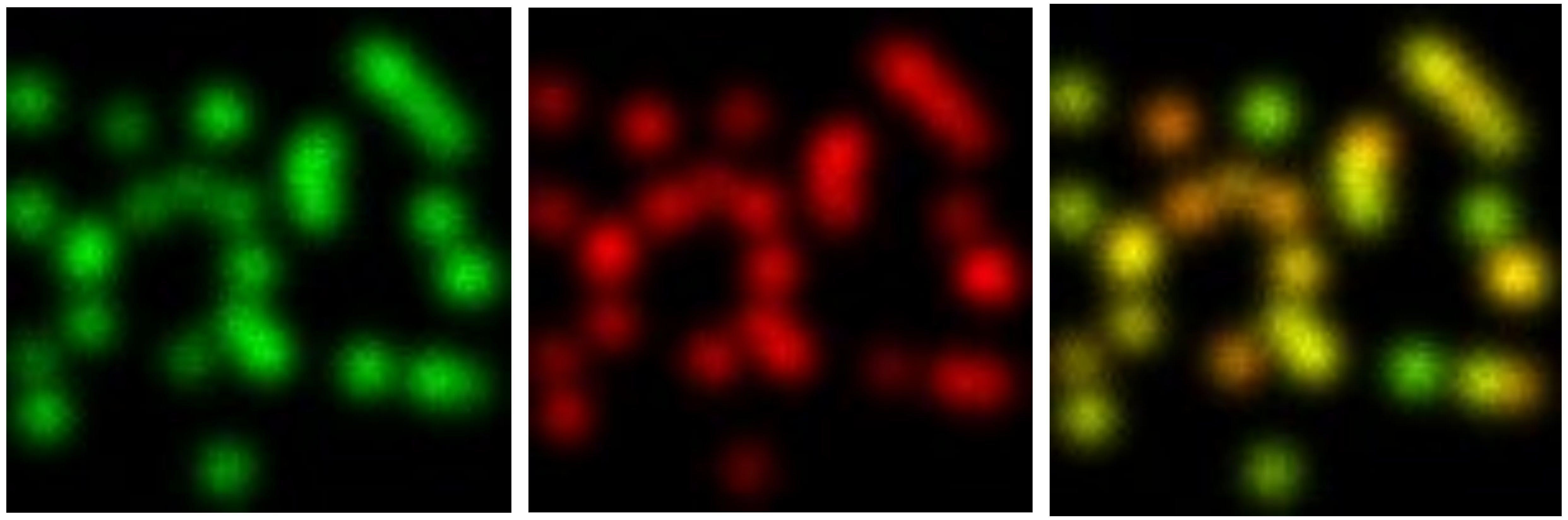
BoBiAC

Boston Bioimage Analysis Course | 2025

Introduction to Colocalizationin Fluorescence Microscopy

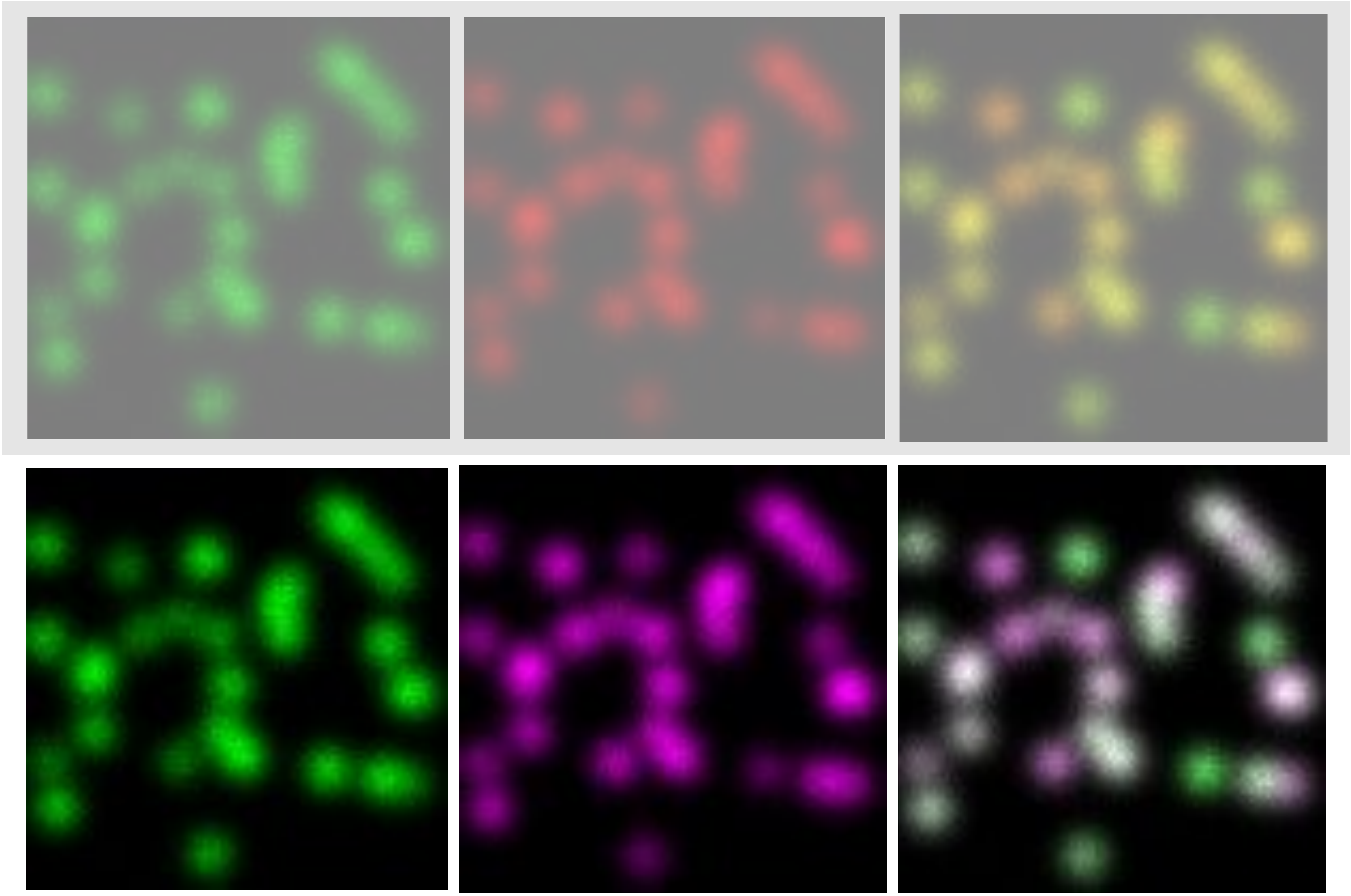
What Colocalization IS NOT in Fluorescence Microscopy



"Yellow" is **not** colocalization

Why?

What Colocalization IS NOT in Fluorescence Microscopy



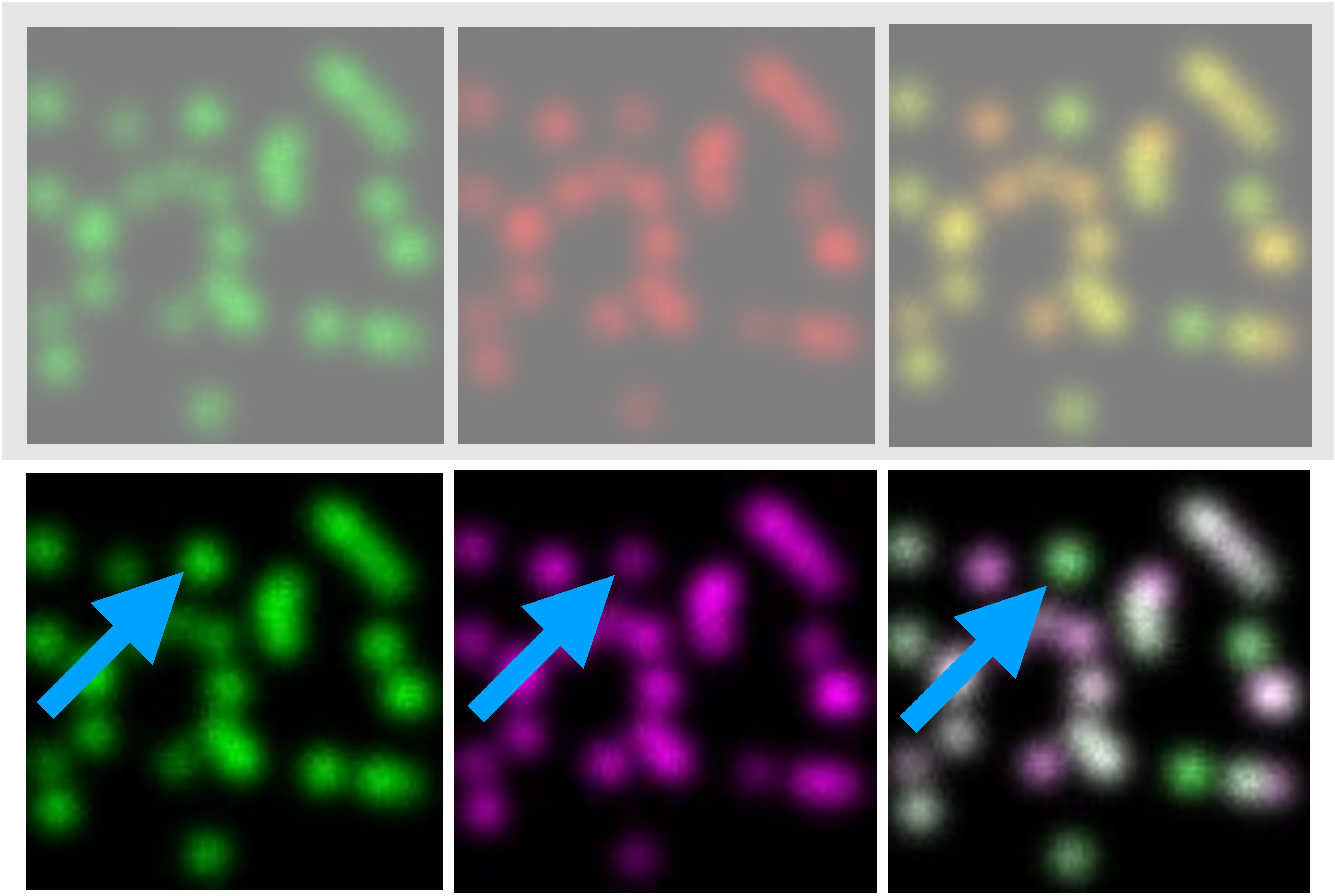
"Yellow" is **not** colocalization

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?

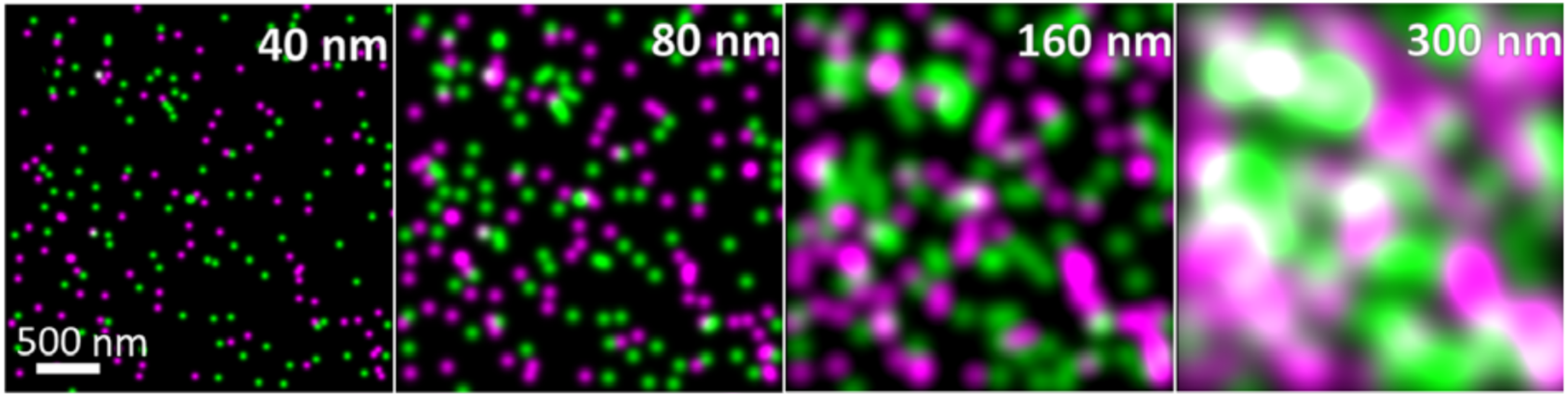
1. you should never see yellow because you should **not use red and green** together.
2. You can visualize overlap only if the signal is high in both channels.
3. How to quantify?

