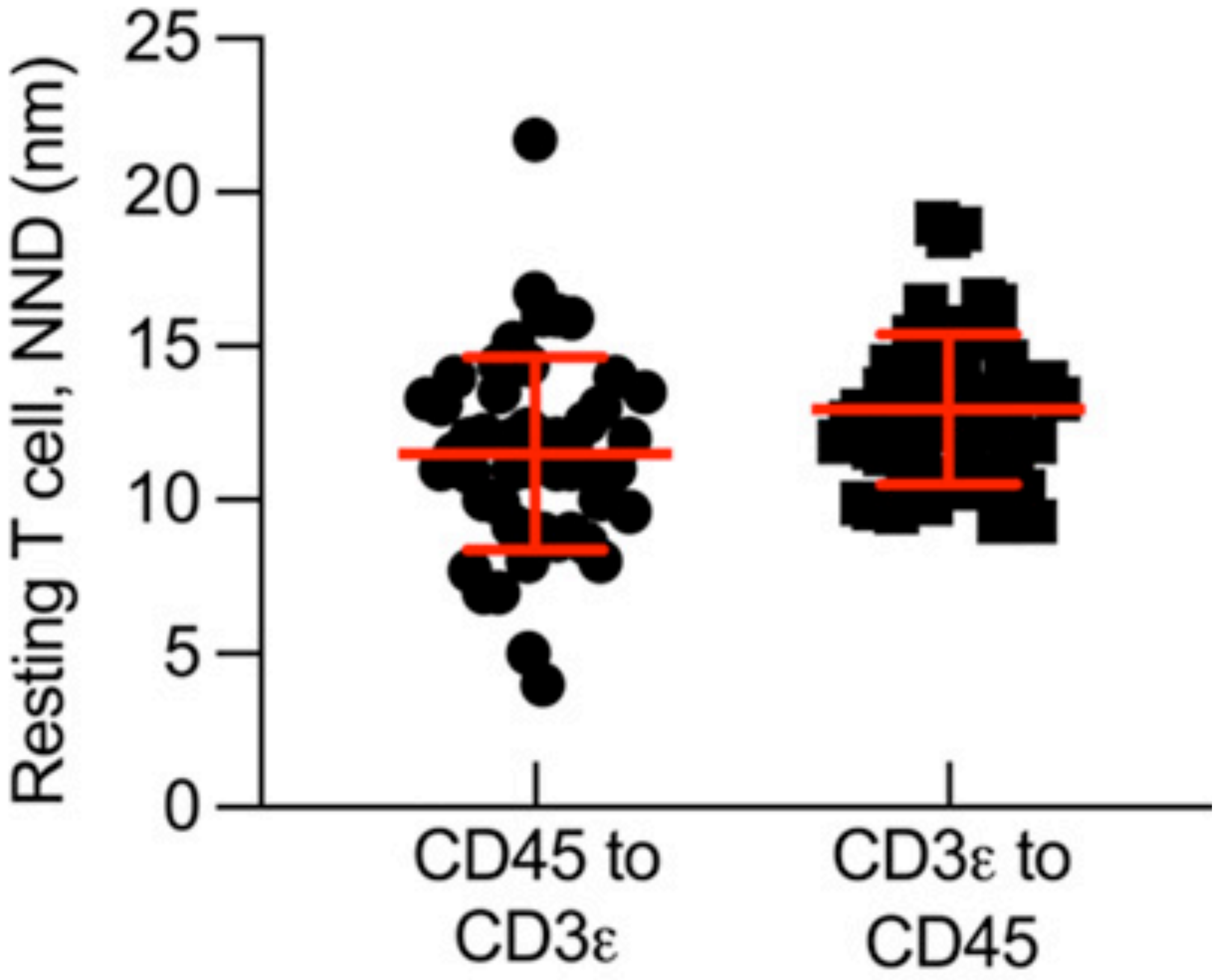




Object-based

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



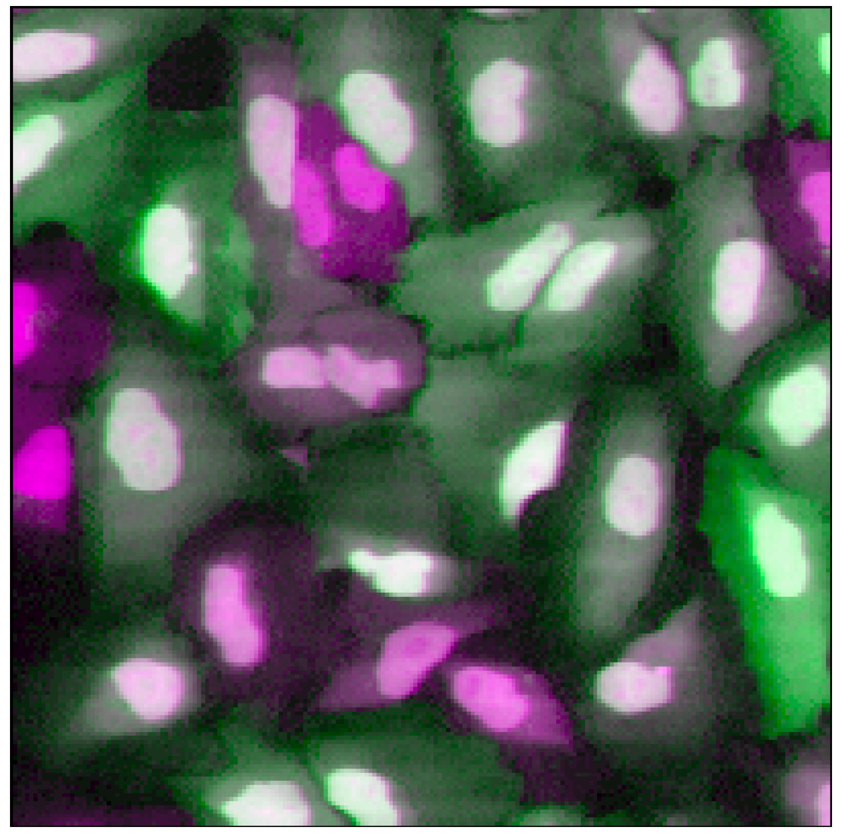


Wang S, Arena ET, Becker JT, Bement WM, Sherer NM, Eliceiri KW, Yuan M. Spatially Adaptive Colocalization Analysis in Dual-Color Fluorescence Microscopy. IEEE Trans Image Process. 2019 Apr 4. doi: 10.1109/TIP.2019.2909194. Epub ahead of print. PMID: 30951467.

Modified from: Simao Coelho *et al.*, Ultraprecise single-molecule localization microscopy enables in situ distance measurements in intact cells.*Sci. Adv.***6**,eaay8271(2020).DOI:10.1126/sciadv.aay8271

Object-based coloc: workflow

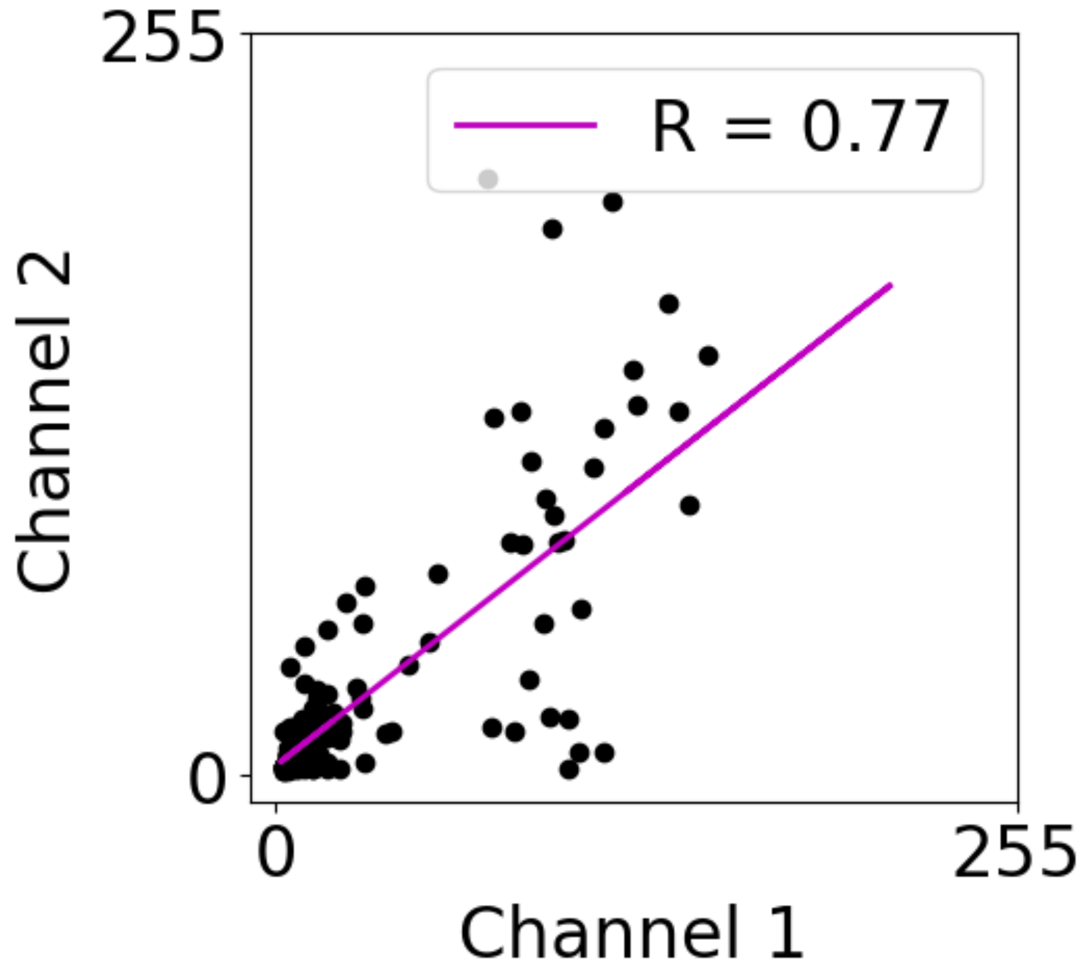
Bleedthrough
Chromatic aberration
Controls
Resolution
SNR
…



Segmentation

Thresholding

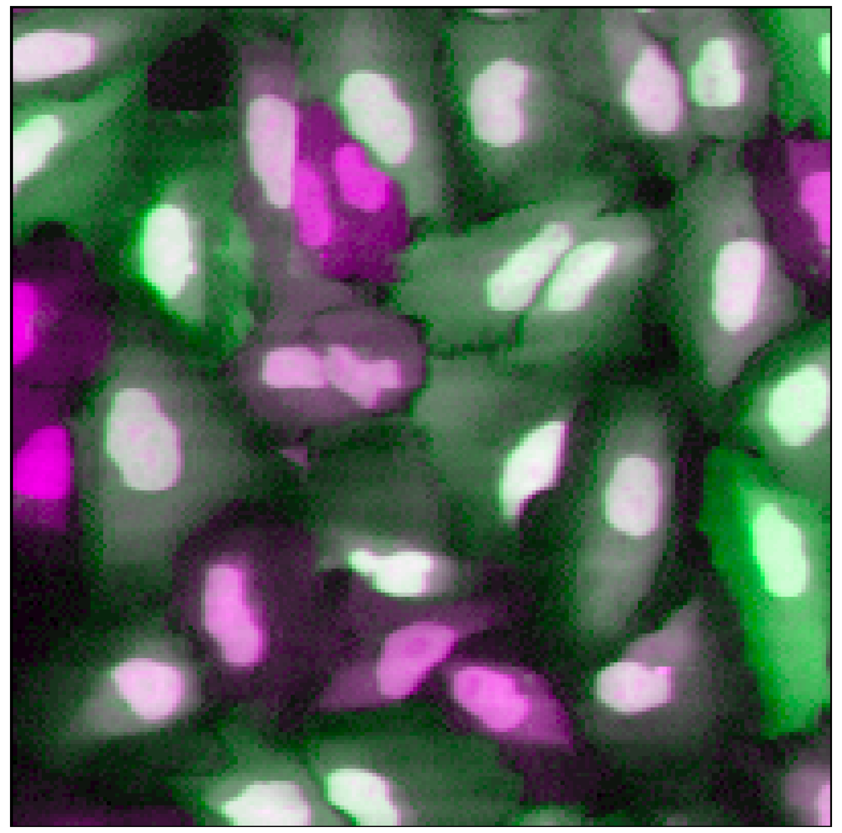
Intensity-based coloc



Data: Image set BBBC014v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [dx.doi.org/10.1038/nmeth.2083].