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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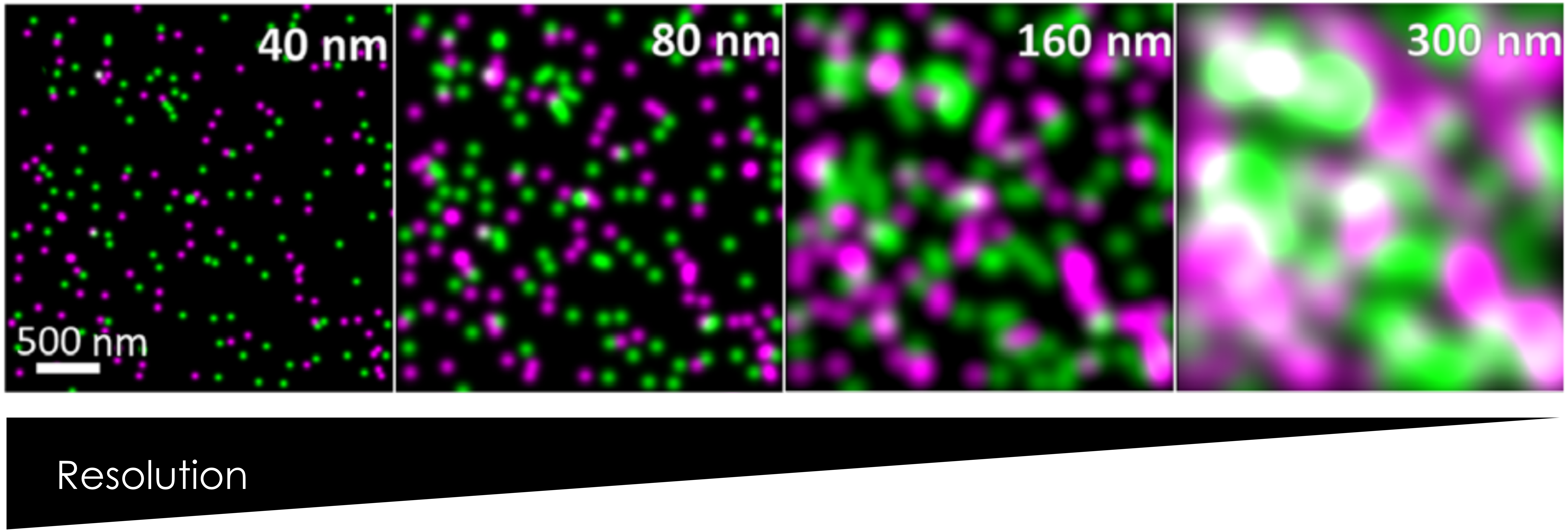
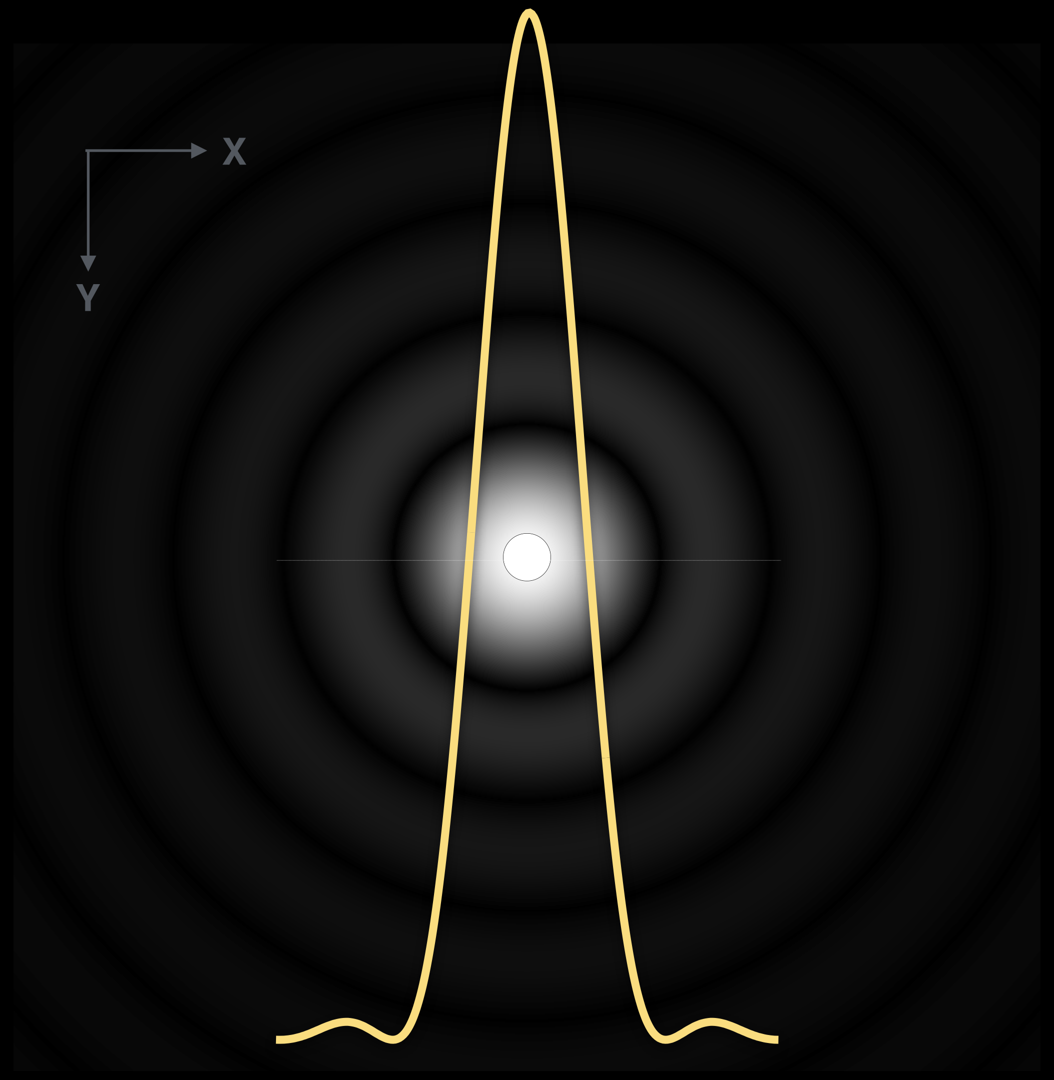


Adapted from Xu et al, 2016

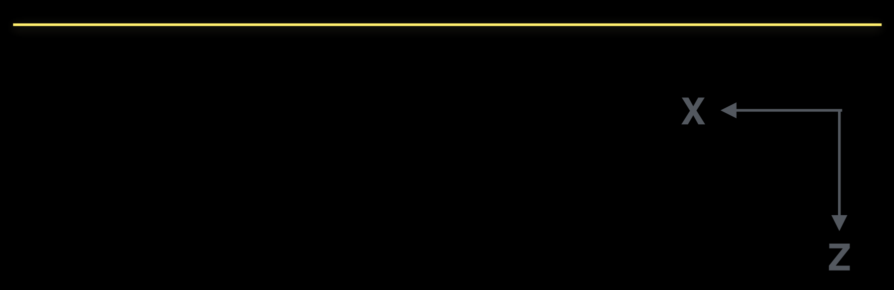
resolution: the ability to distinguish objects that are separate in the sample as separate from one another in the image of the sample

Adapted form Jennifer Waters

The Point Spread Function (PSF)

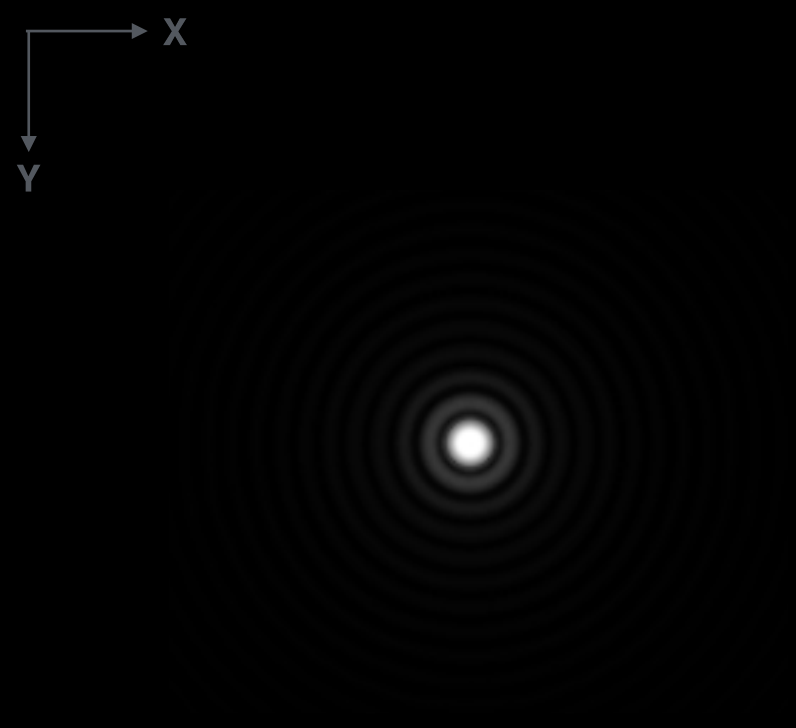


lateral

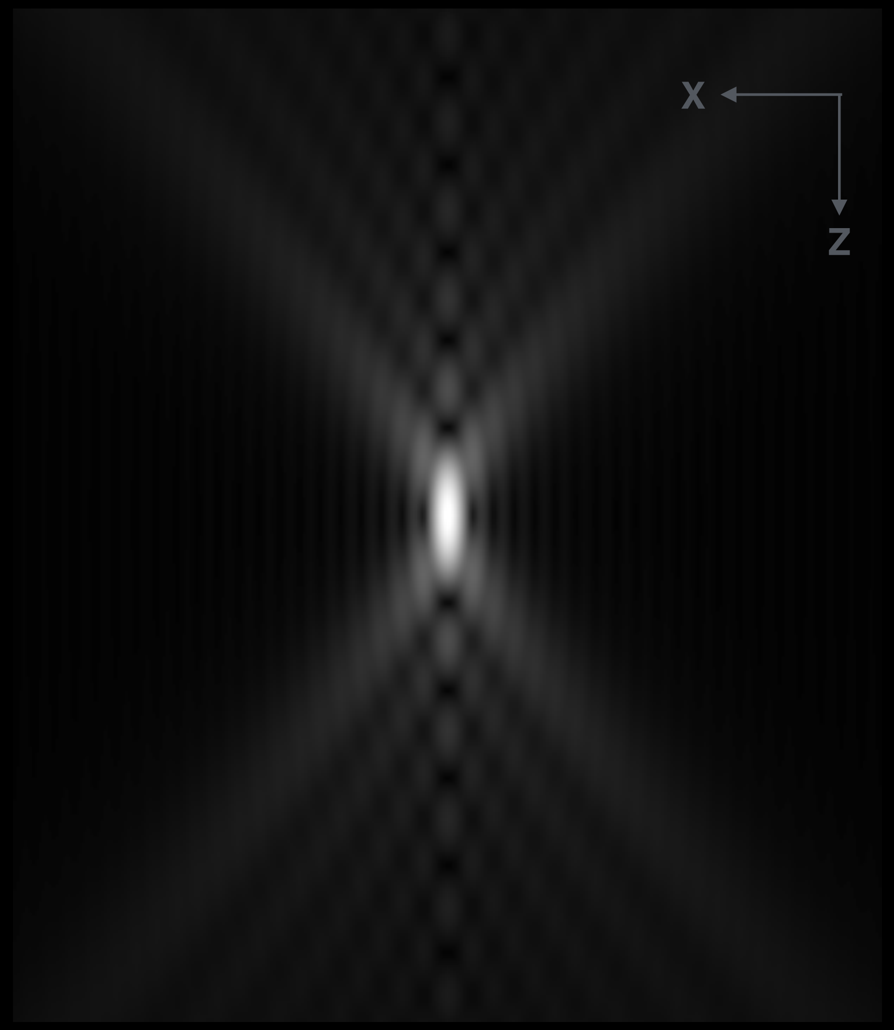


axial

The Point Spread Function (PSF)

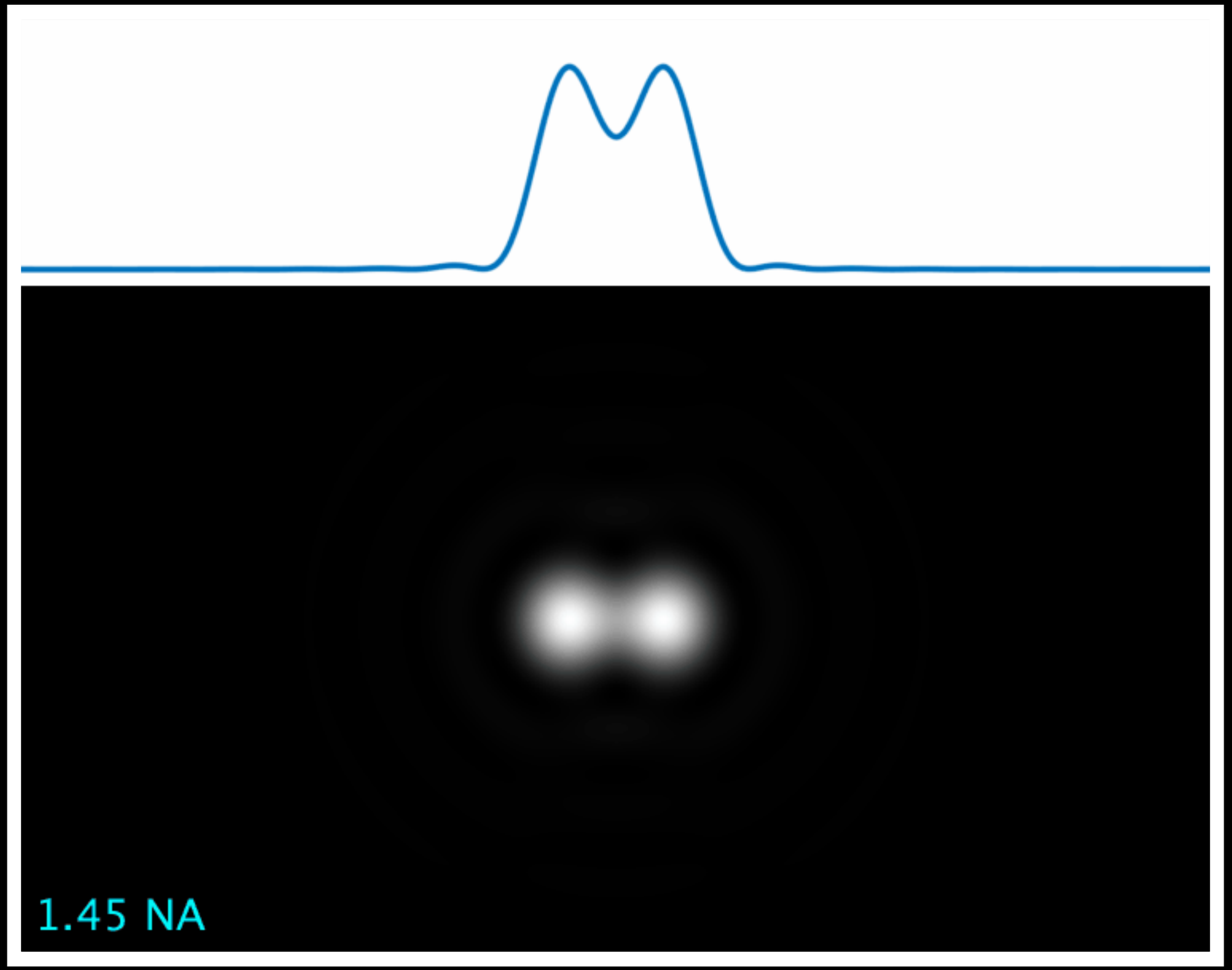


lateral



axial

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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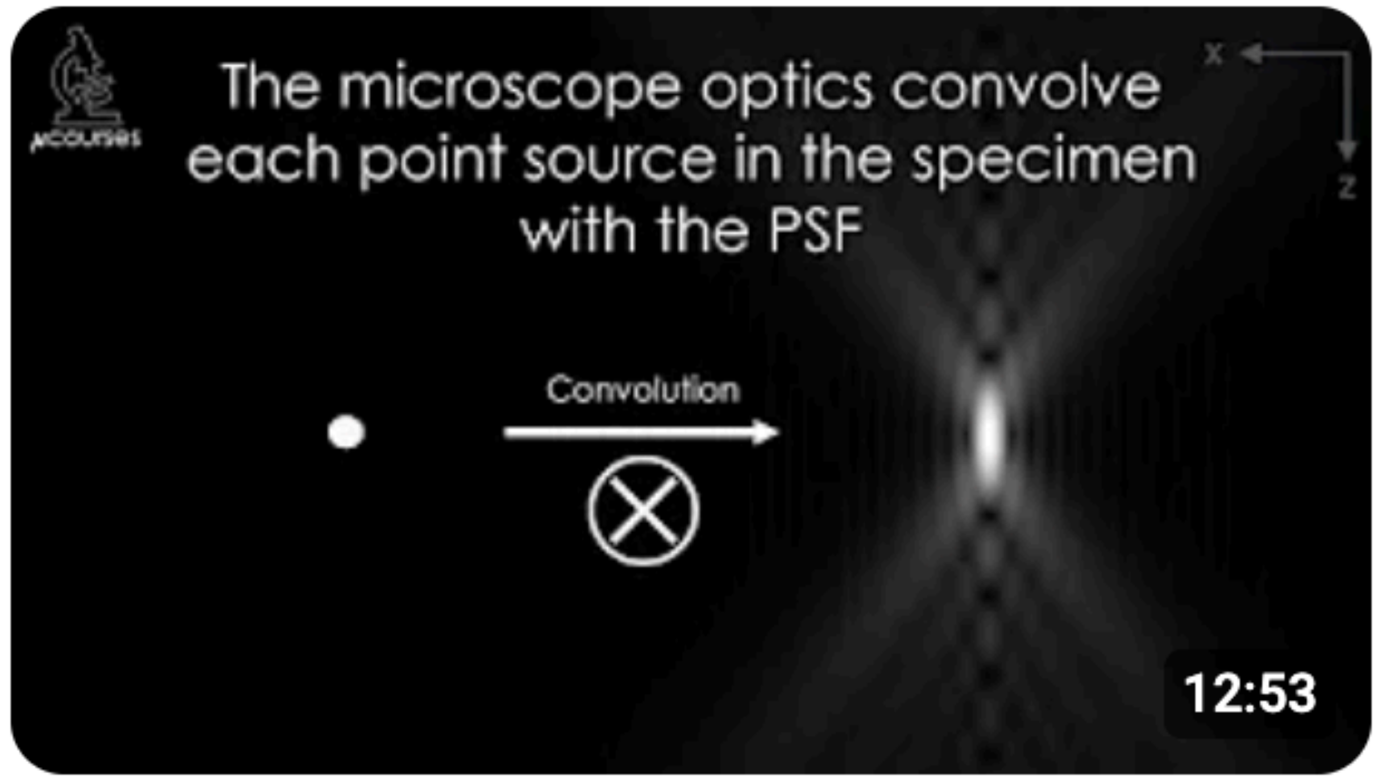
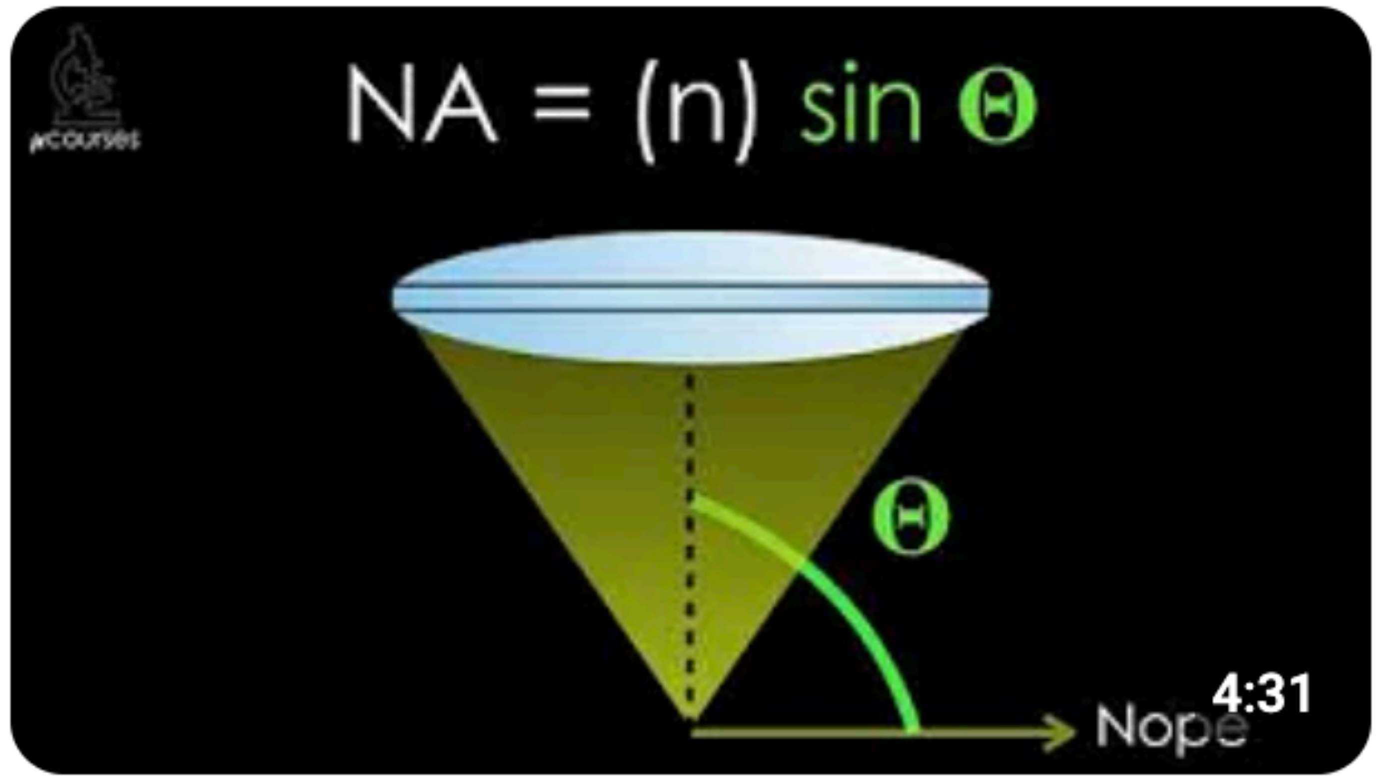


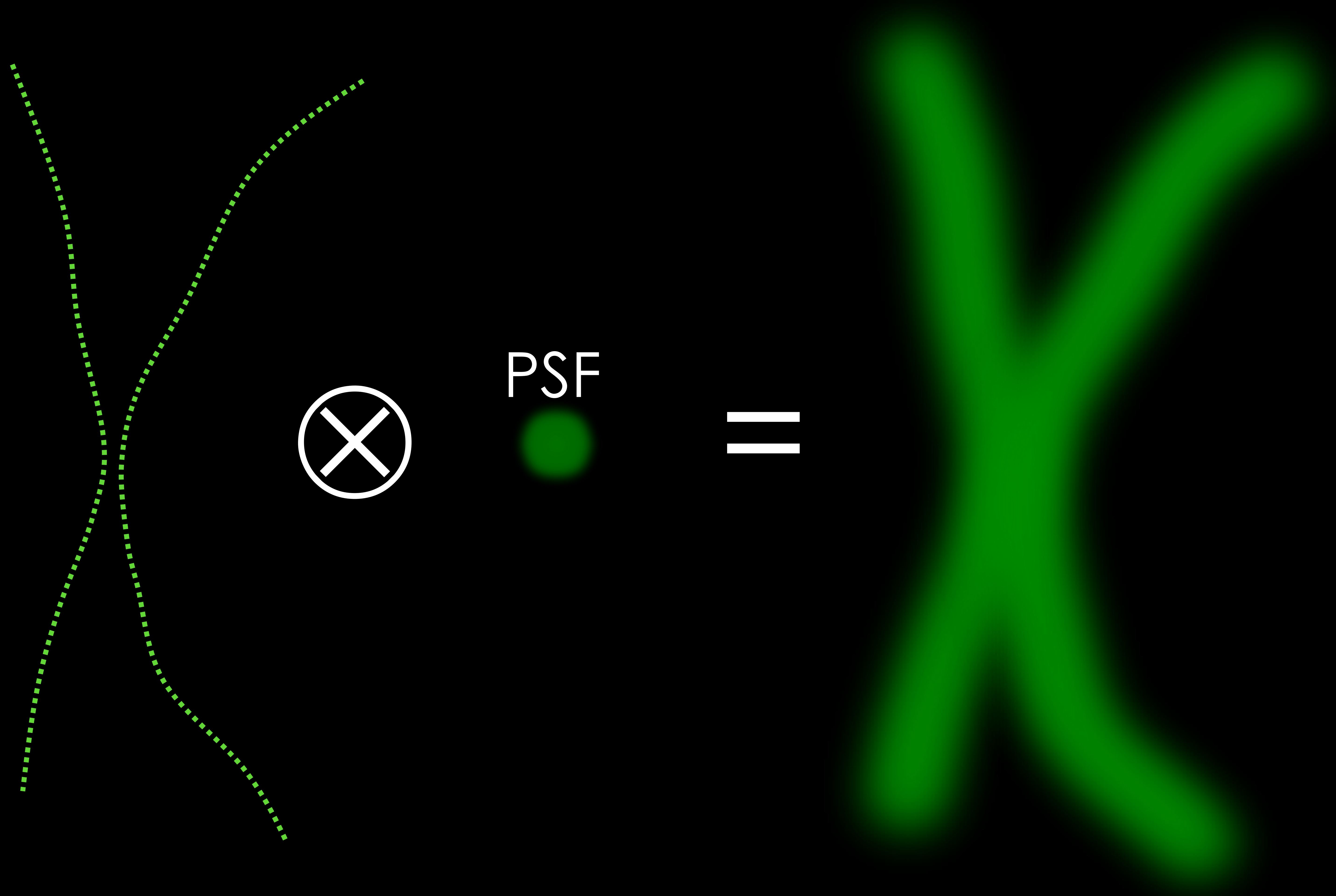
The Point Spread Function

70K views • 5 years ago



Numerical Aperture

82K views • 5 years ago



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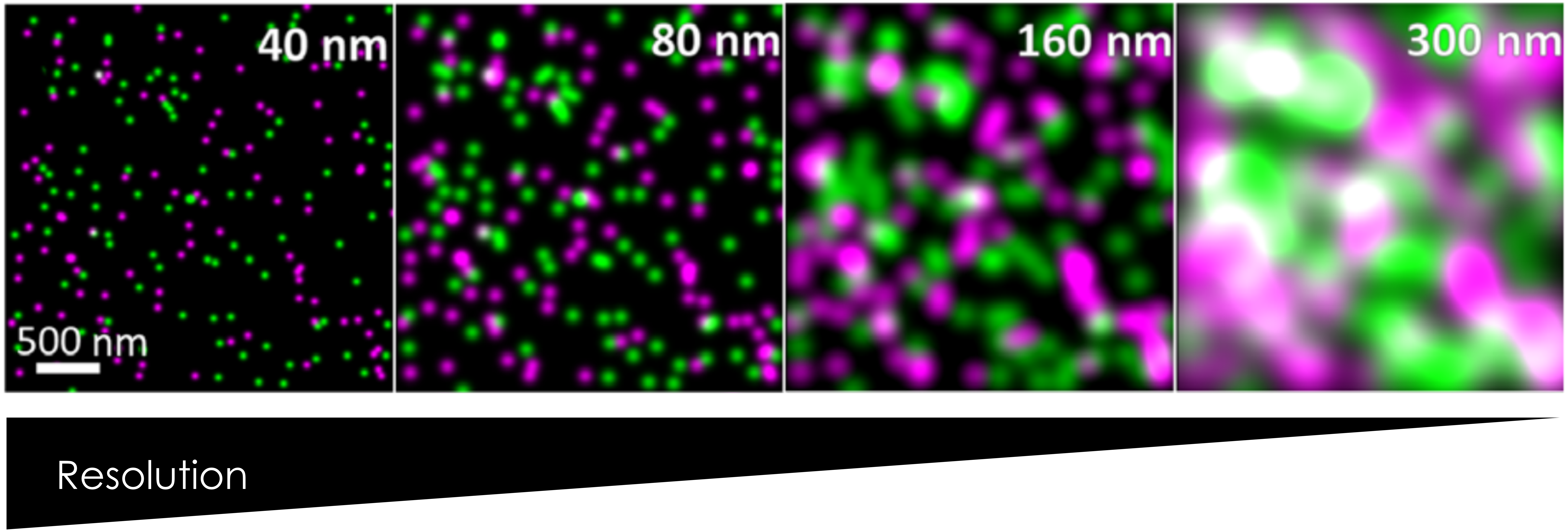

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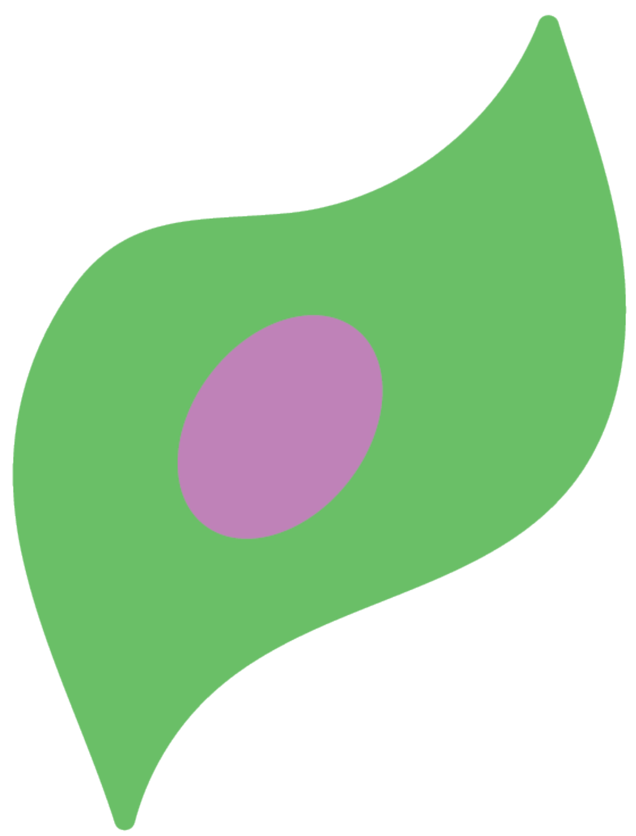
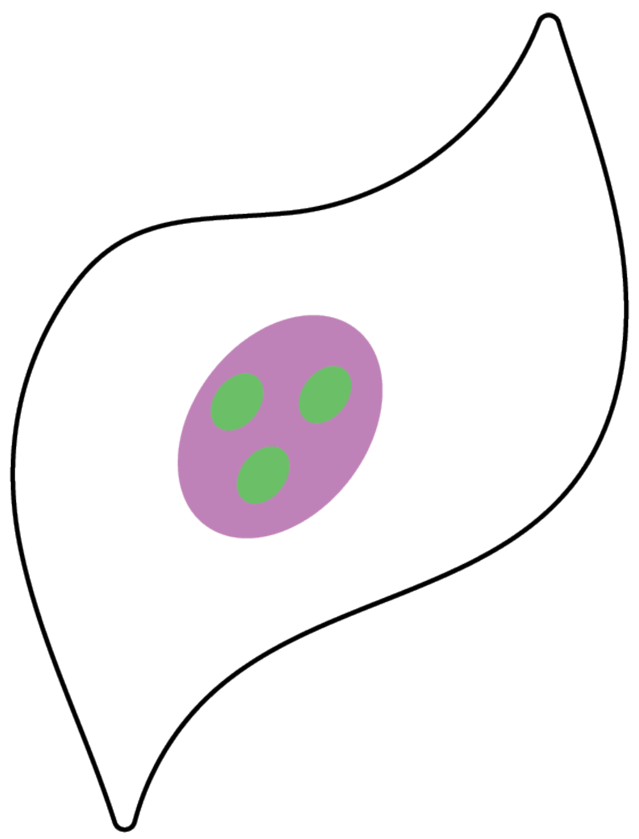
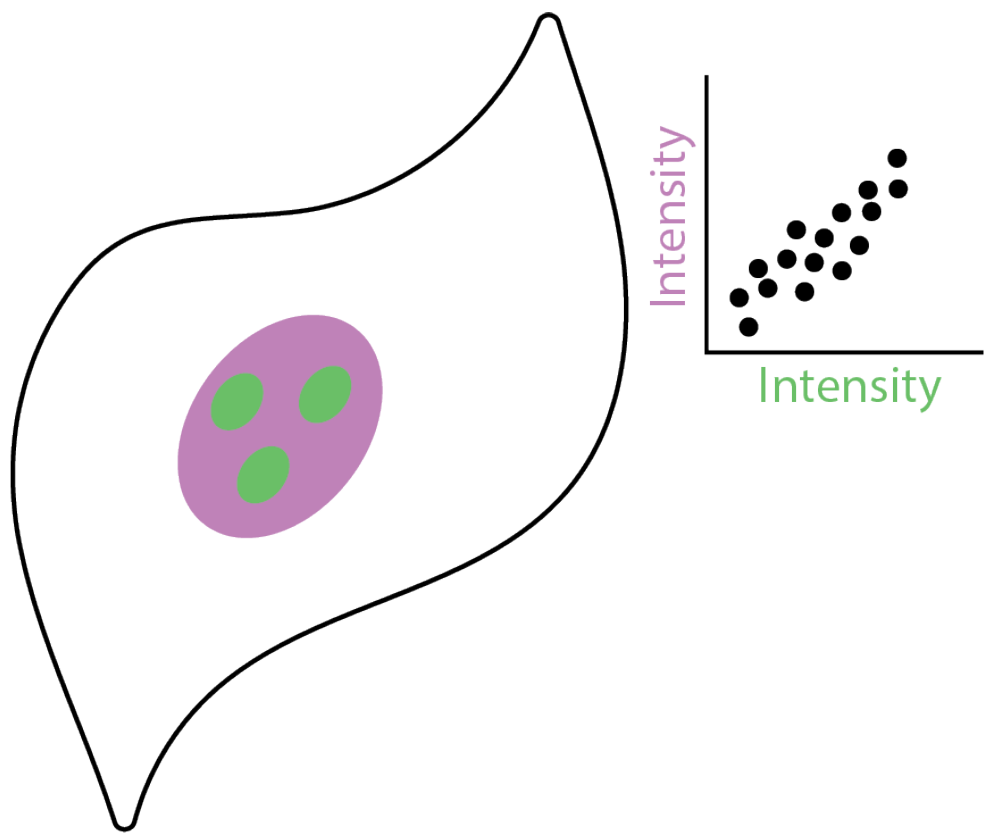
What is Colocalization in Fluorescence Microscopy