Object-based coloc: workflow

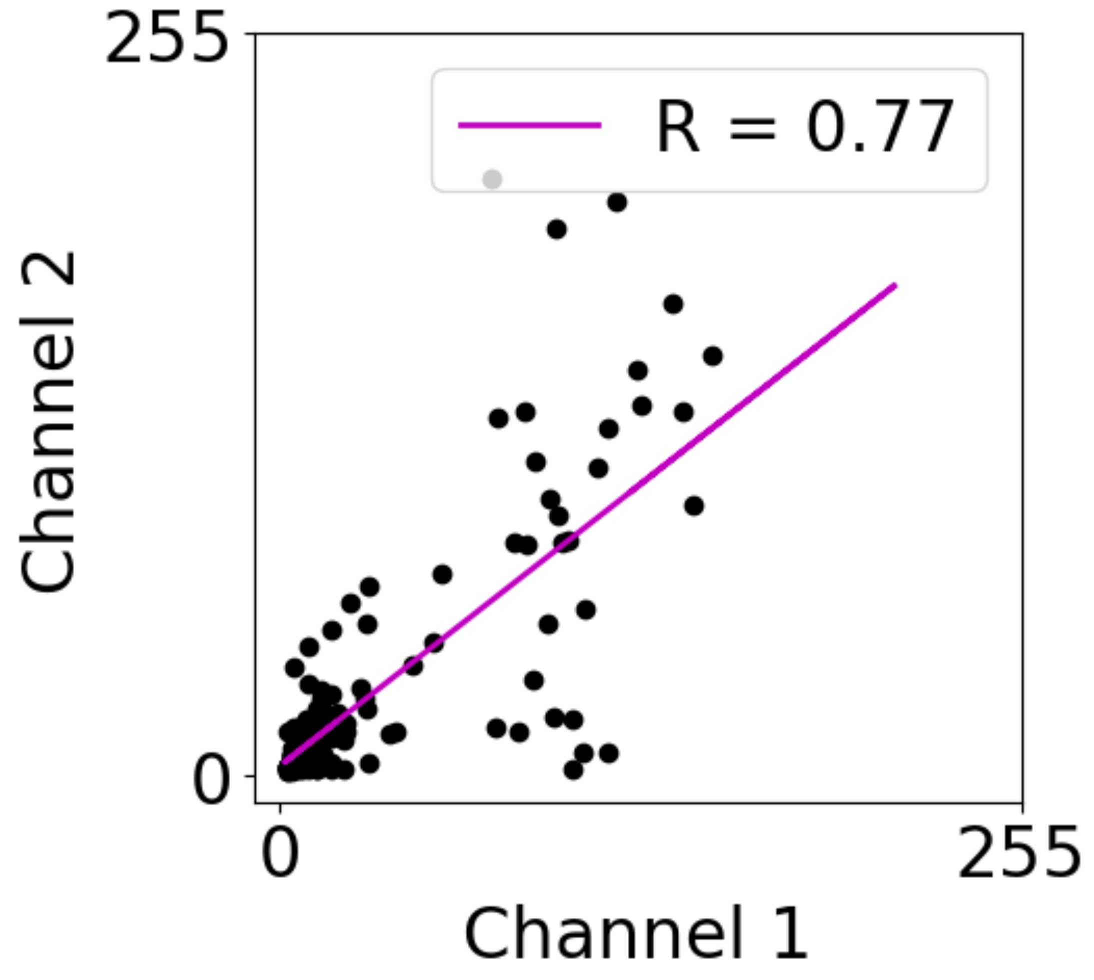
Bleedthrough
Chromatic aberration
Controls
Resolution
SNR
...



Segmentation

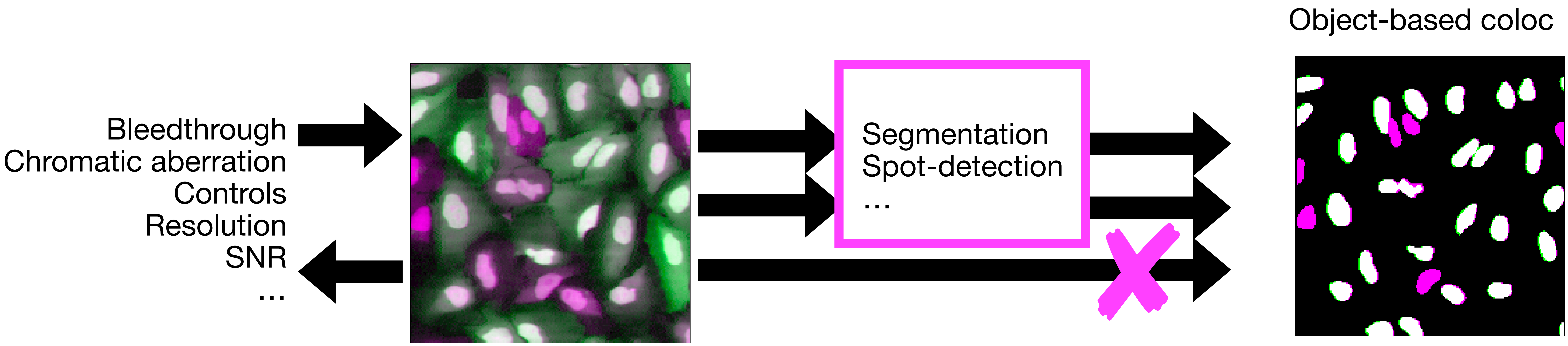
Thresholding

Intensity-based coloc



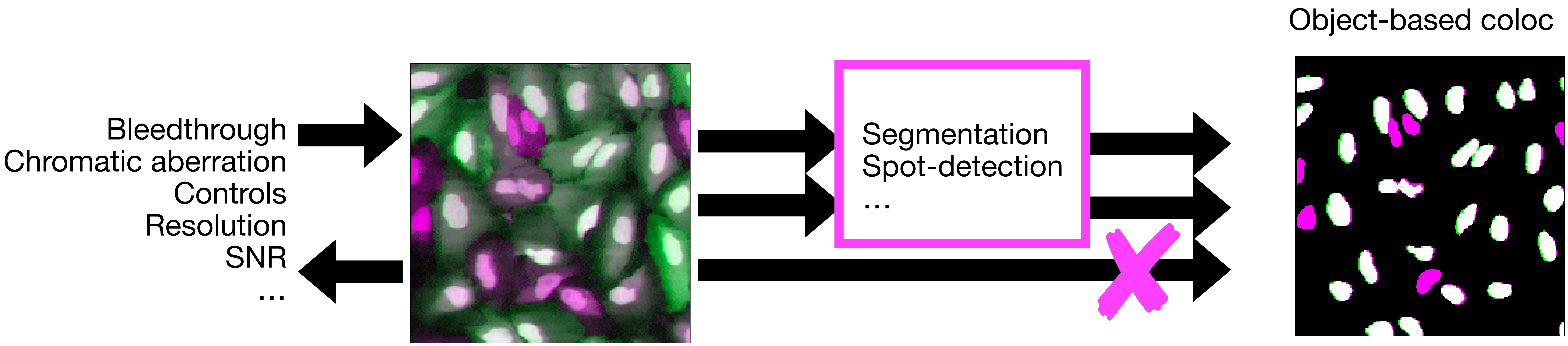
Data: Image set BBBC014v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [dx.doi.org/10.1038/nmeth.2083].

Object-based coloc: workflow



Data: Image set BBBC014v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [dx.doi.org/10.1038/nmeth.2083].

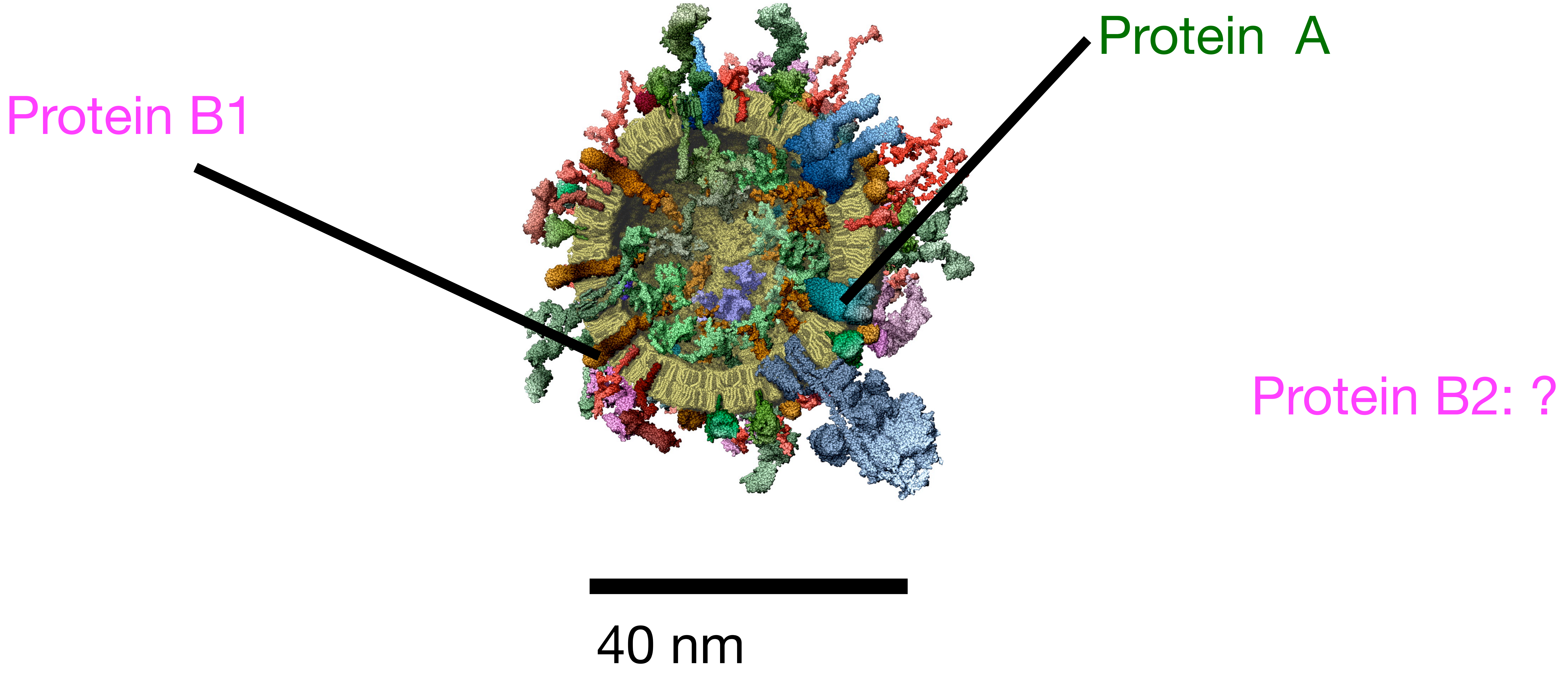
Object-based coloc: workflow



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

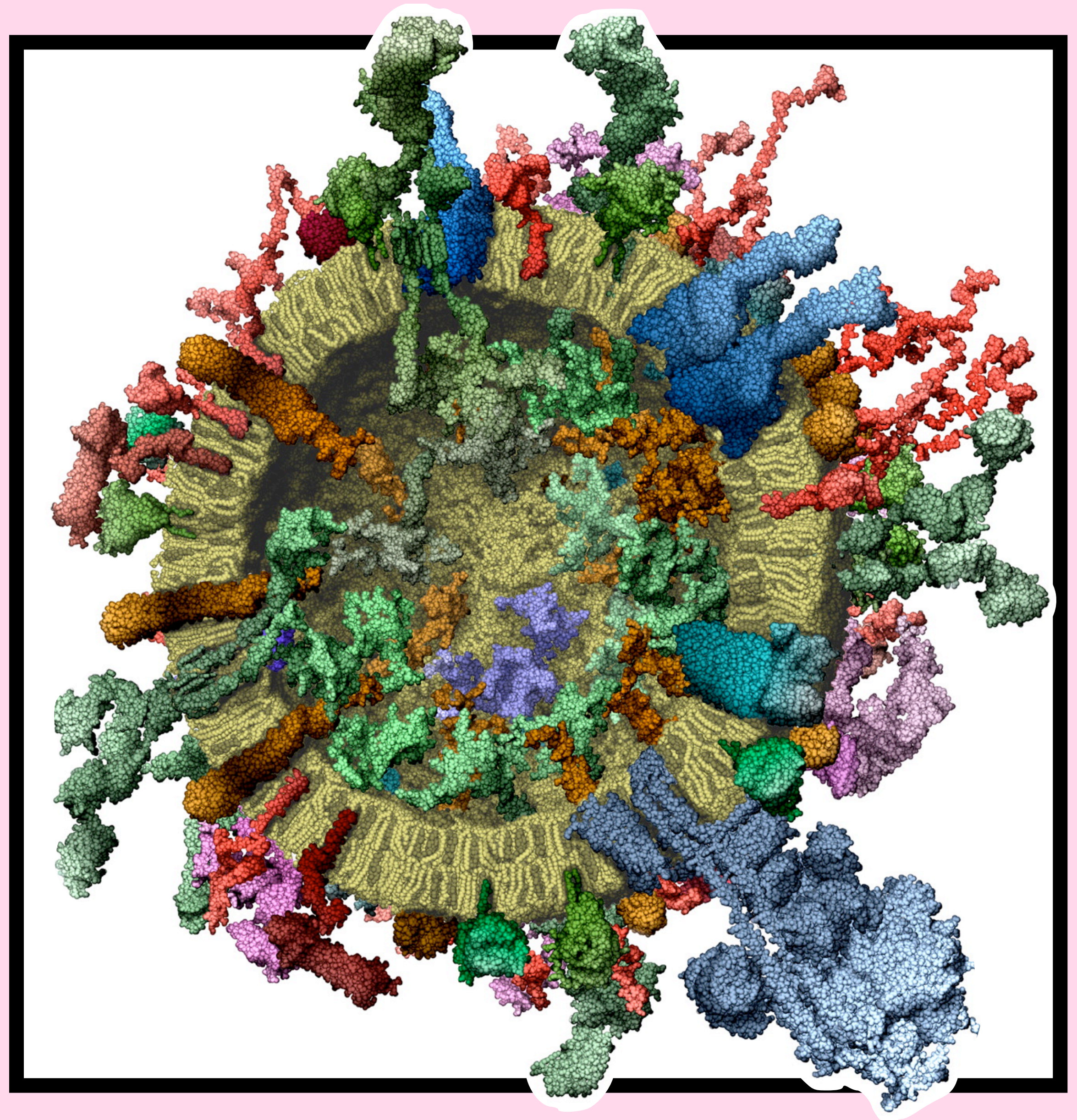
Data: Image set BBBC014v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [dx.doi.org/10.1038/nmeth.2083].

Example: Area



Modified from: **Molecular Anatomy of a Trafficking Organelle;** Takamori, Shigeo et al.; Cell, Volume 127, Issue 4, 831 - 846

Example: Area



~ PSF

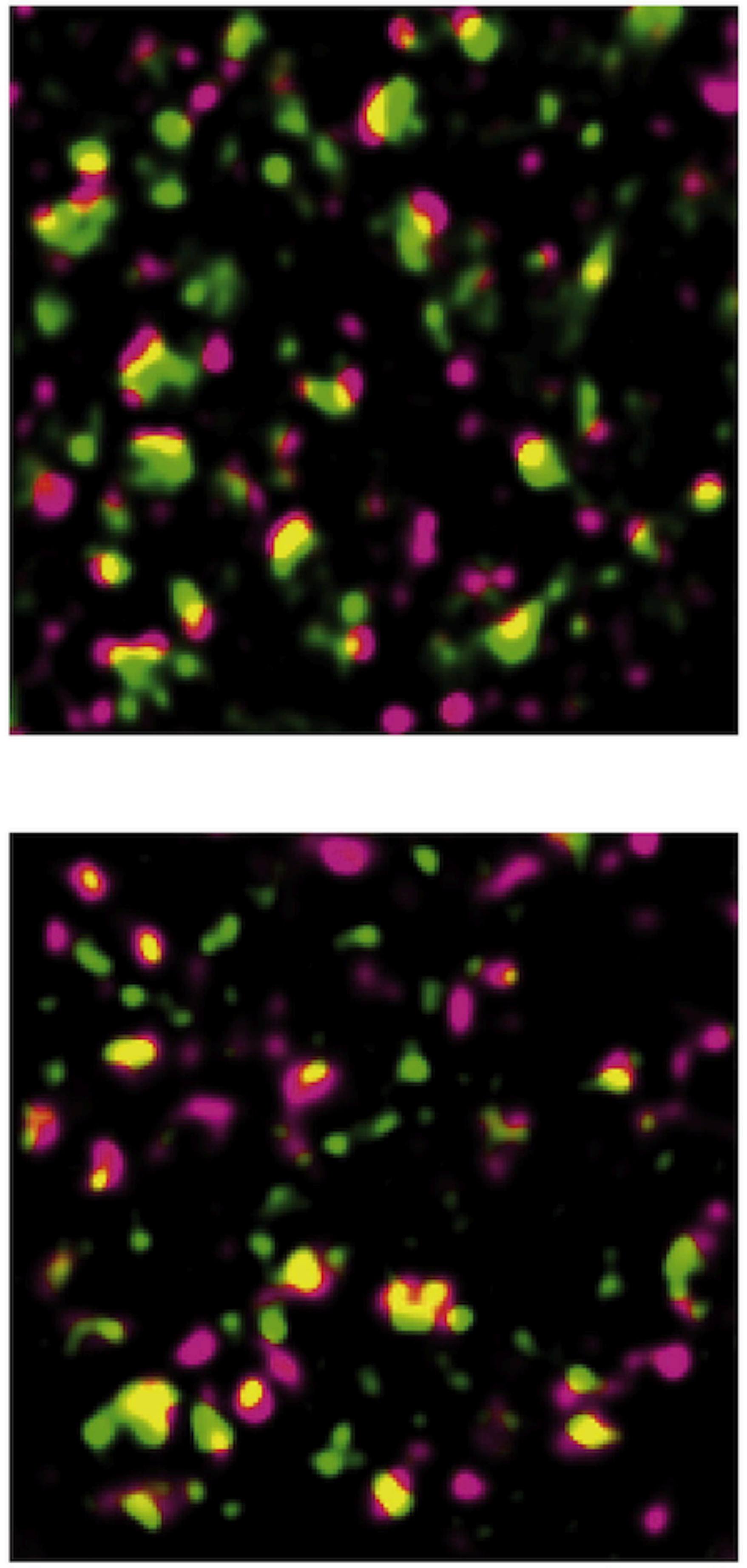
px: 60 nm

Modified from: **Molecular Anatomy of a Trafficking Organelle;** Takamori, Shigeo et al.; Cell, Volume 127, Issue 4, 831 - 846

Example: Area

Original

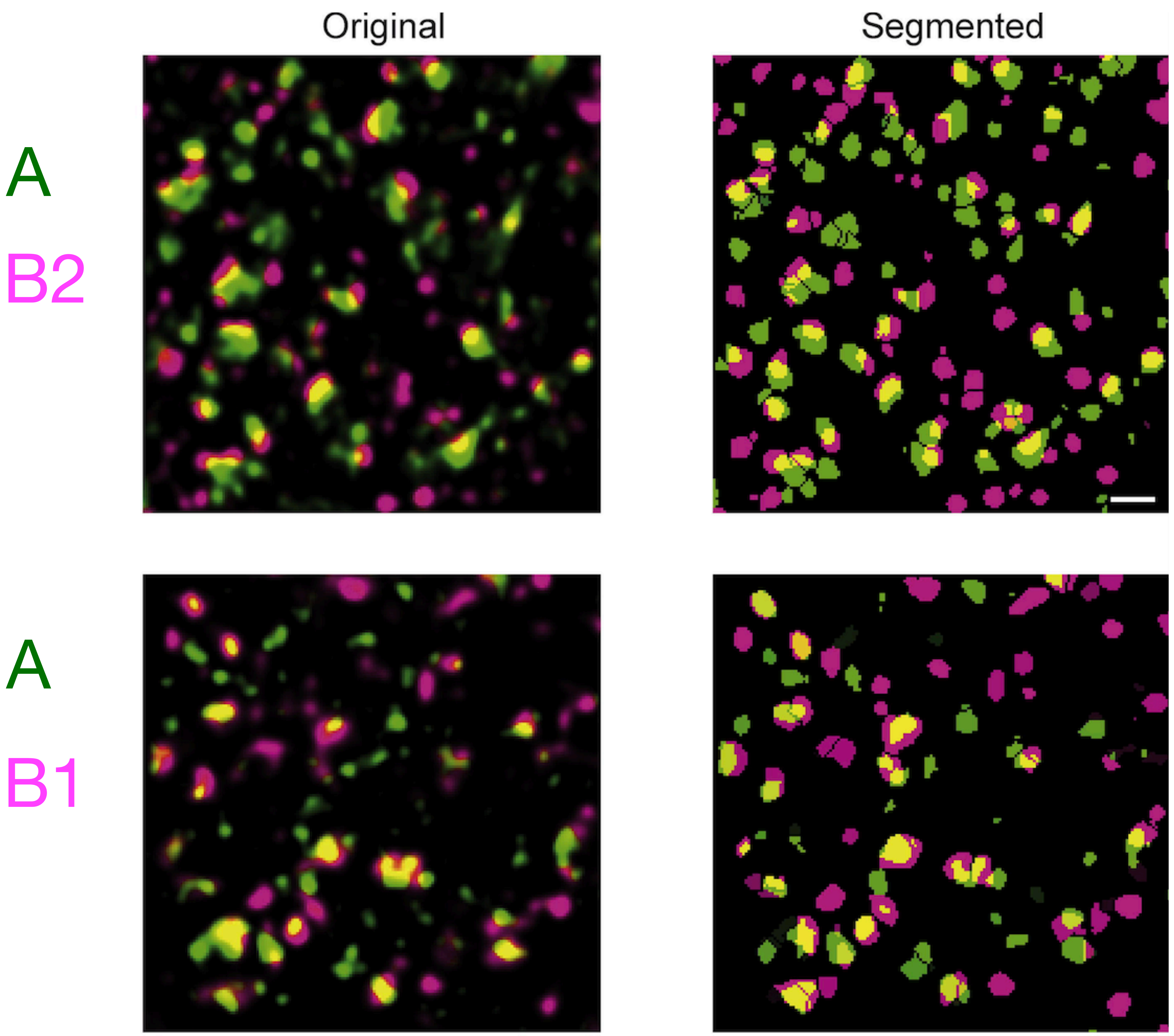
A
B2



A
B1

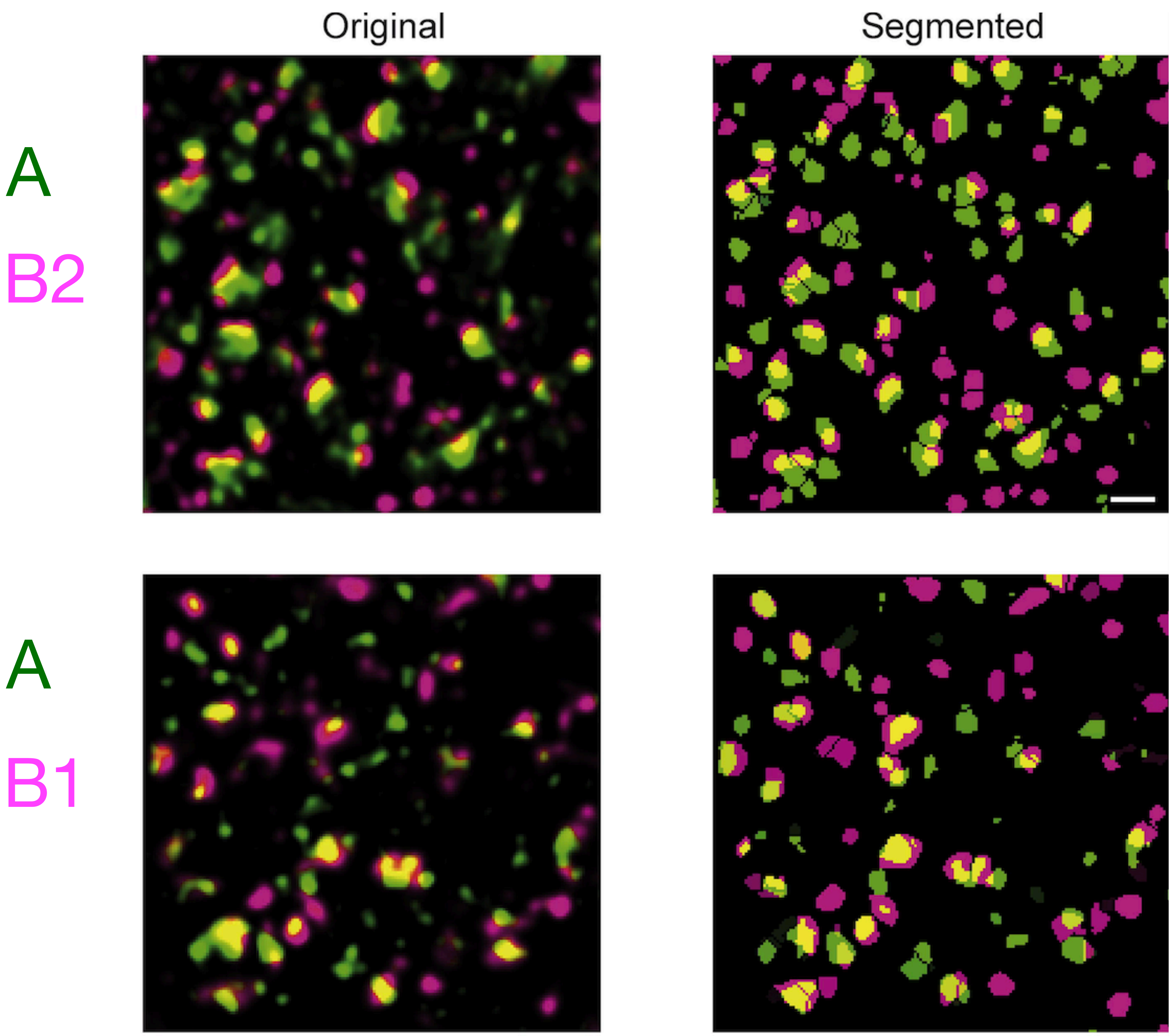
Modified from: Gilles *et al*.; **DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis**, Methods, Volume 115, 2017, Pages 55-64