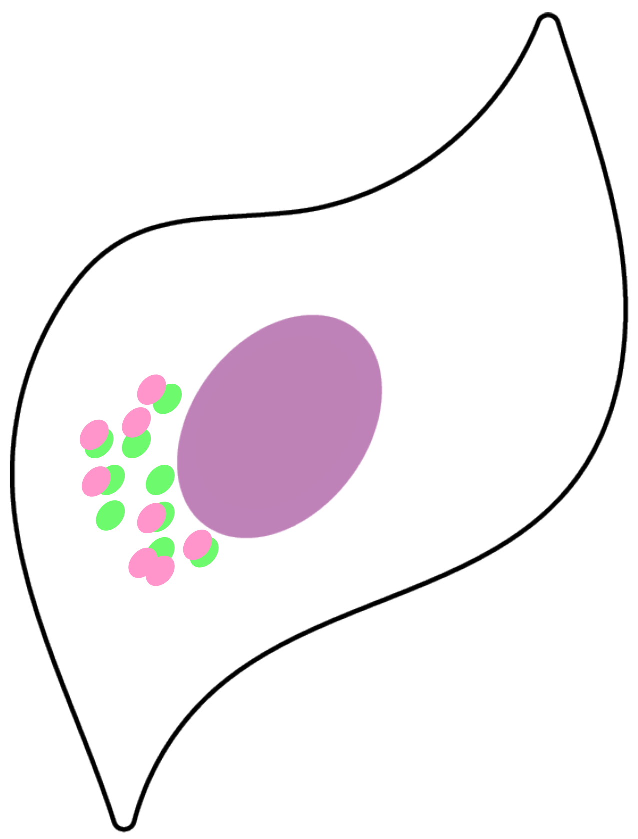
Co-expression: The presence of two or more fluorescent signals in the same cell, indicating that the corresponding proteins or molecules are expressed in the same biological sample.



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.



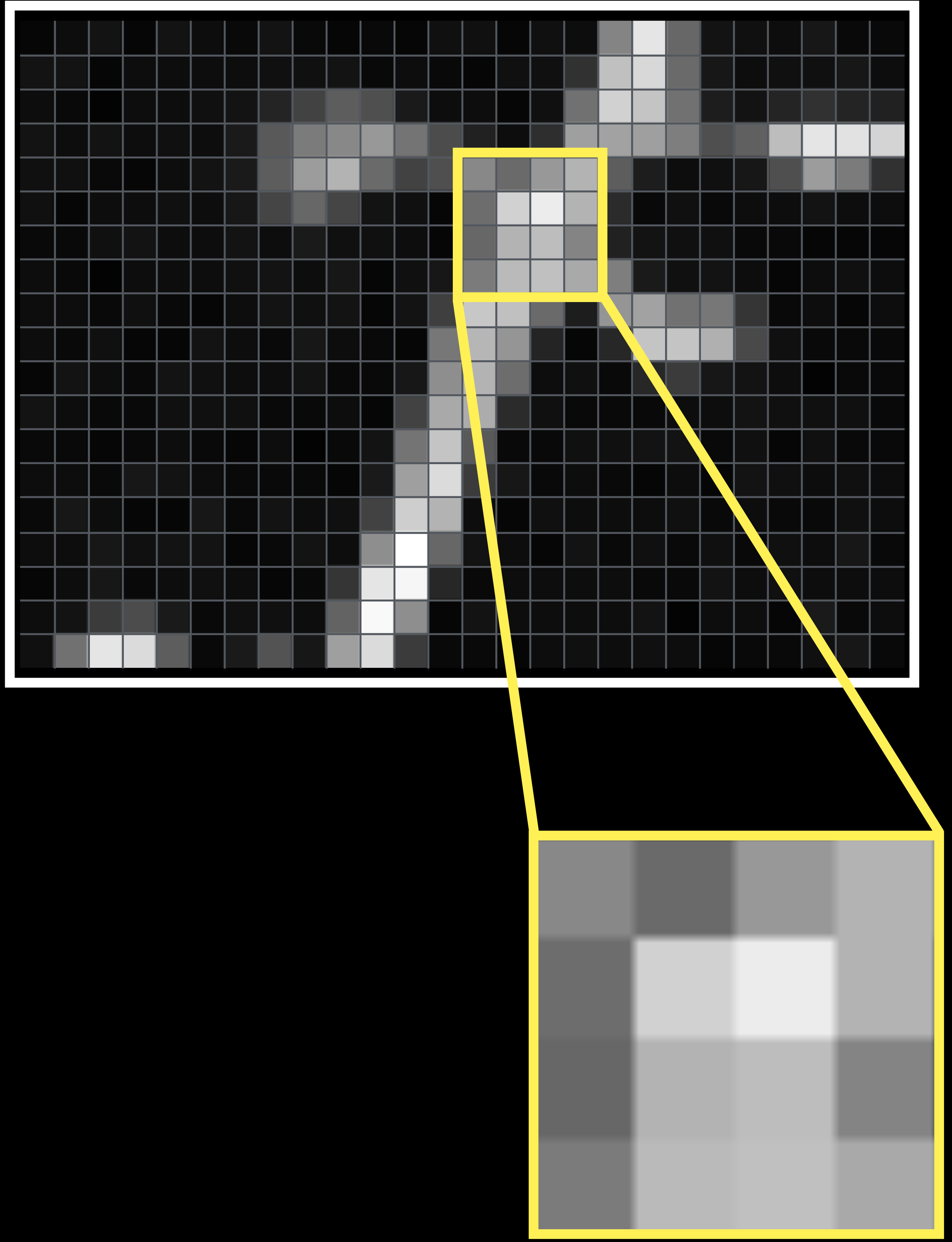
Correlation: A quantitative measure of how the intensity of two fluorescent signals changes together across the sample, helping to determine if their distributions are related.



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
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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?

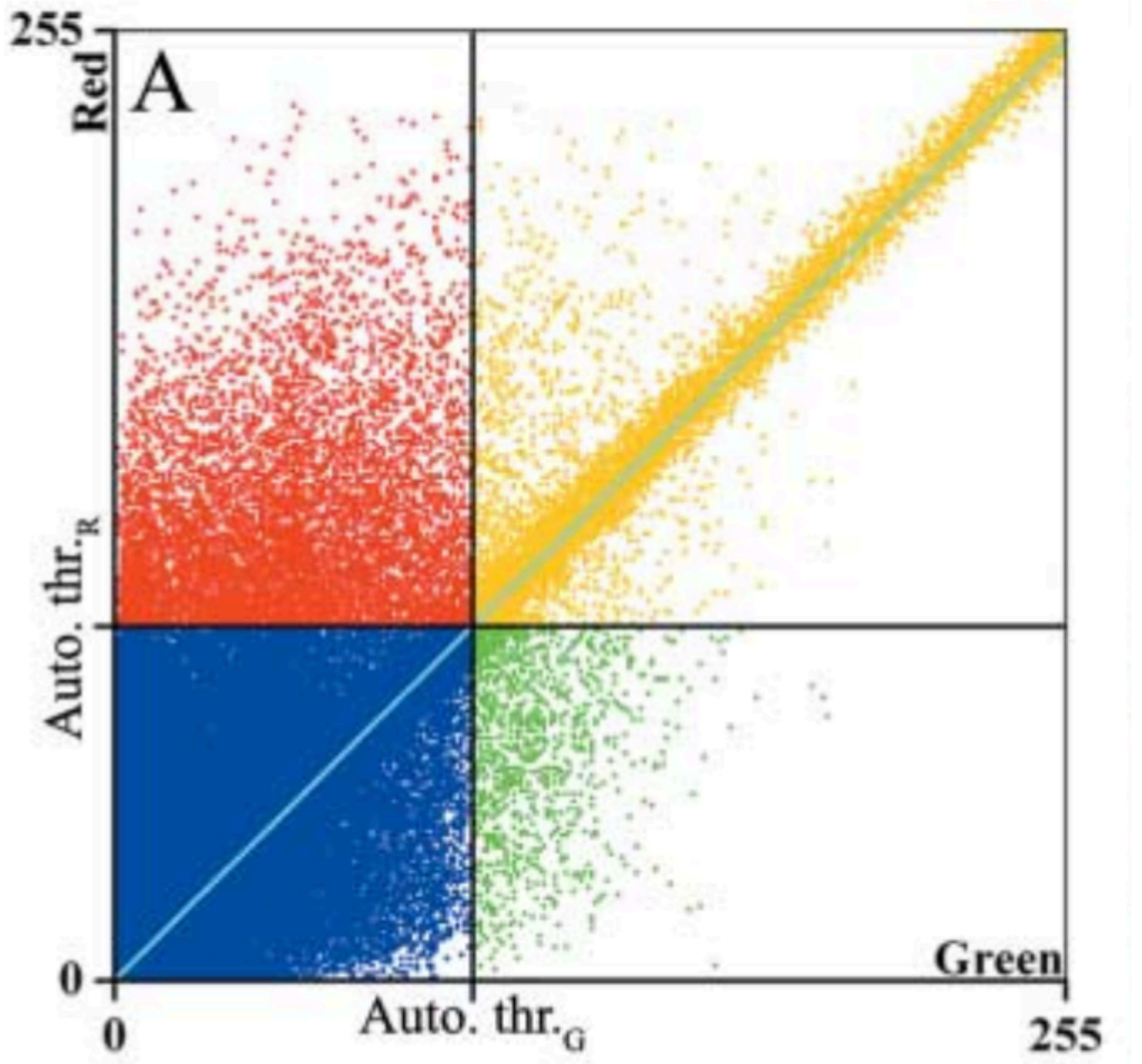
- Pixel Intensity-based methods for co-occurrence & correlation
- Object-based methods for co-expression & co-distribution (spatial statistics)

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' colocalization coefficients)
- Intensity correlation (e.g. Pearson's or Spearman's correlation coefficients)
- Cross-correlation



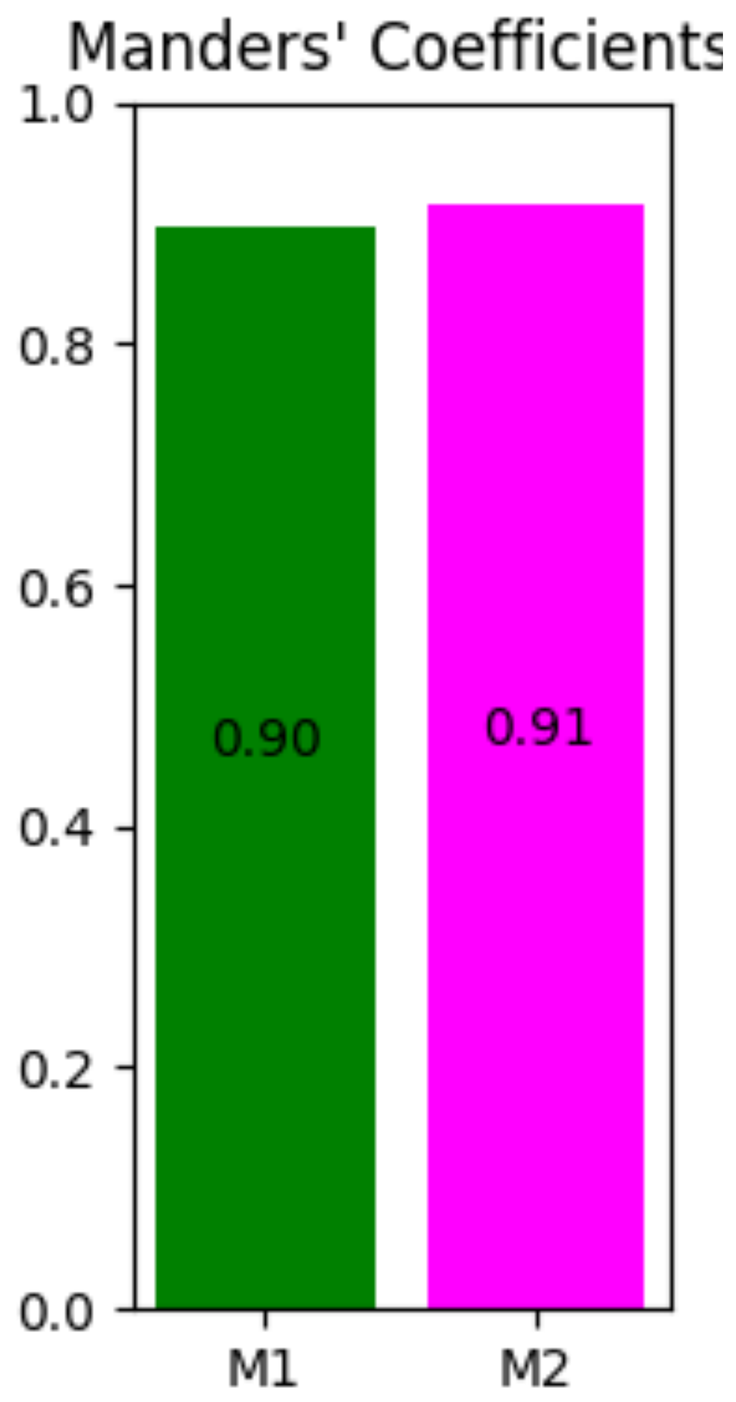
Adapted form S Bolte , F P Cordelières, 2006