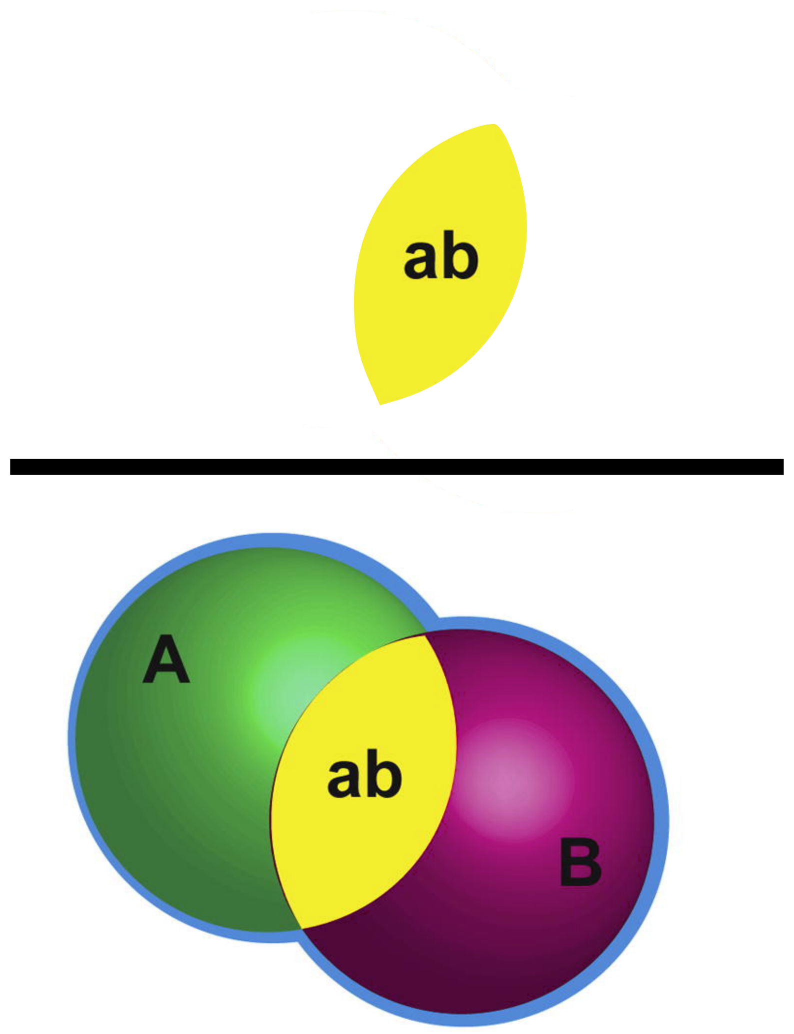
Example: Area

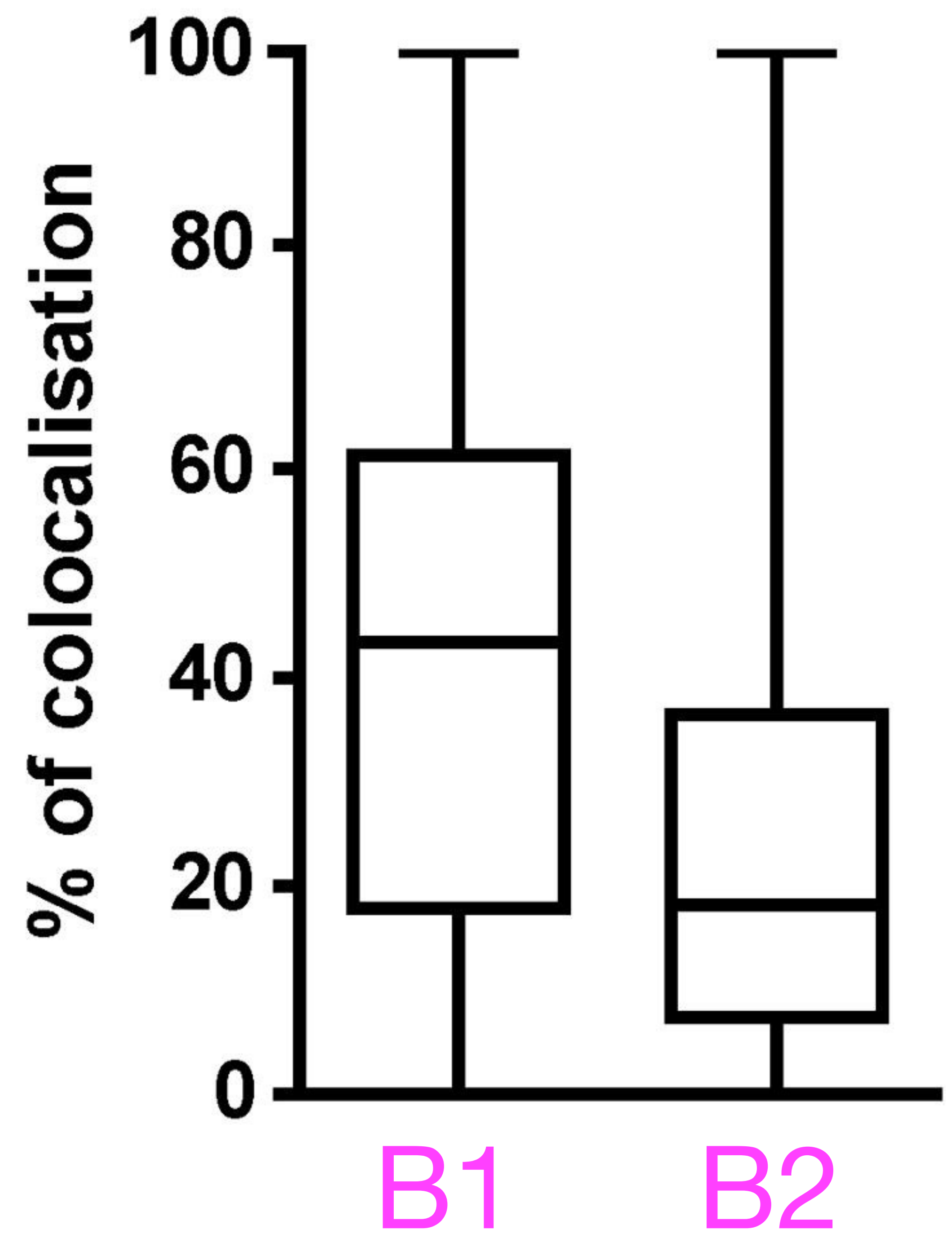


Modified from: Gilles *et al*.; **DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis**, Methods, Volume 115, 2017, Pages 55-64

Example: Area

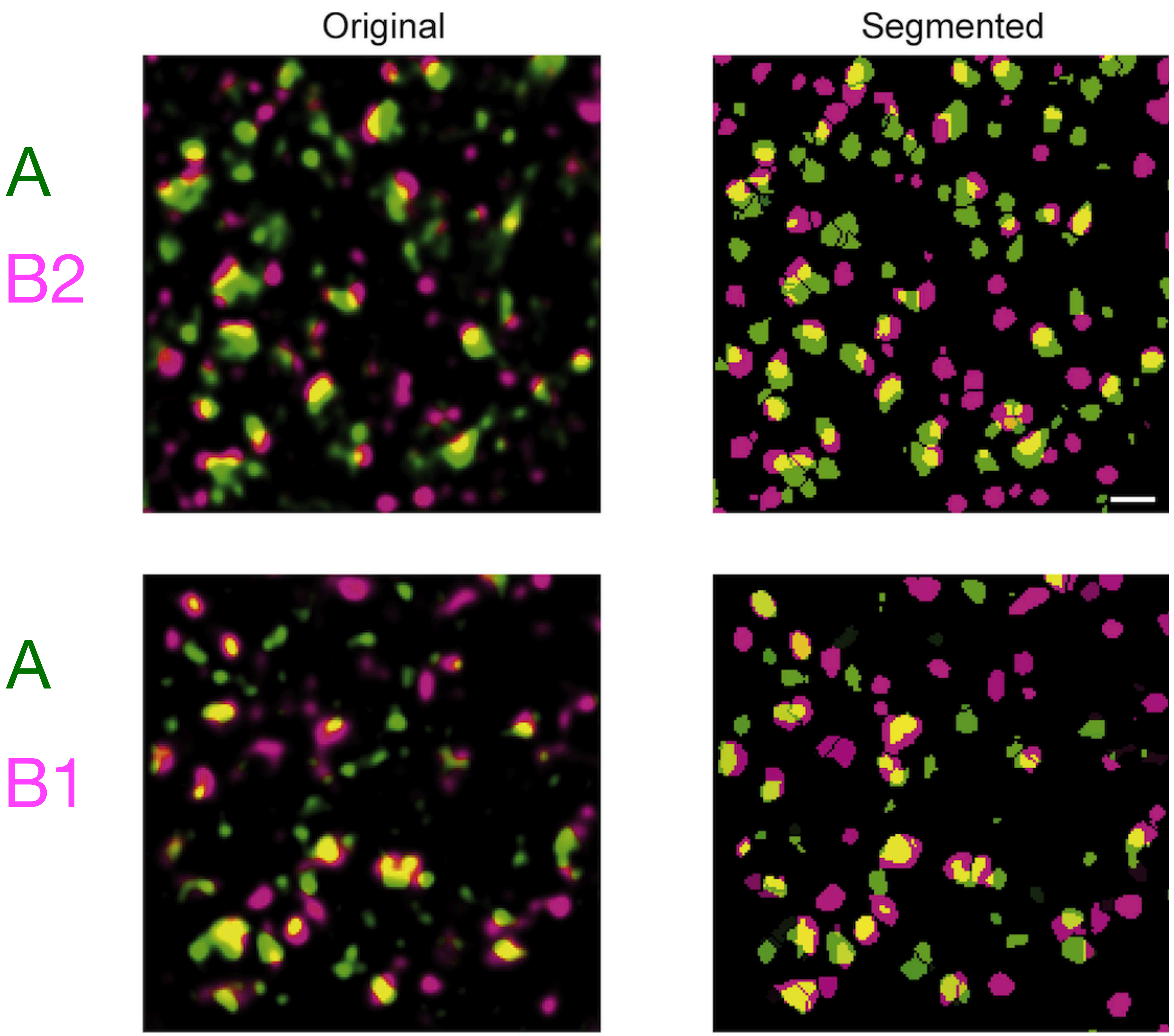


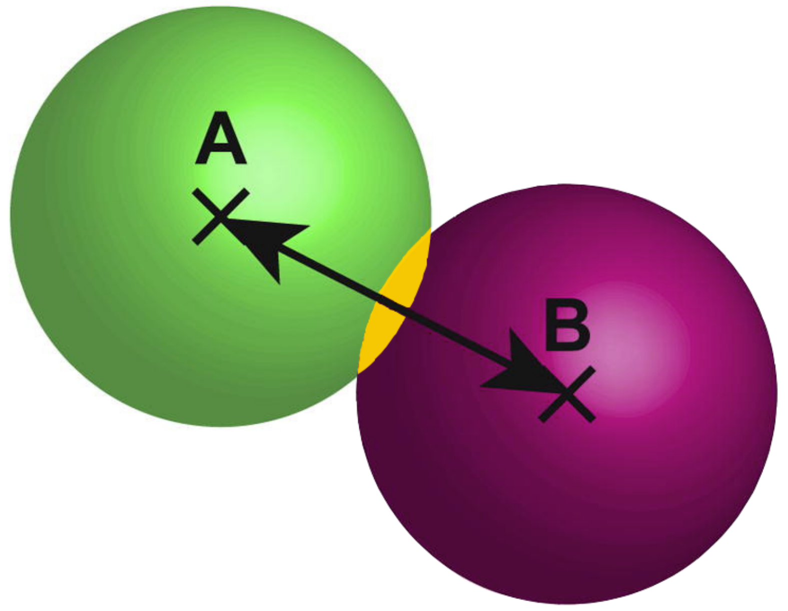




Modified from: Gilles *et al.*; **DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis**, Methods, Volume 115, 2017, Pages 55-64

Example: Points



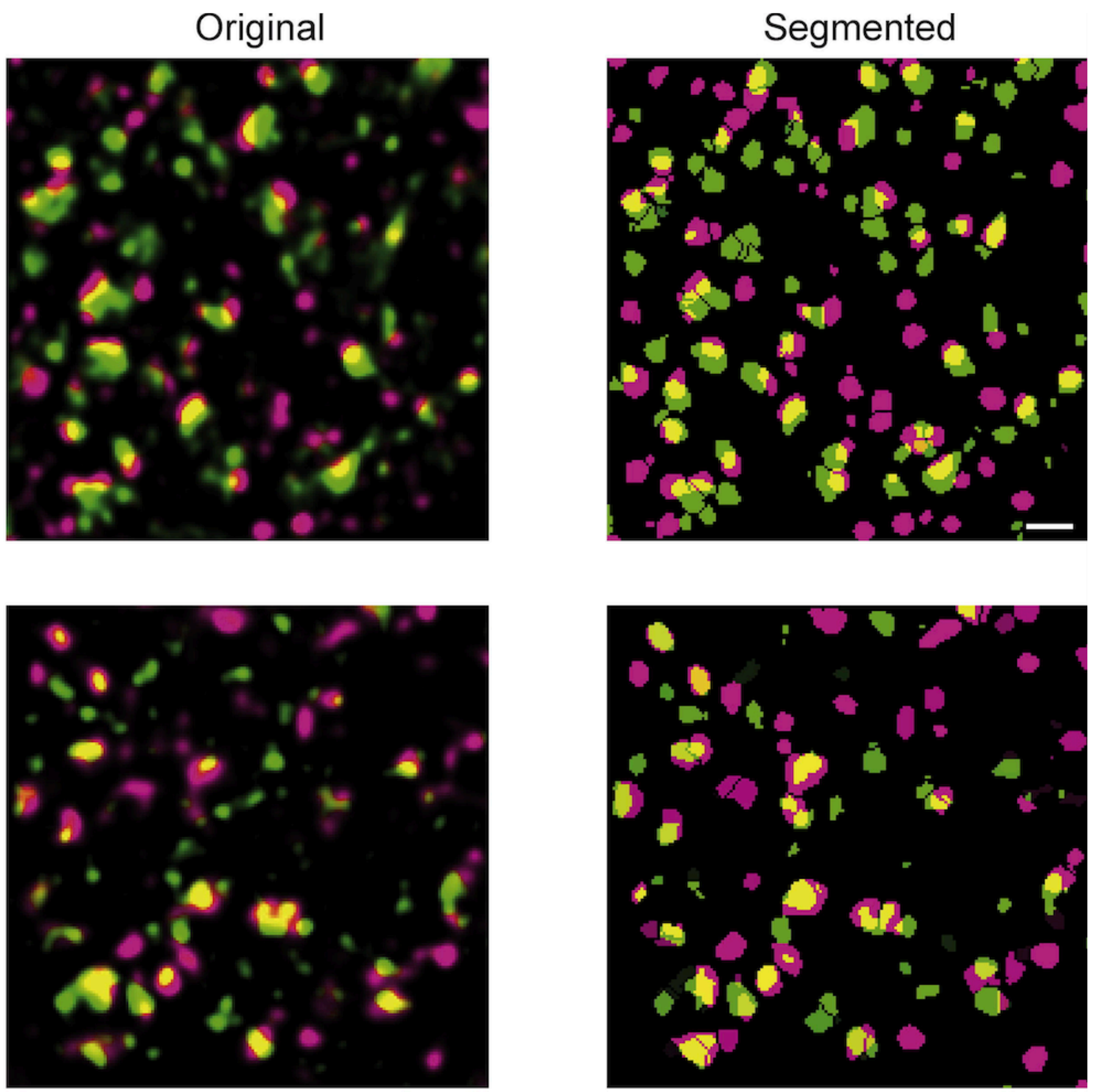


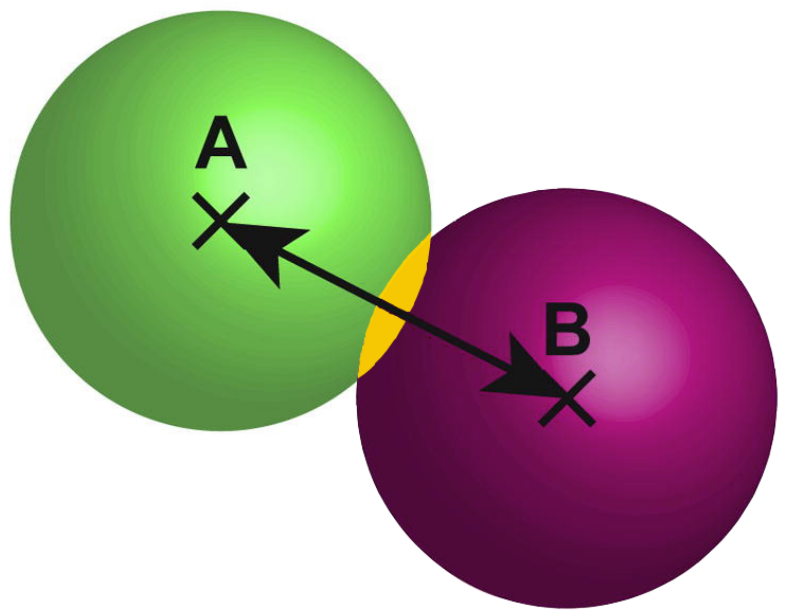
Modified from: Gilles *et al*.; **DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis**, Methods, Volume 115, 2017, Pages 55-64

Example: Points

A
B2

A
B1

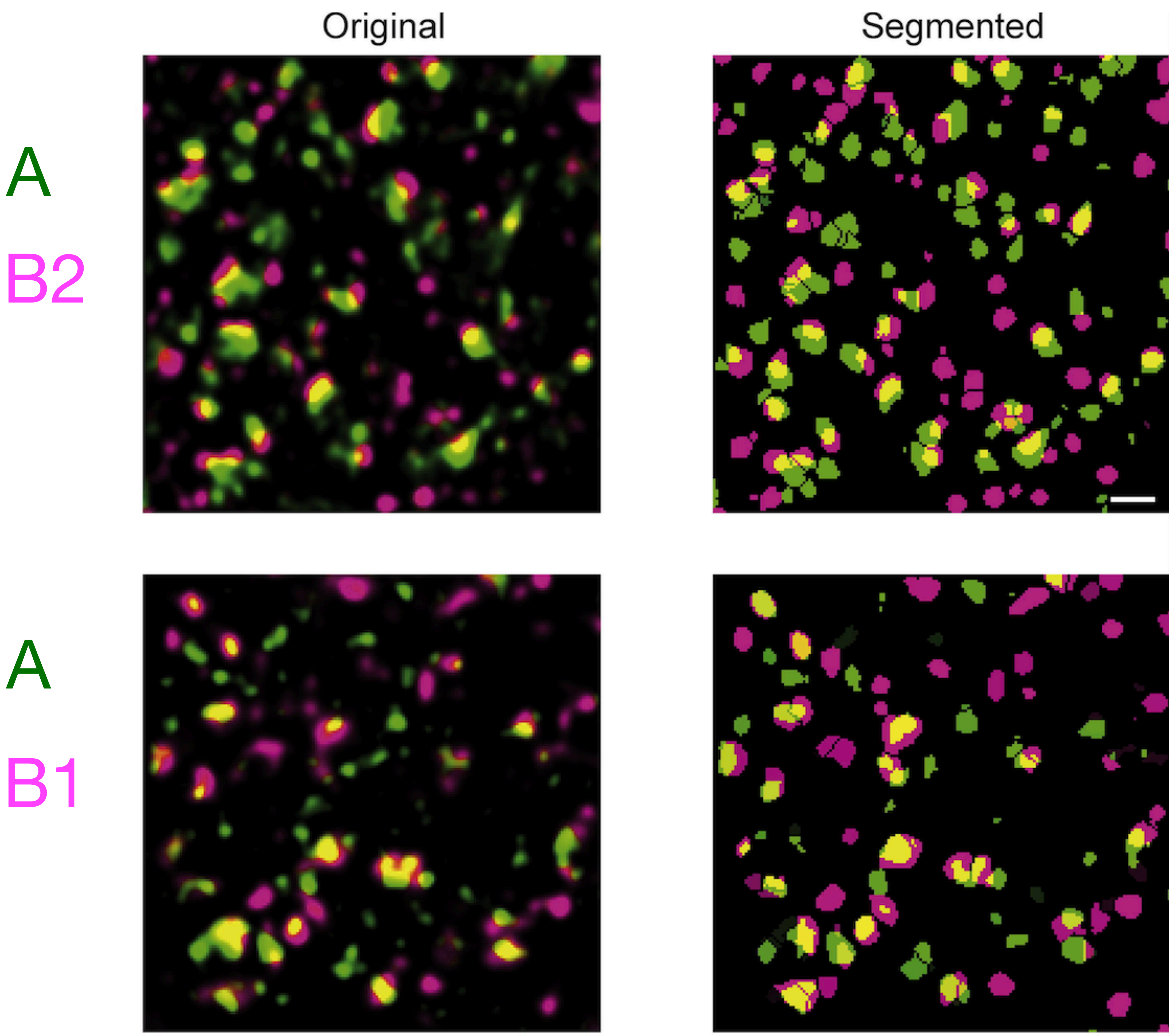


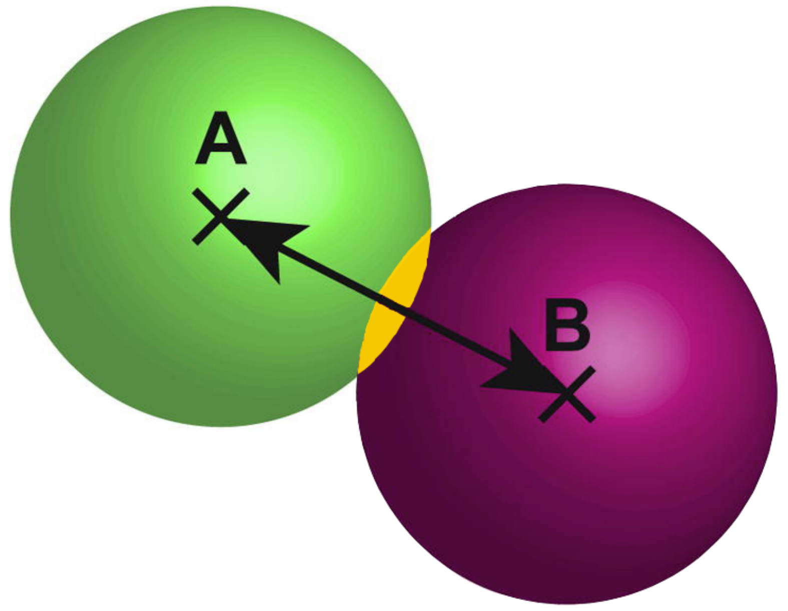


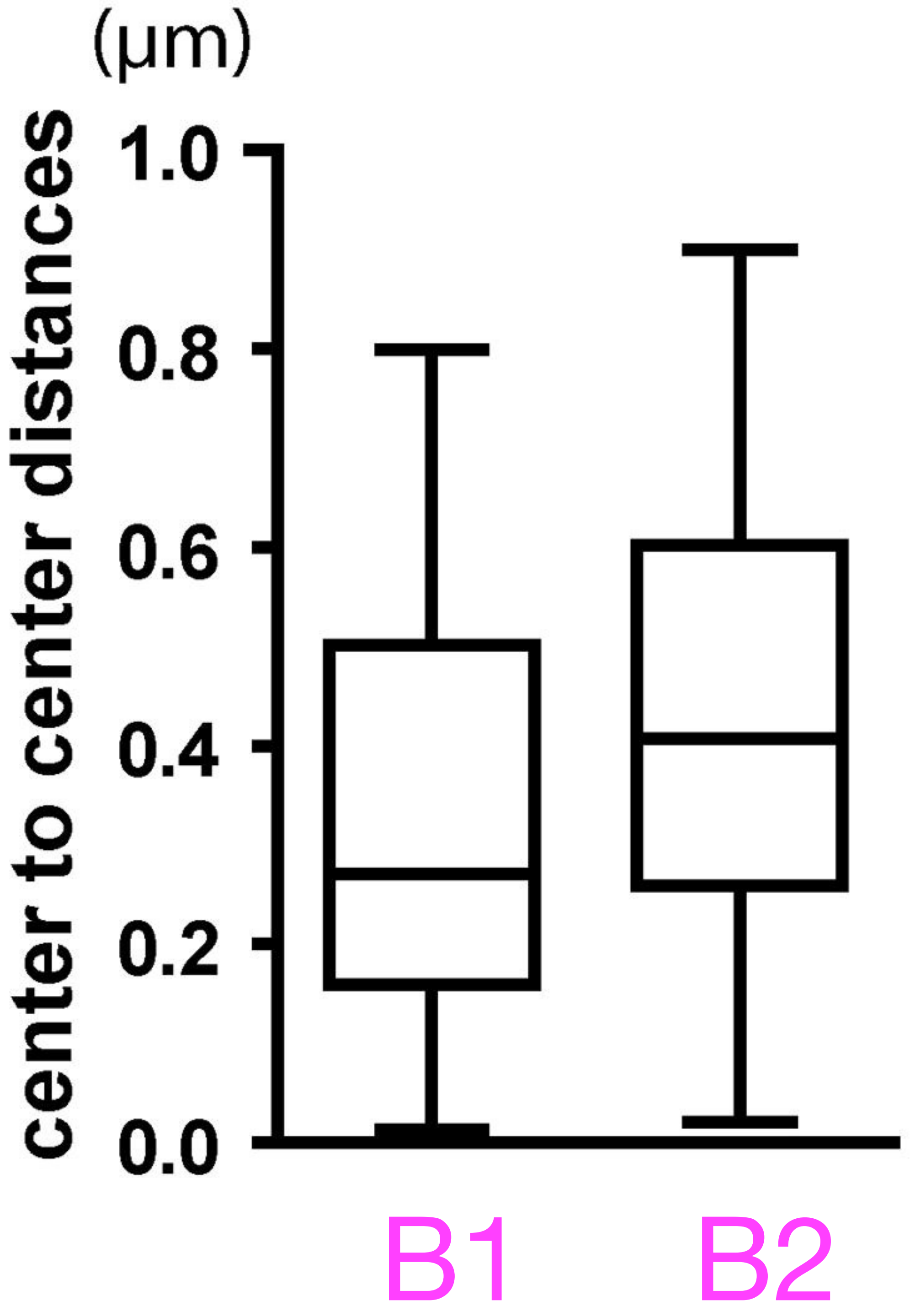
Remember:
Objects can
sometimes be
interconverted!

Modified from: Gilles *et al*.; **DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis**, Methods, Volume 115, 2017, Pages 55-64