Manders' colocalization coefficients

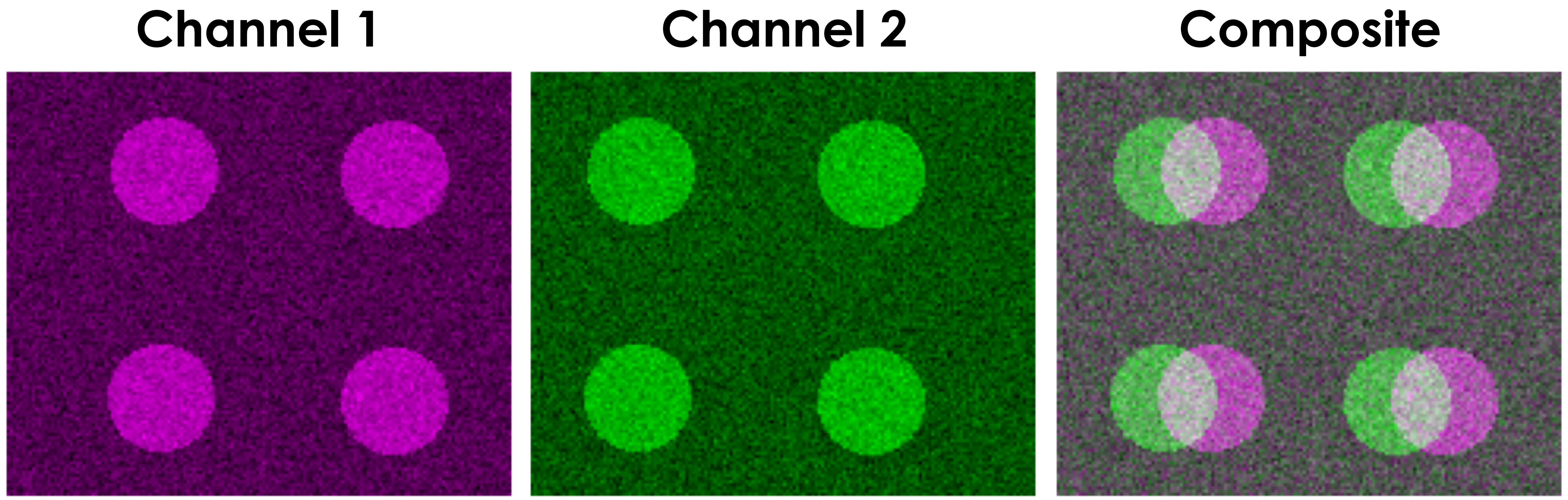
$$M_1 = \frac{\sum_i R_i^{coloc}}{\sum_i R_i} \text{ and } M_2 = \frac{\sum_i G_i^{coloc}}{\sum_i G_i}$$

Pearson's correlation coefficient

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

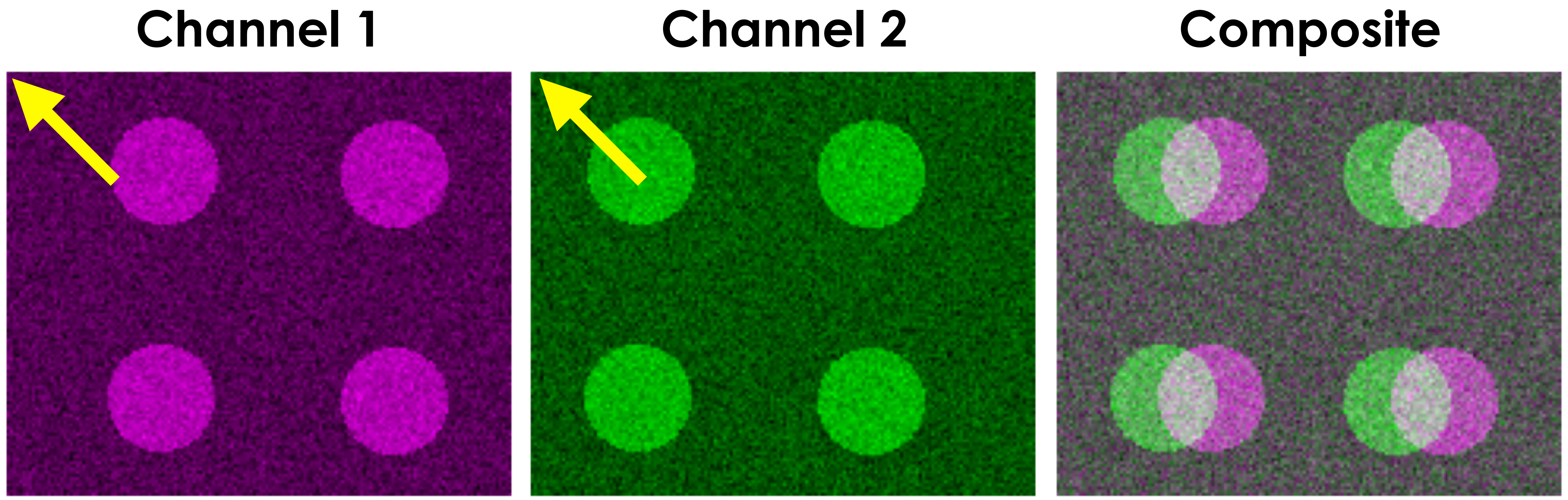


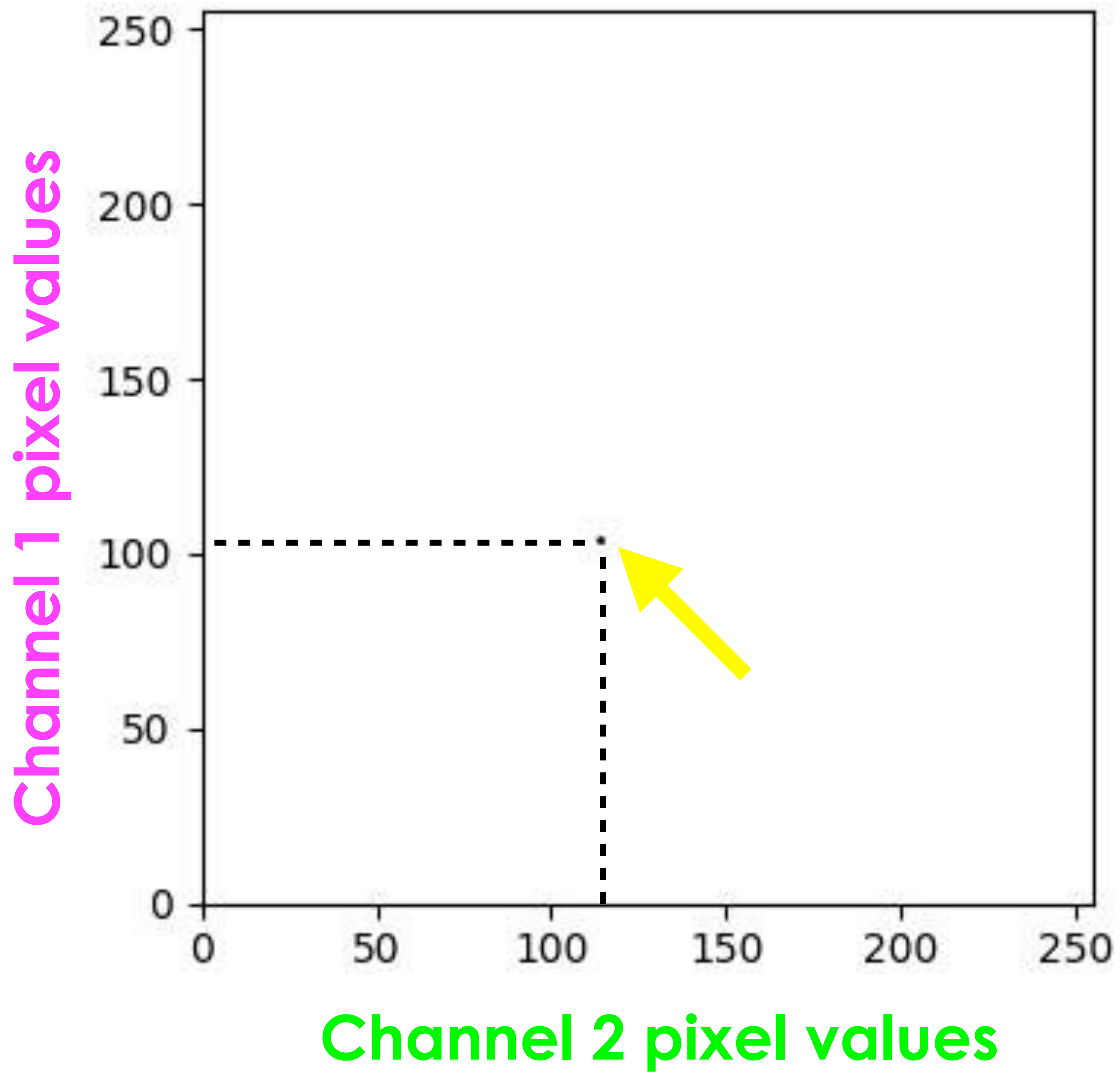
Scatter Plot



Scatter Plot

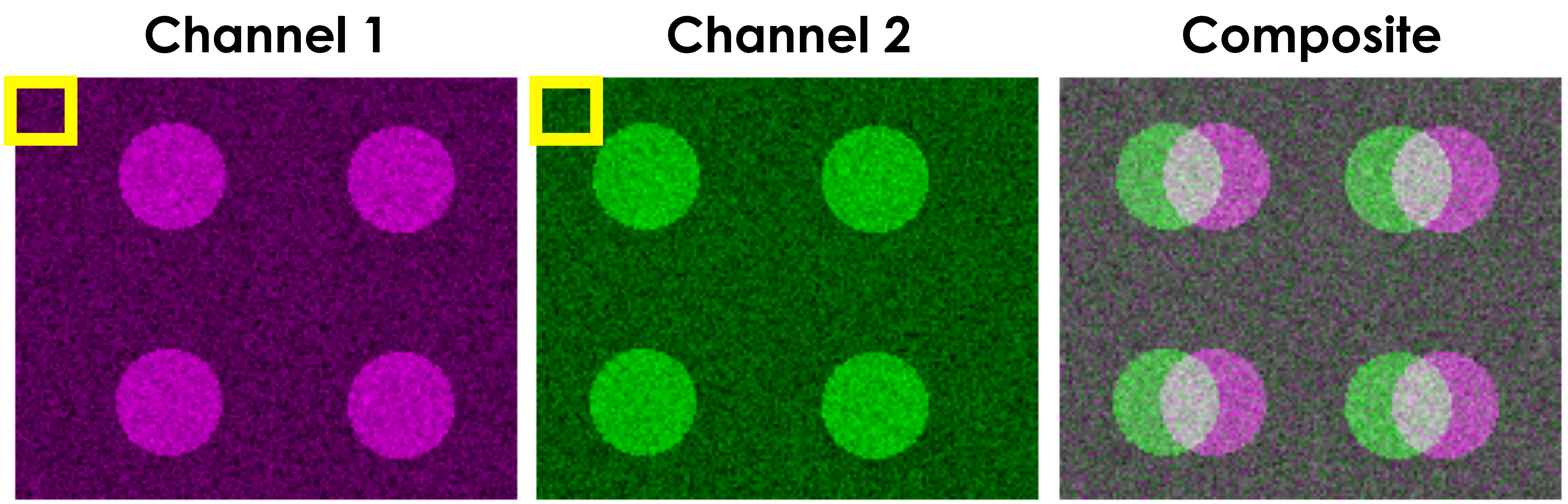
1 pixel

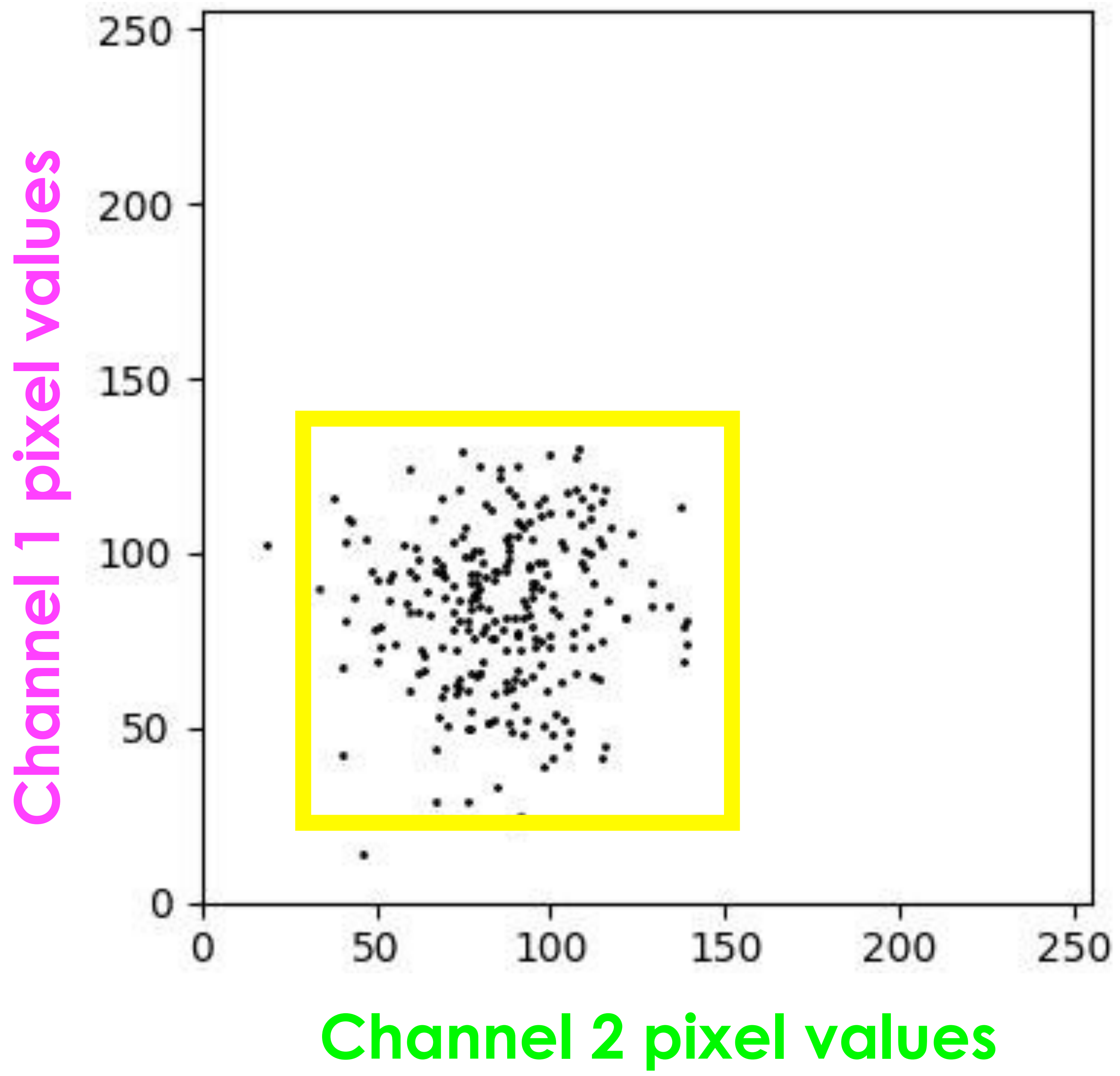




Scatter Plot

more pixels

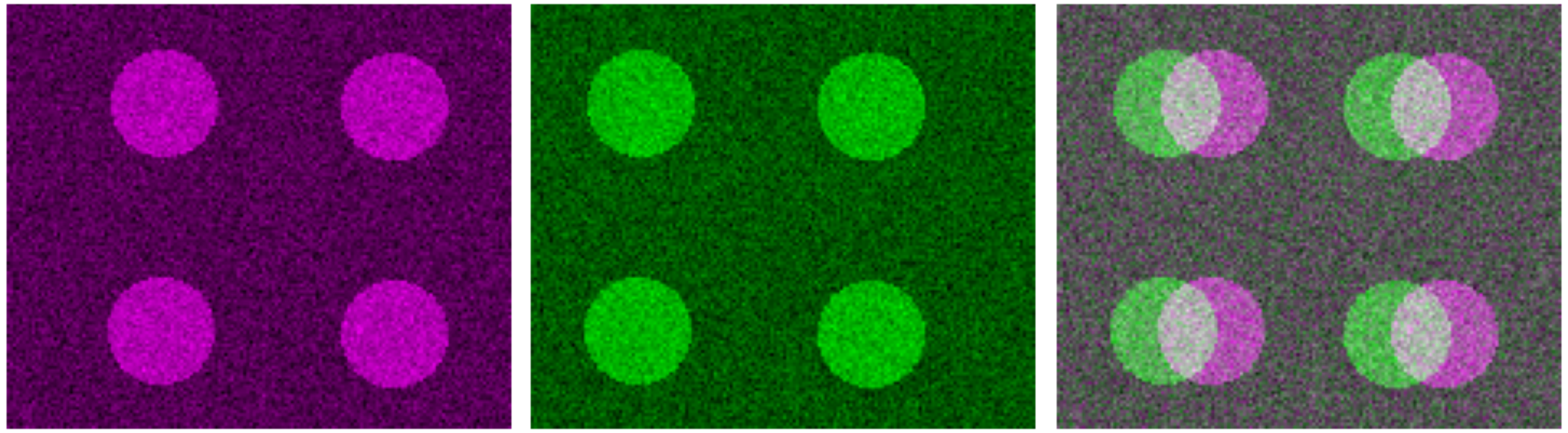


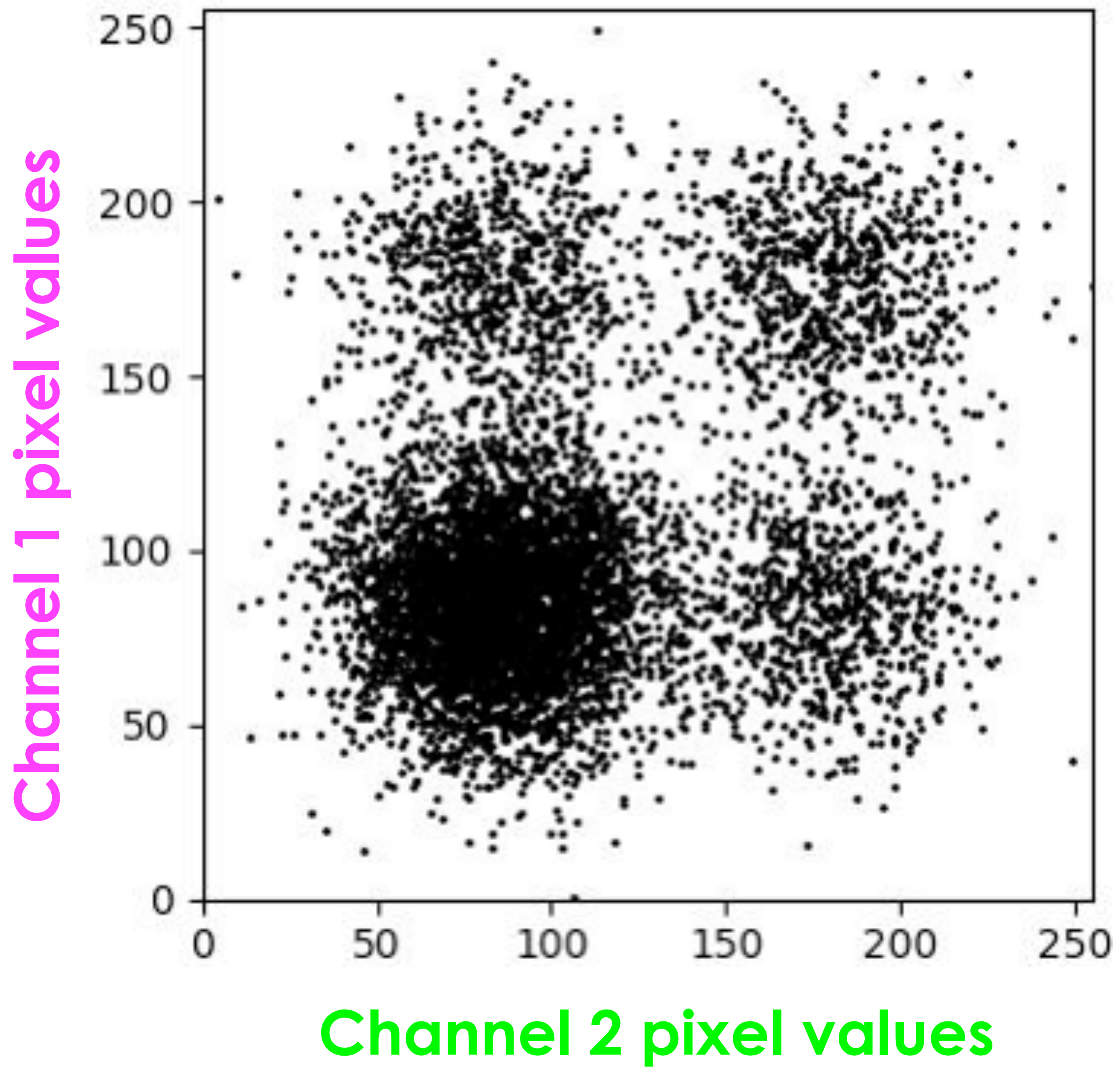


Scatter Plot

all pixels

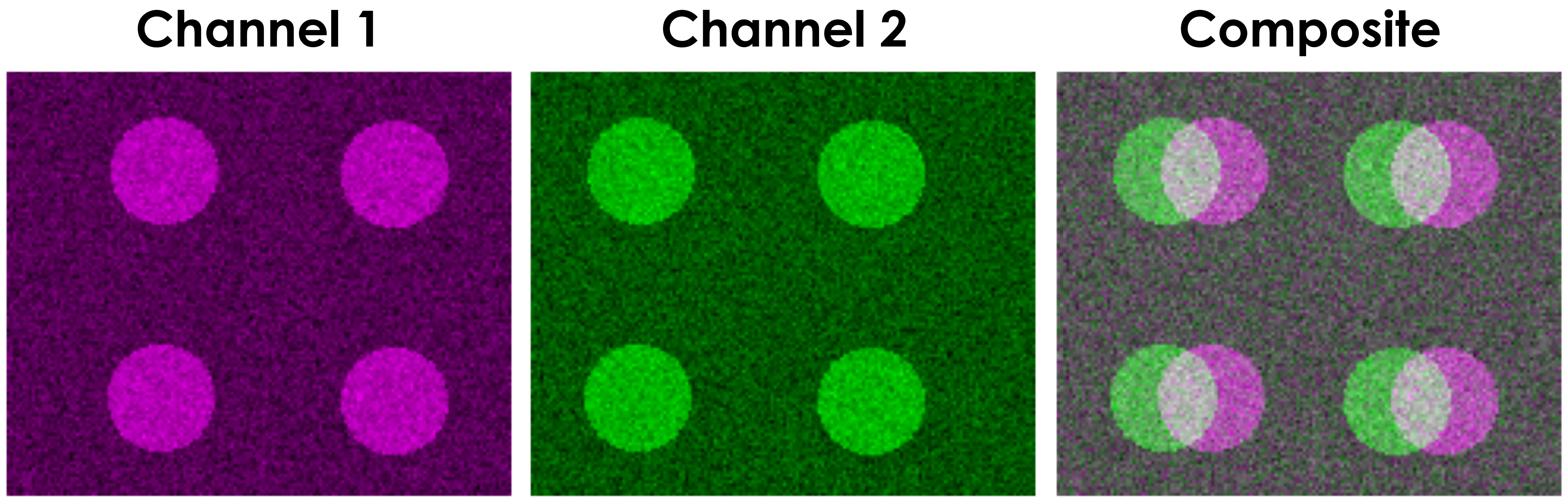
Channel 1 Channel 2 Composite

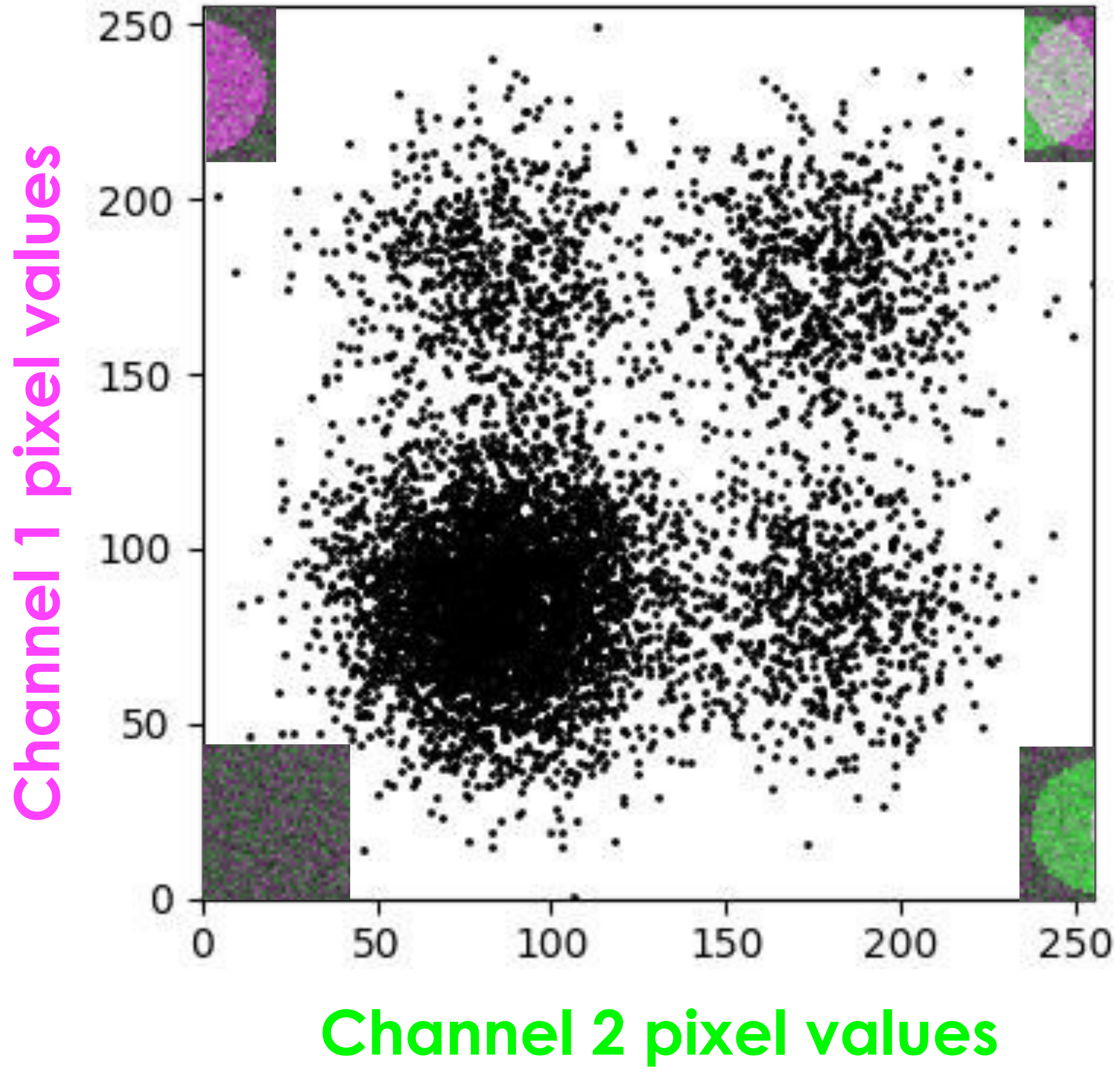




Scatter Plot

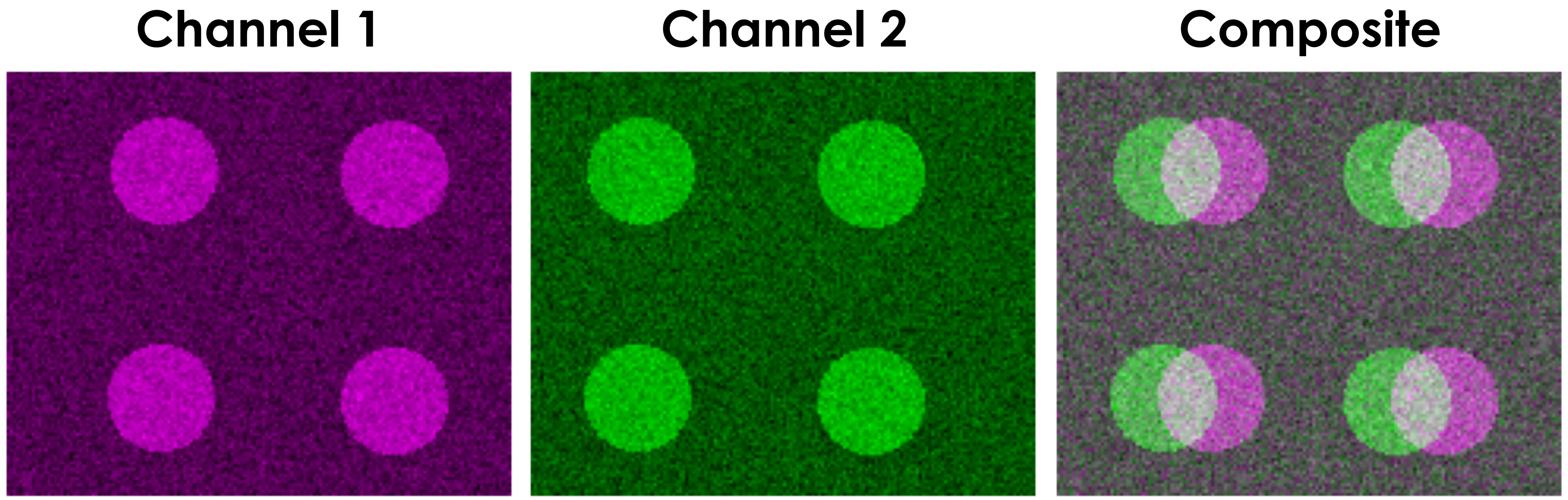
all pixels

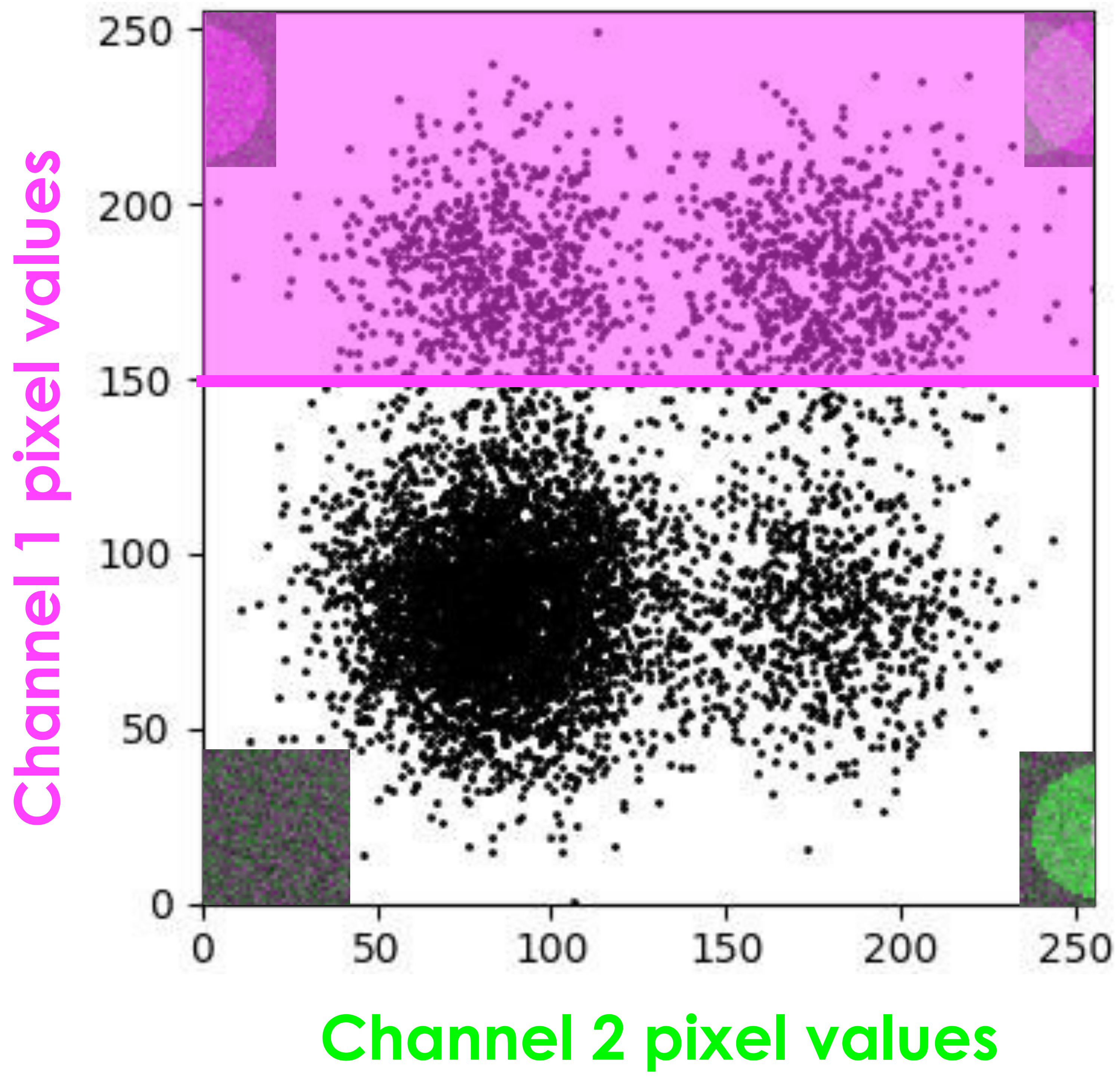




Scatter Plot

visualize thresholds

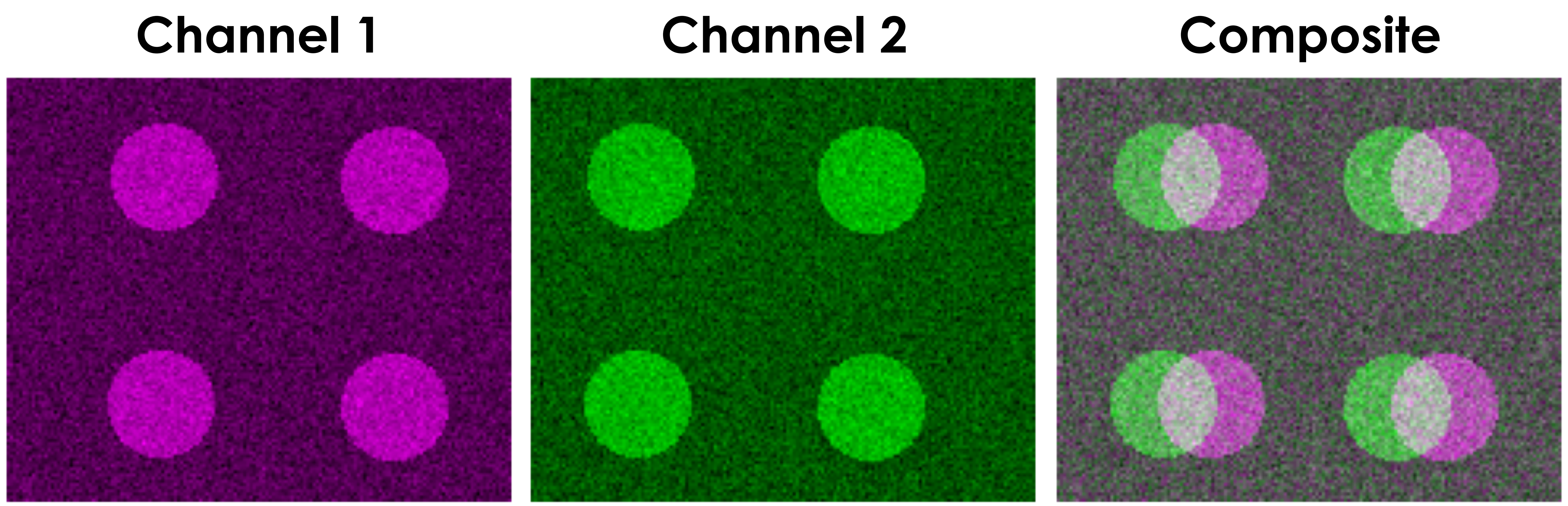


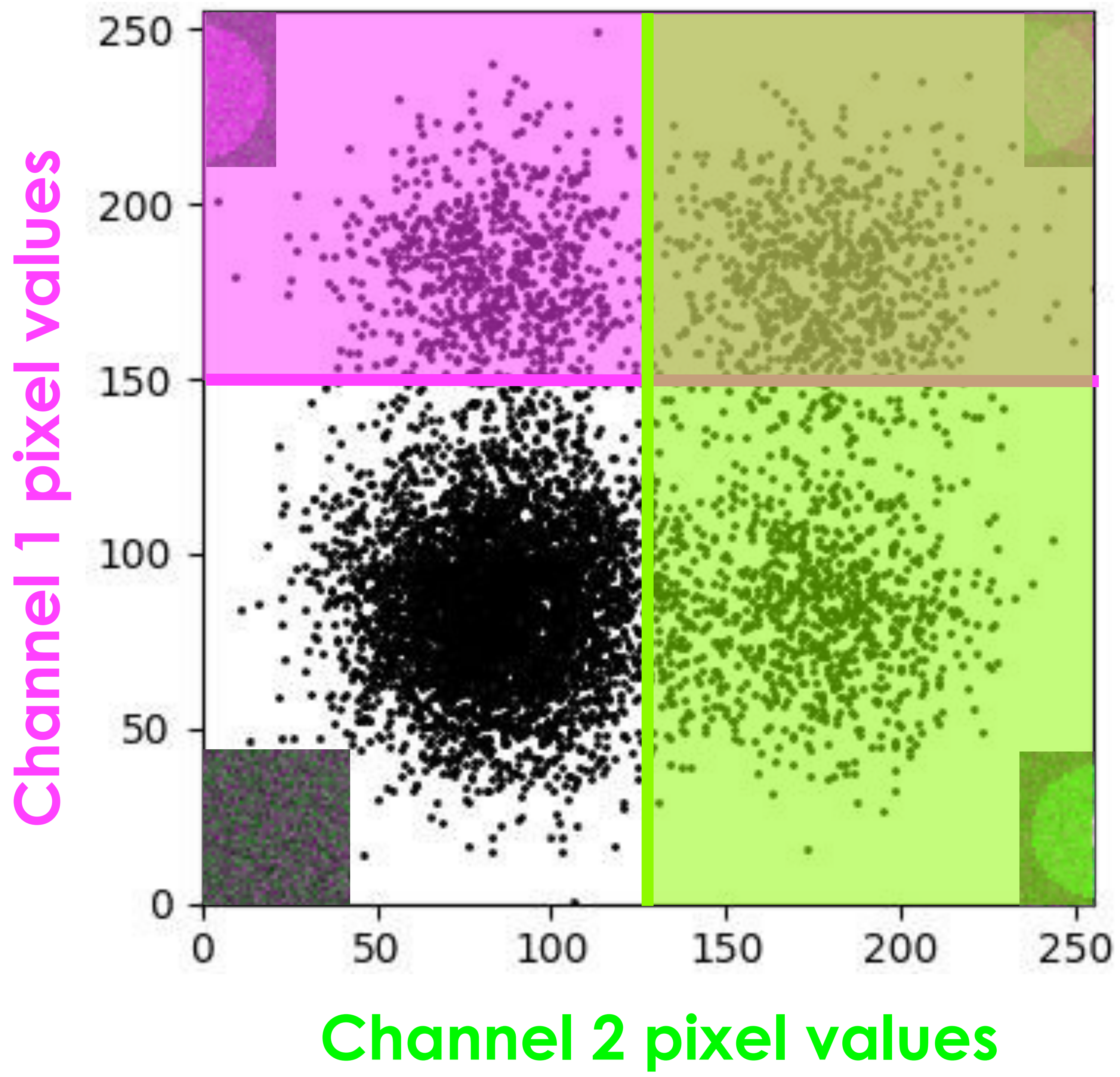


Channel 1 threshold = 150