Example: Points

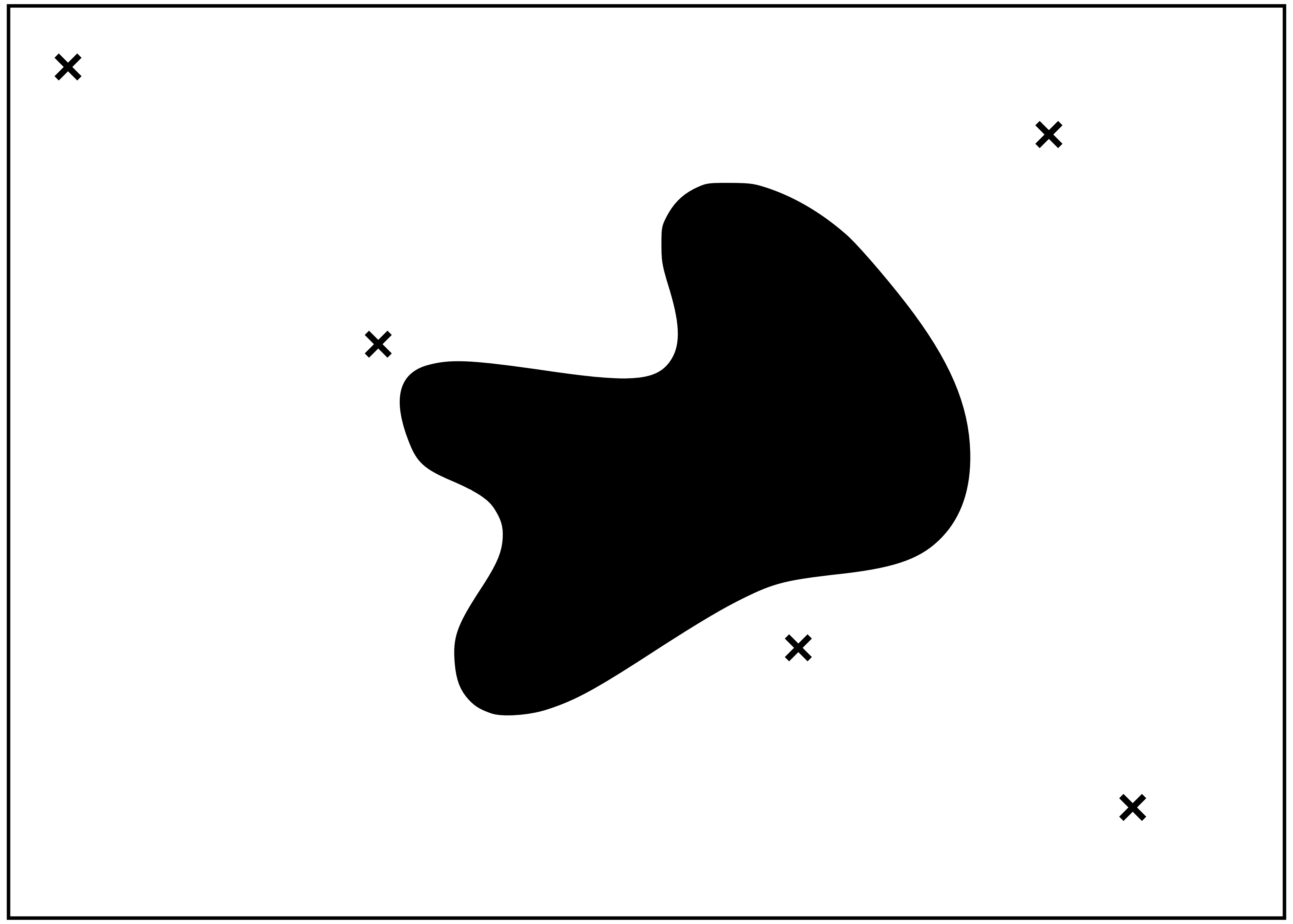




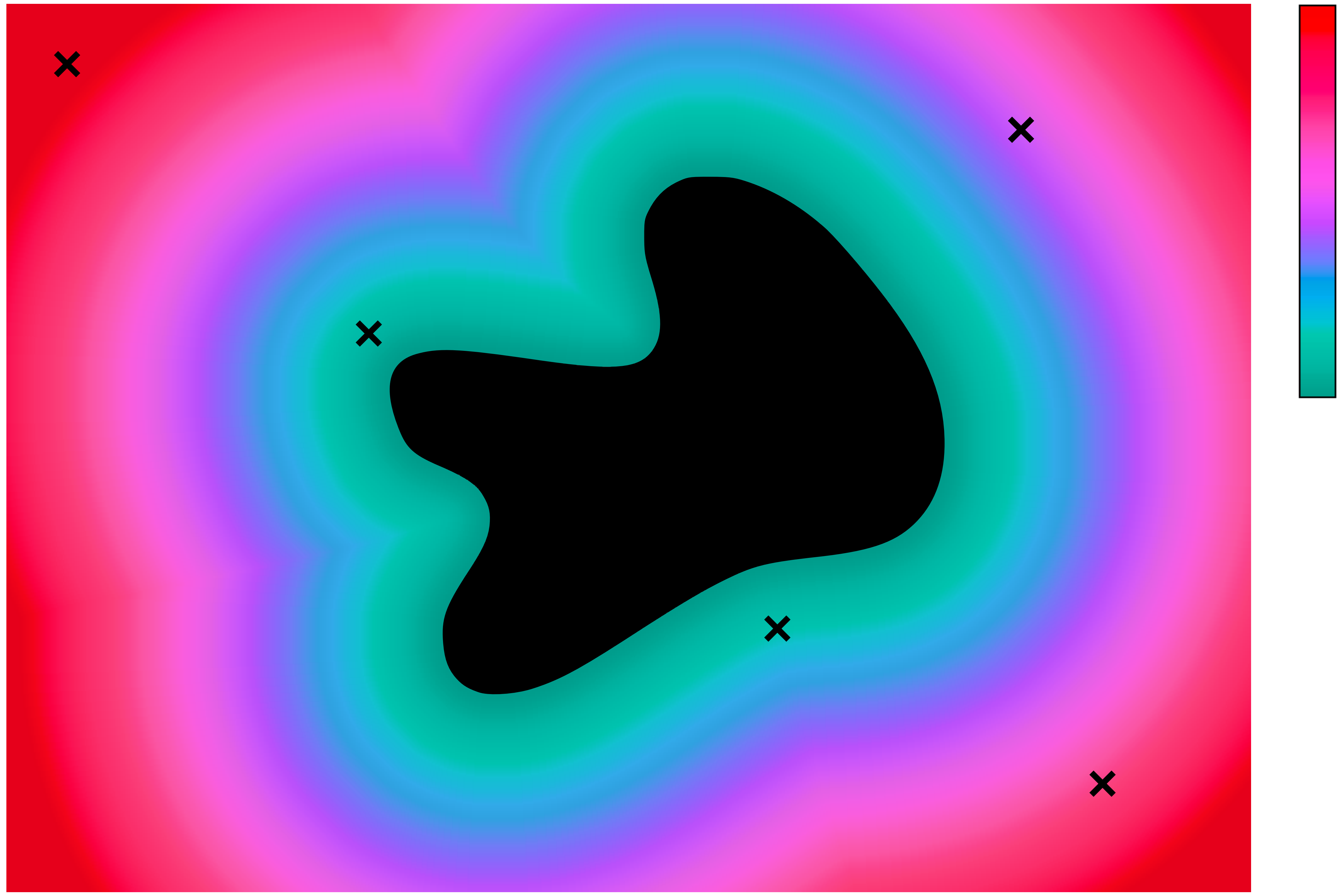


Modified from: Gilles *et al*.; **DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis**, Methods, Volume 115, 2017, Pages 55-64

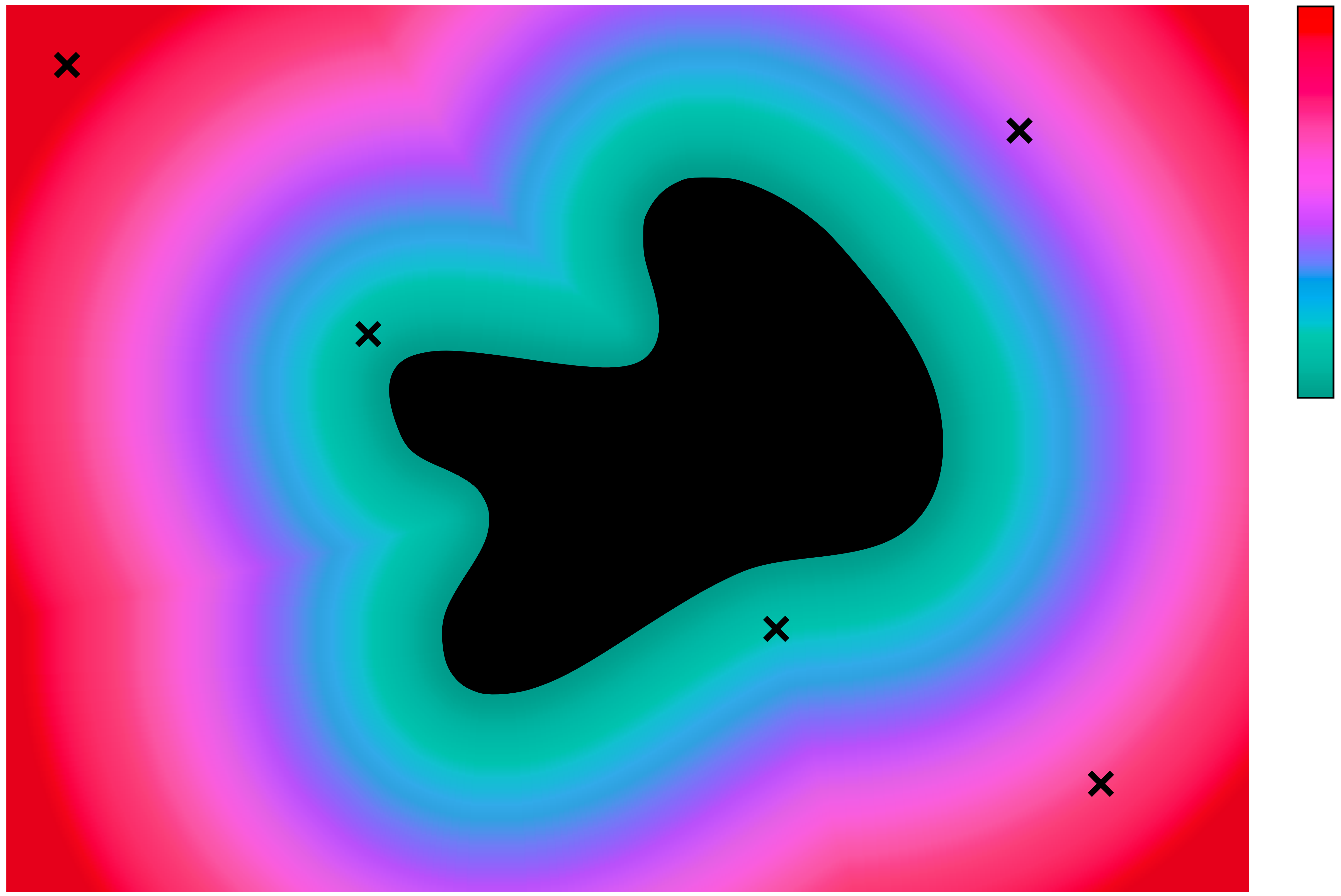
Example: Points to Edges

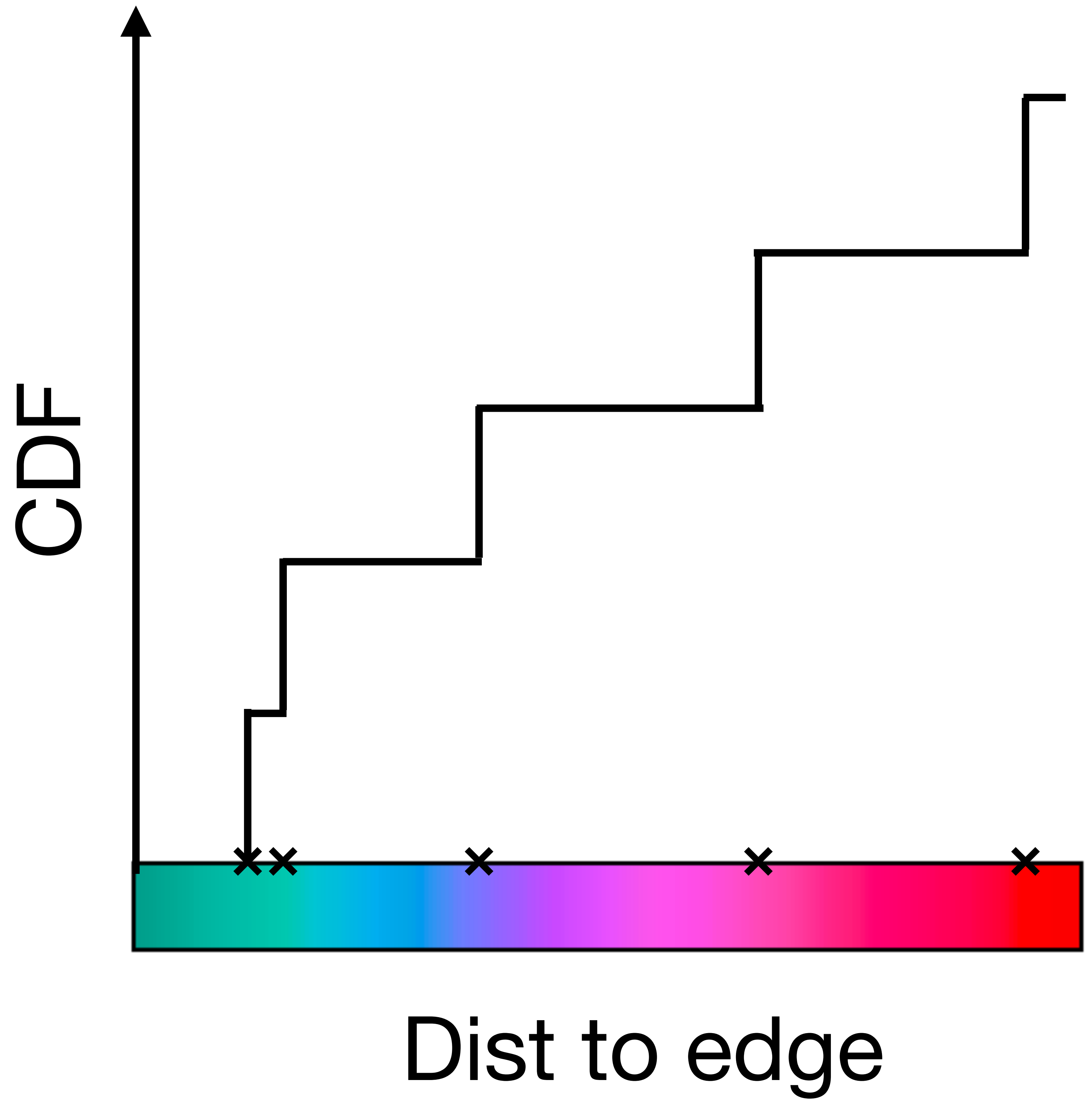


Example: Points to Edges



Example: Points to Edges



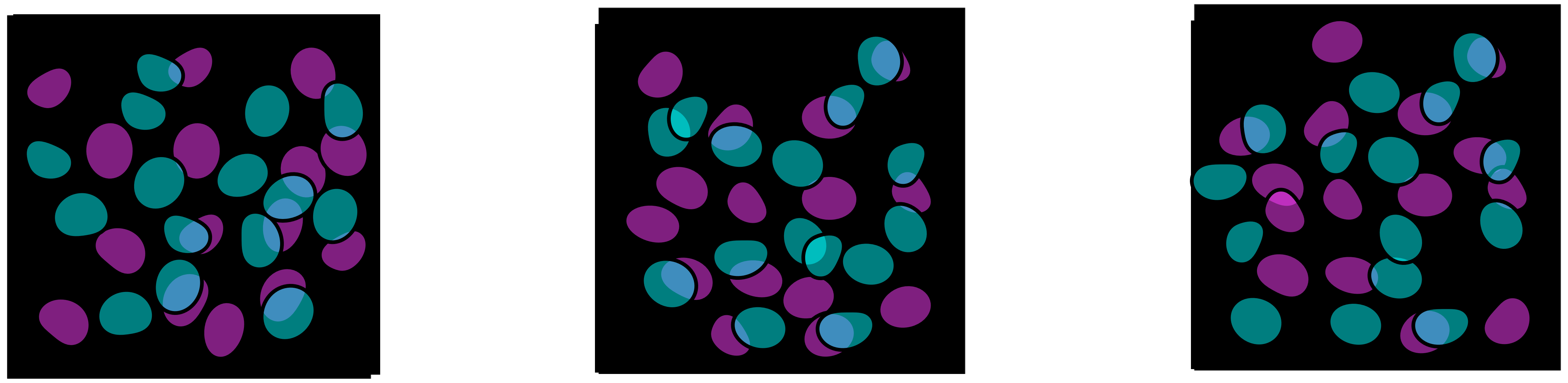


Statistical validation: Comparison to known controls

Which hypothesis explains the condition better:

+ CTRL: Hypothesis A

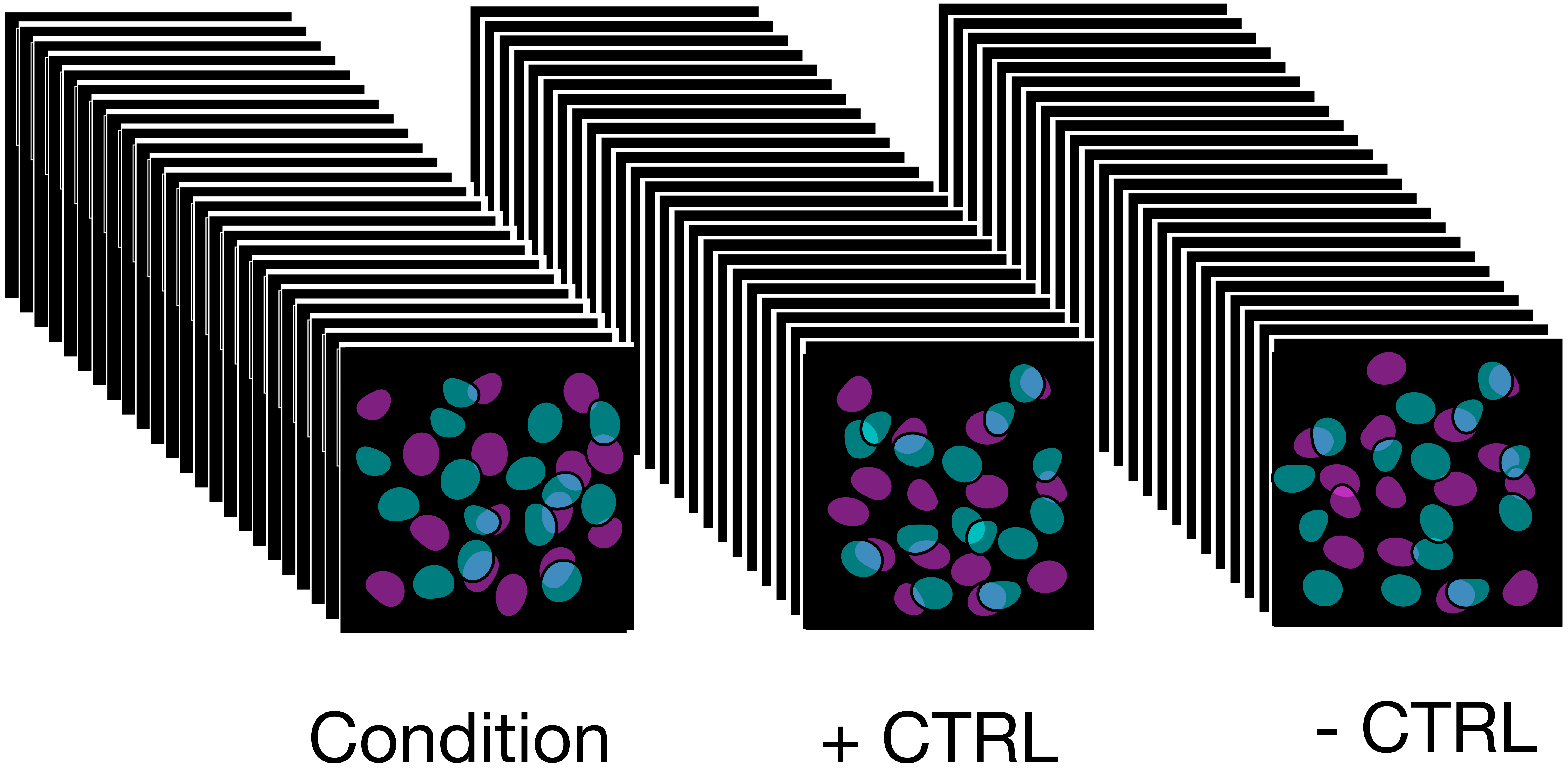
- CTRL: Hypothesis B



Condition + CTRL - CTRL

Modified from: Gilles *et al*.; **DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis**, Methods, Volume 115, 2017, Pages 55-64

Statistical validation: Comparison to known controls



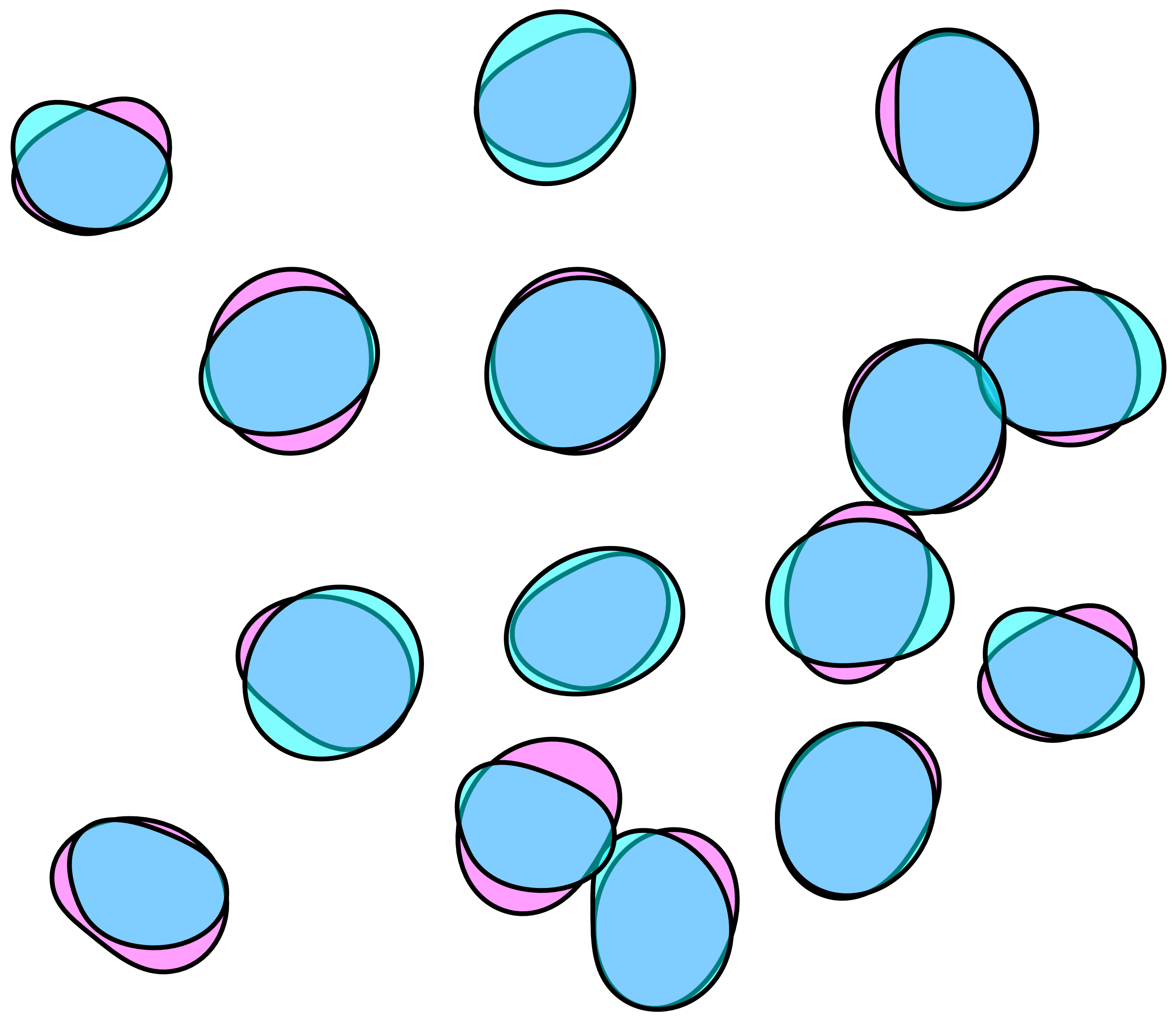
A large enough sample size allows us to robustly compare samples

Modified from: Gilles *et al*.; **DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis**, Methods, Volume 115, 2017, Pages 55-64

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

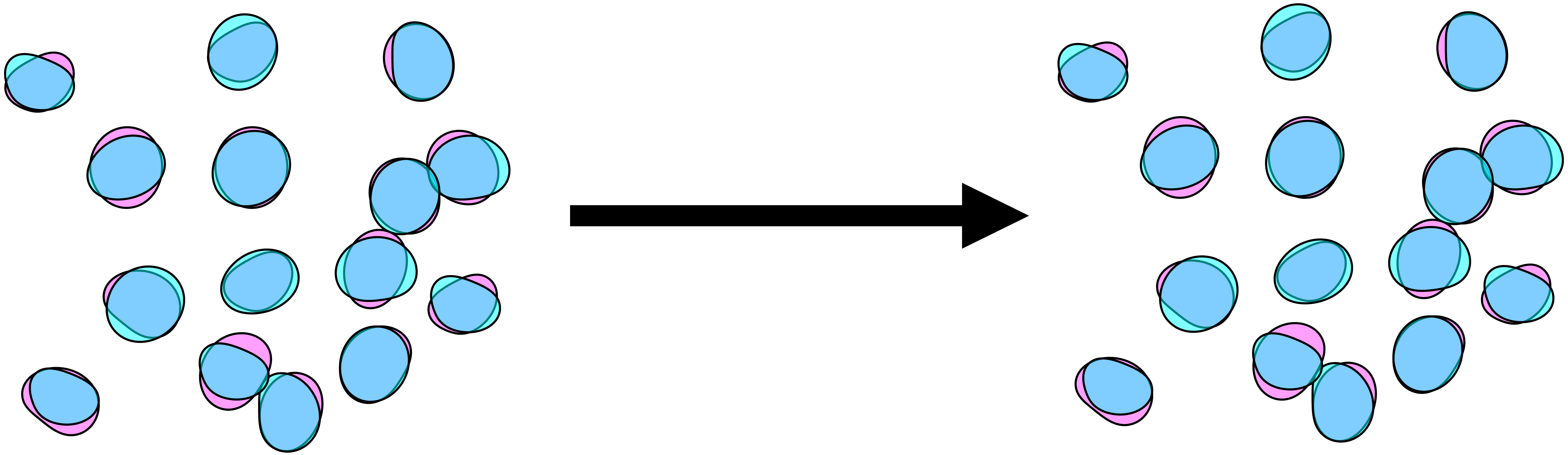
Statistical validation



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

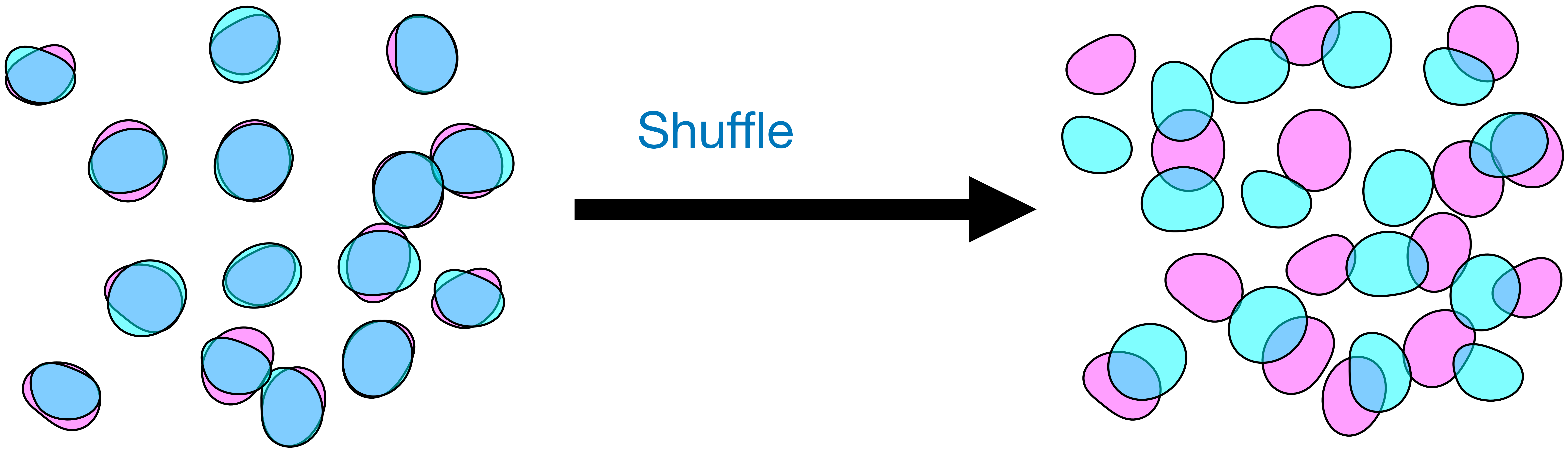


Q: Is the overlap between cyan and magenta pure chance?