Scatter Plot

visualize thresholds





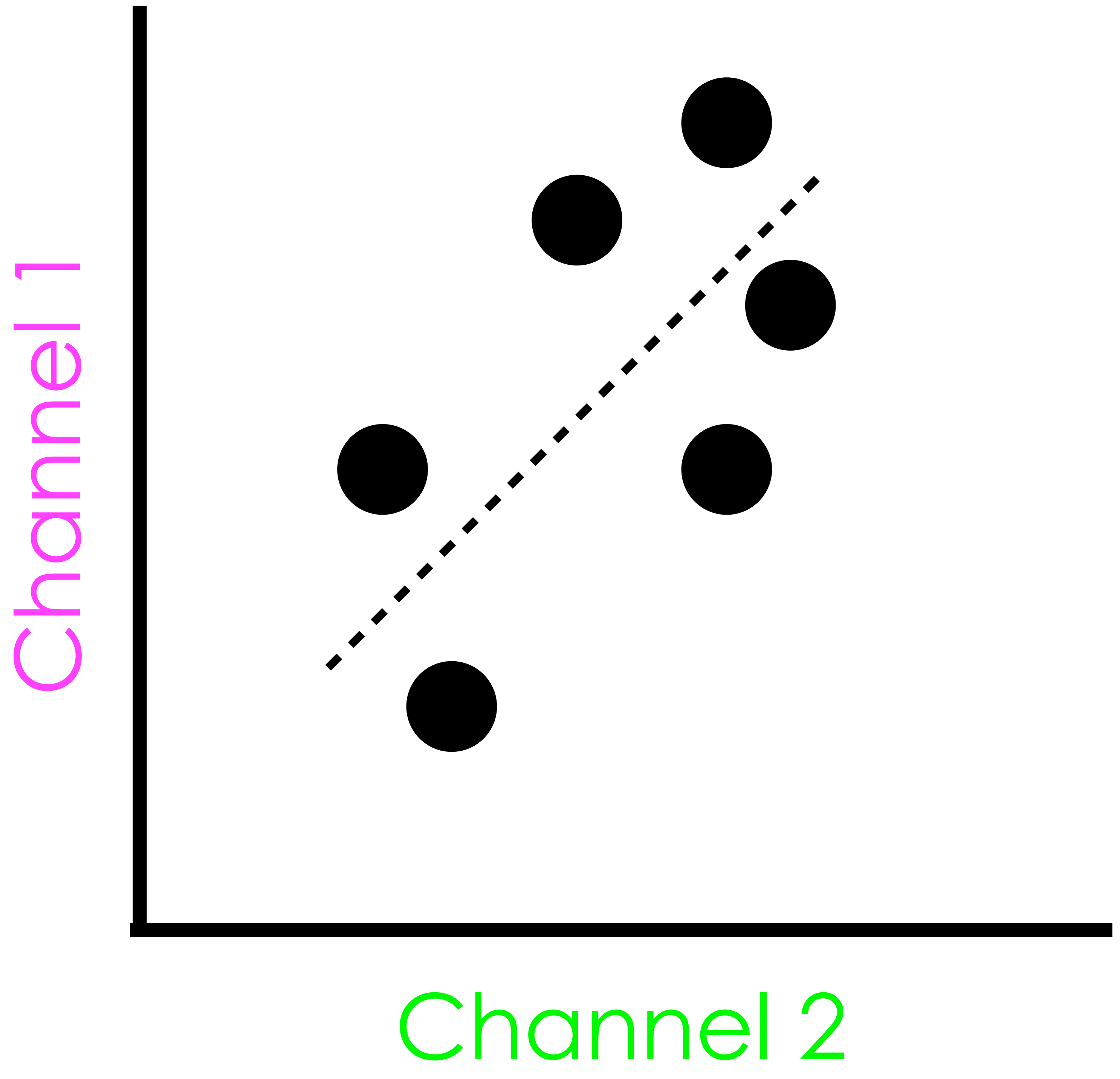
Channel 1 threshold = 150

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 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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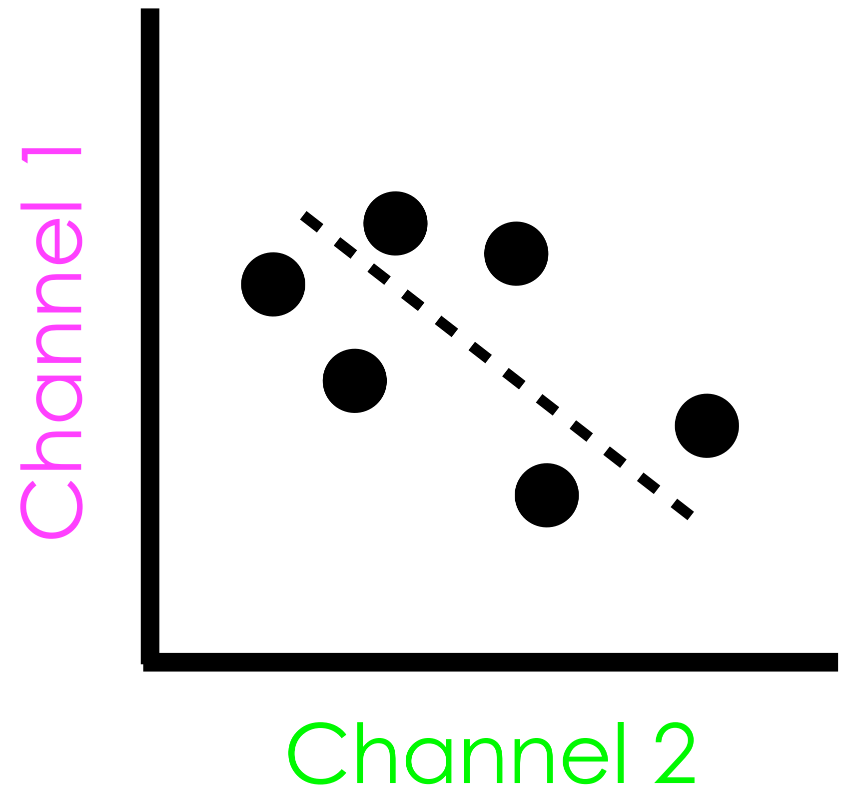
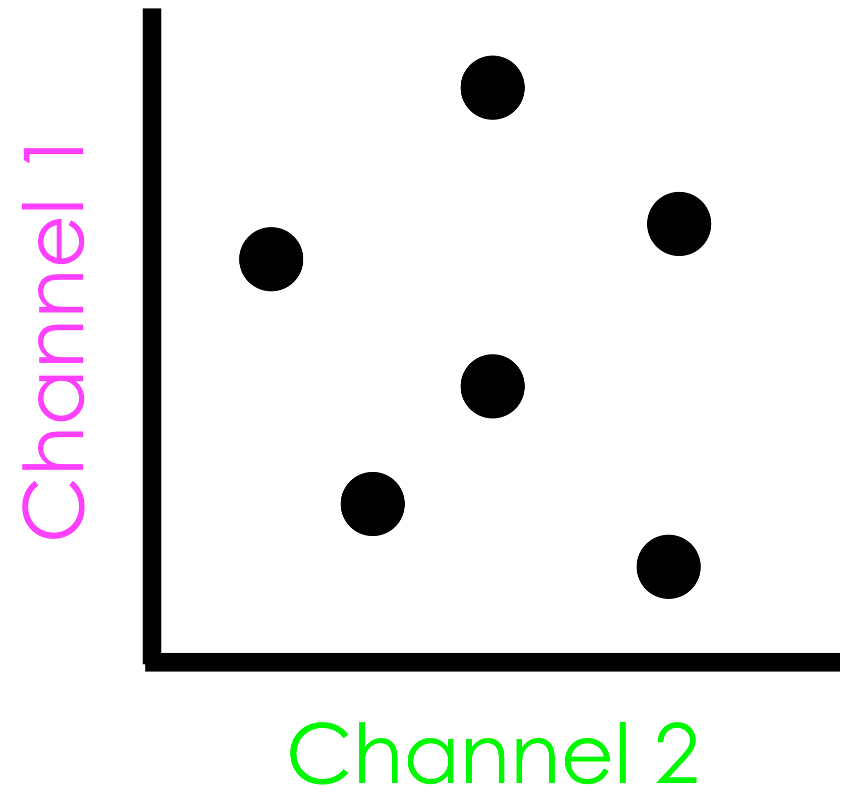
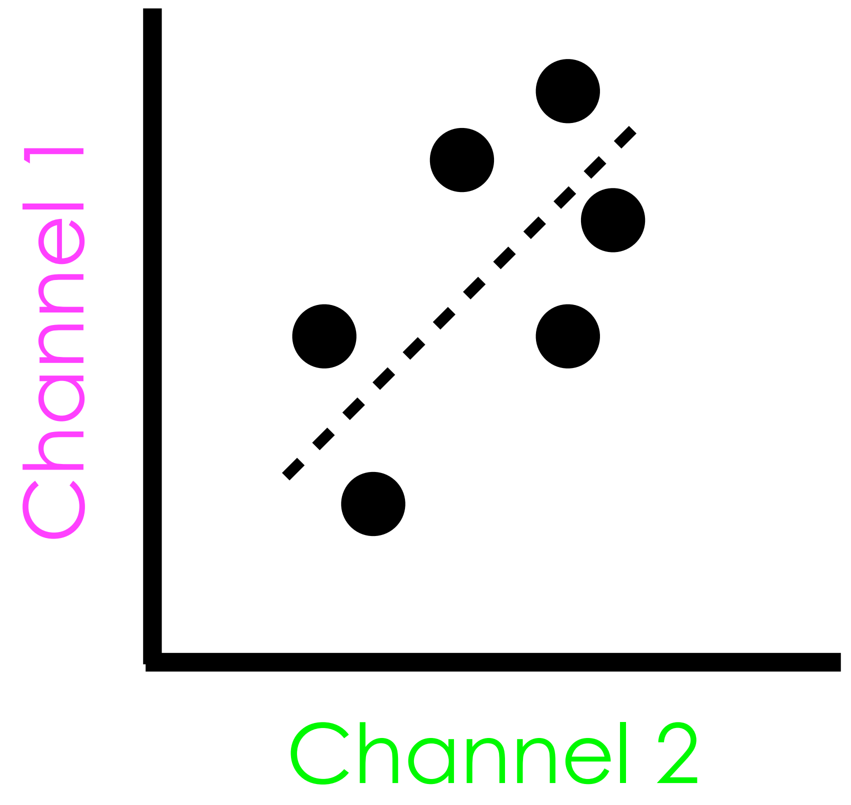


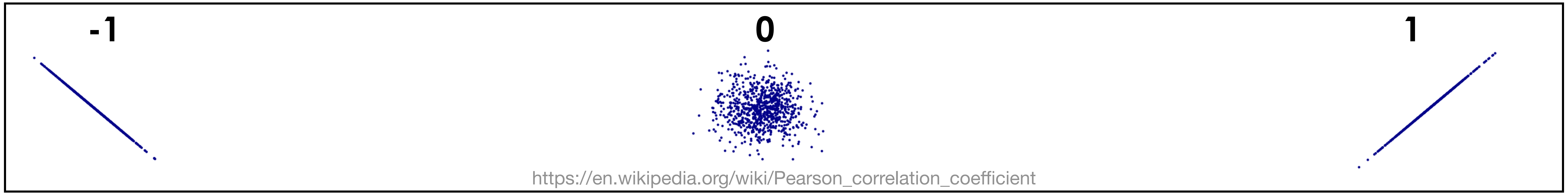
r_P ~ -1



r_P ~ 0



r_P ~ 1



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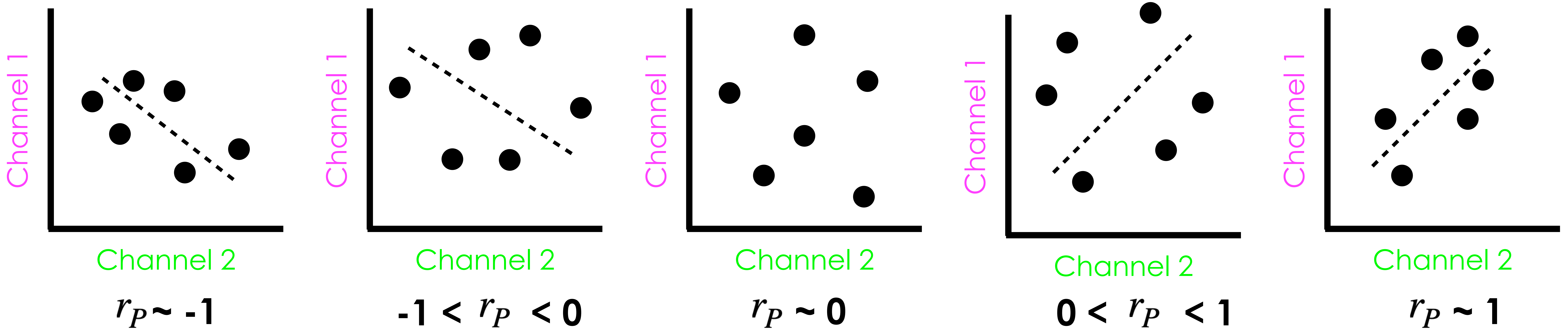

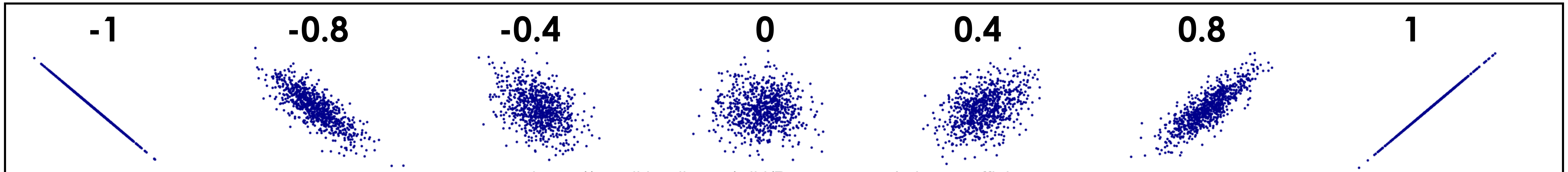

https://en.wikipedia.org/wiki/Pearson_correlation_coefficient

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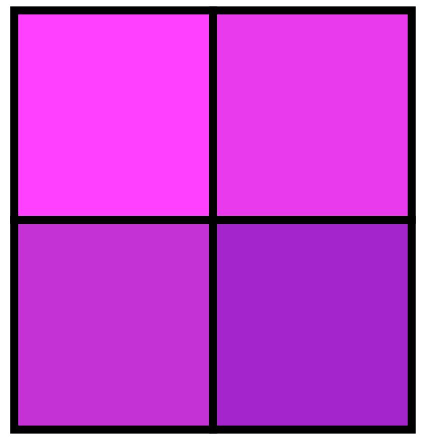
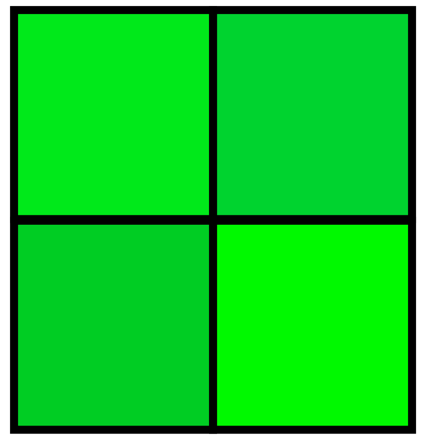


200	190