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

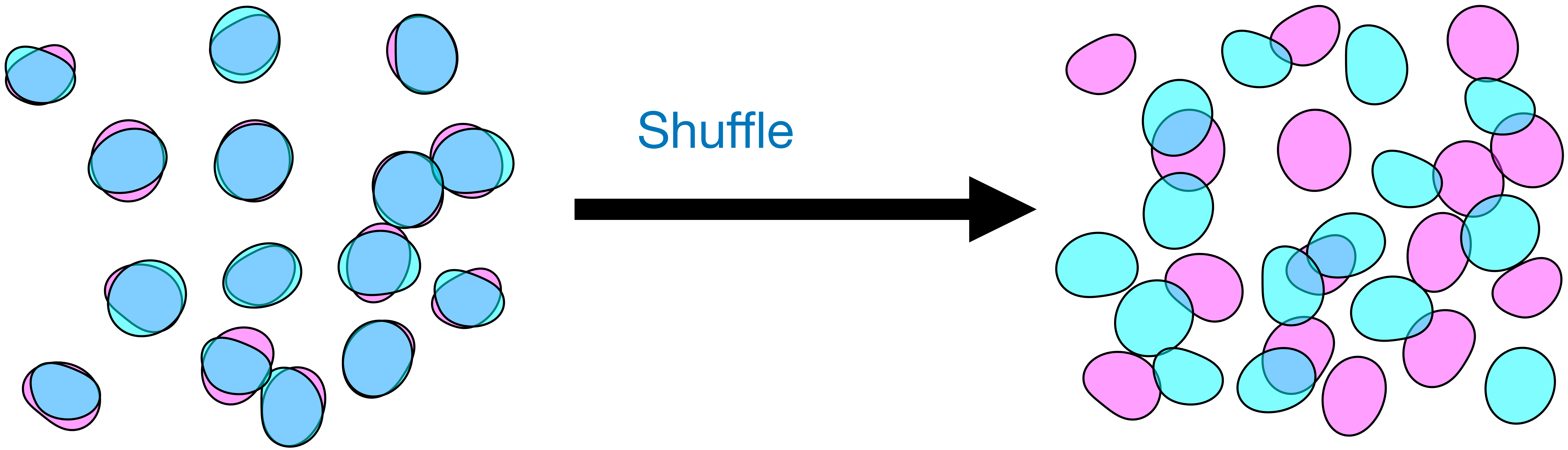
> Simulate what H0 would look like

Statistical validation



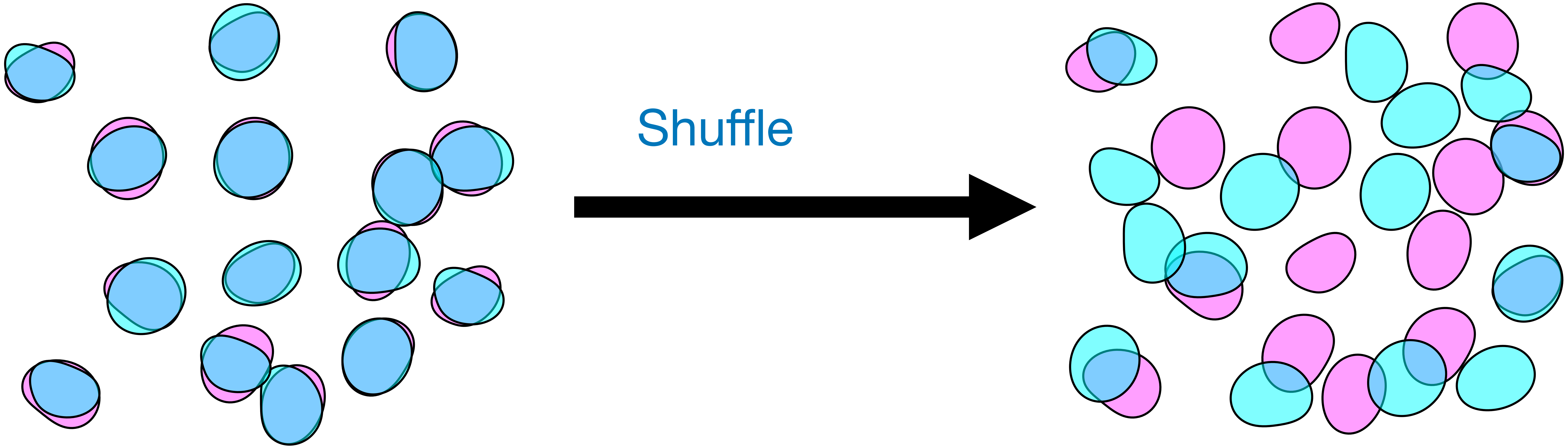
Randomly shuffle blue objects to simulate H0

Statistical validation



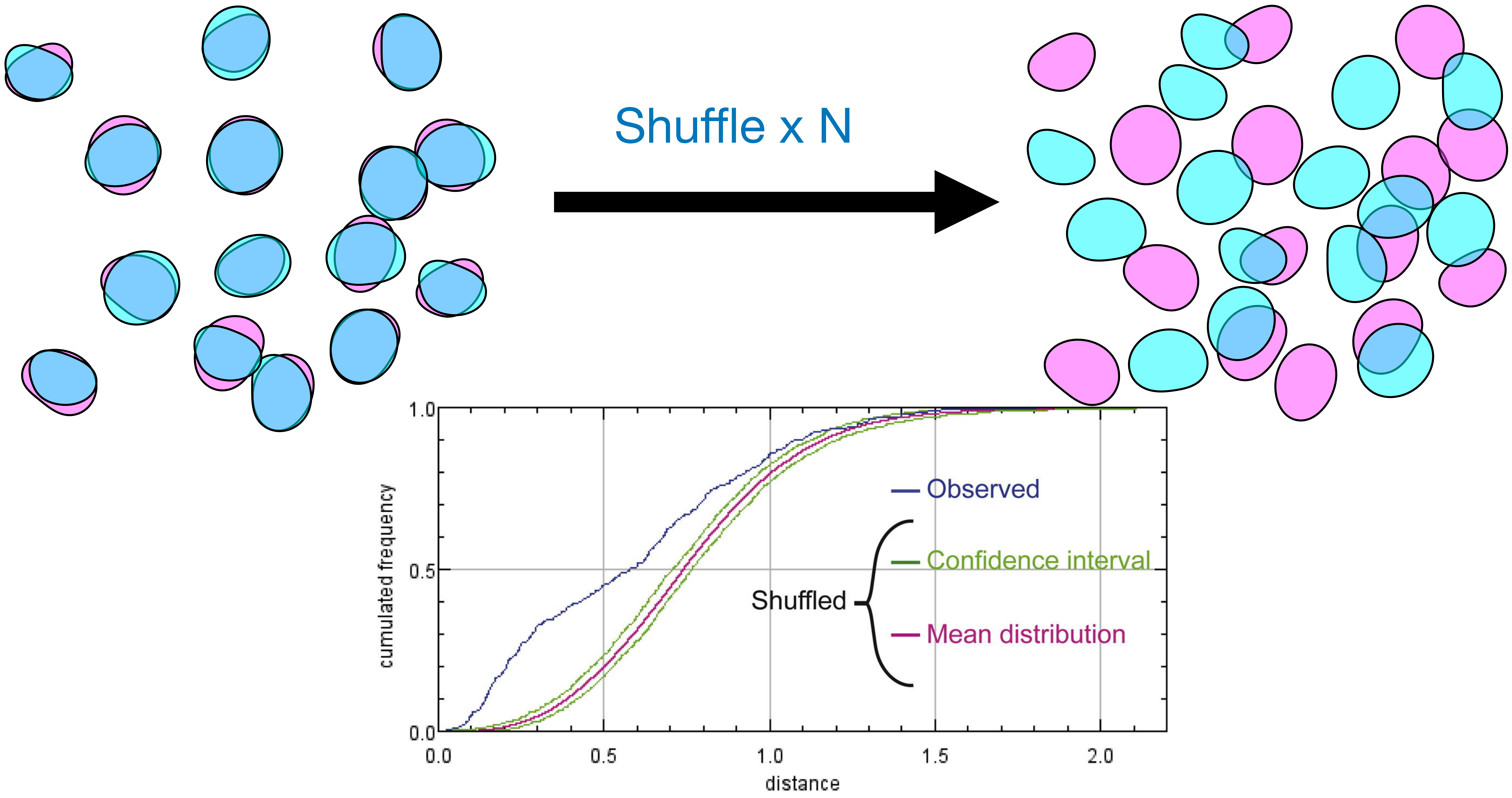
Randomly shuffle blue objects to simulate H0

Statistical validation



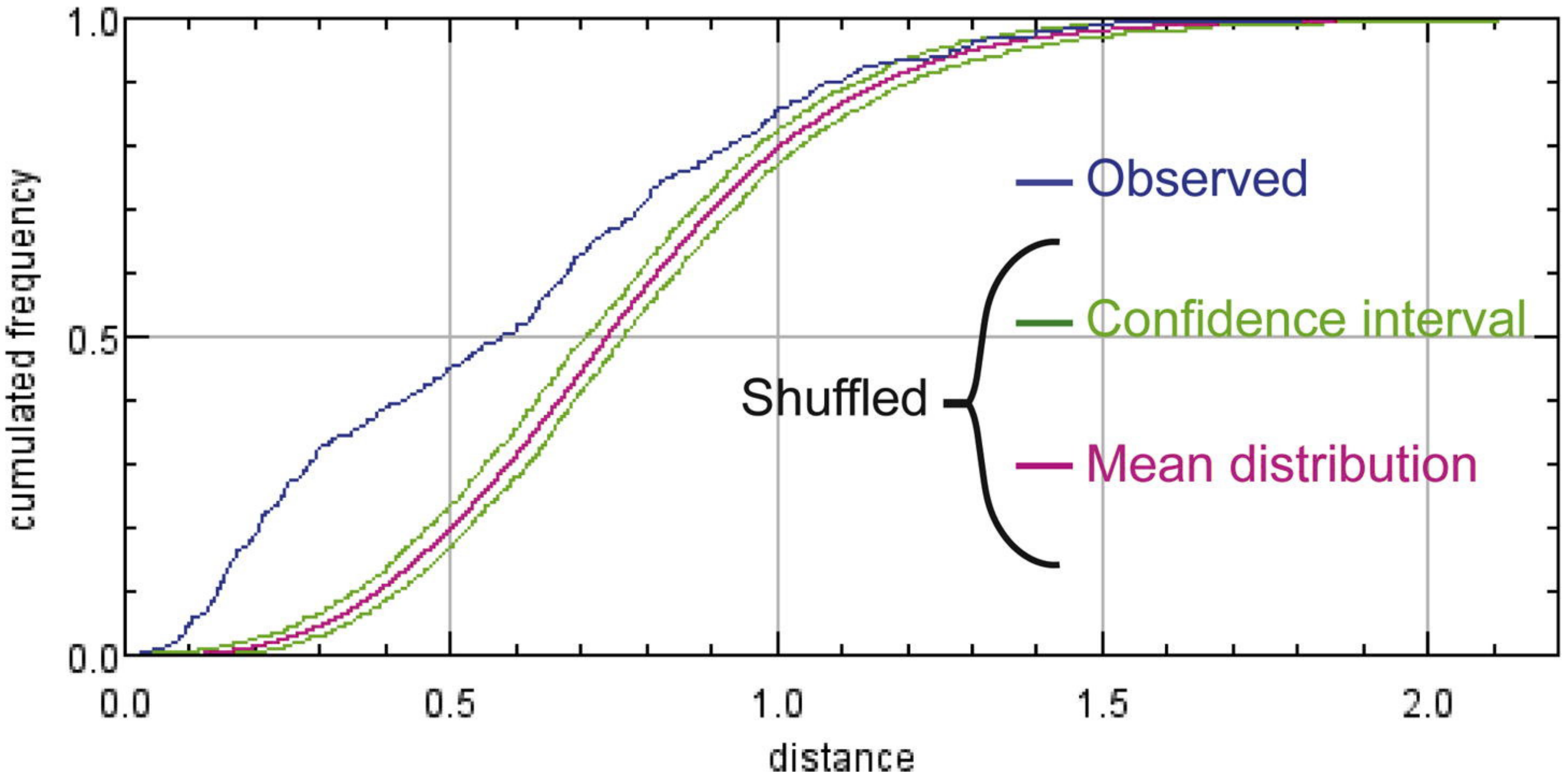
Randomly shuffle blue objects to simulate H0

Statistical validation



Modified from: Gilles *et al*.; **DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis**, Methods, Volume 115, 2017, Pages 55-64

Statistical validation



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