90	80



100	90
70	152

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

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

$(R_i - R_{avg})$ =

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

=

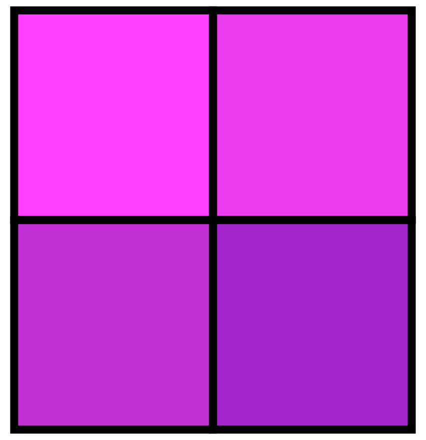
-180	-650
1650	-2940

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

-180	-650
1650	-2940

= **-2120**

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



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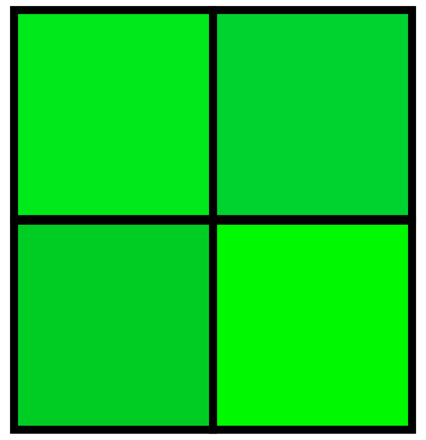


100	90
70	152

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

$(R_i - R_{avg})$ =

60	50
-50	-60

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

60²	50²
(-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	49²

=

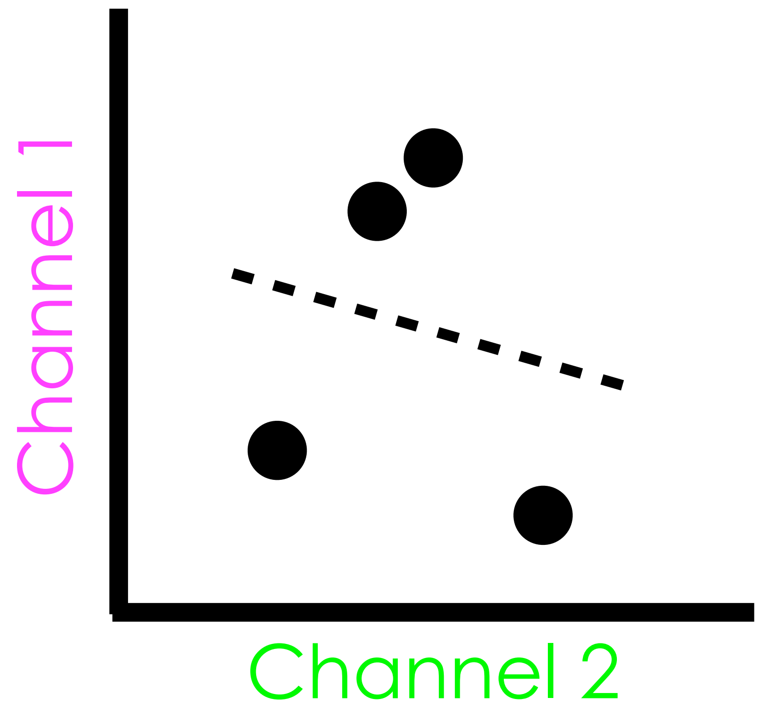
9	169
1089	2401

= 3668

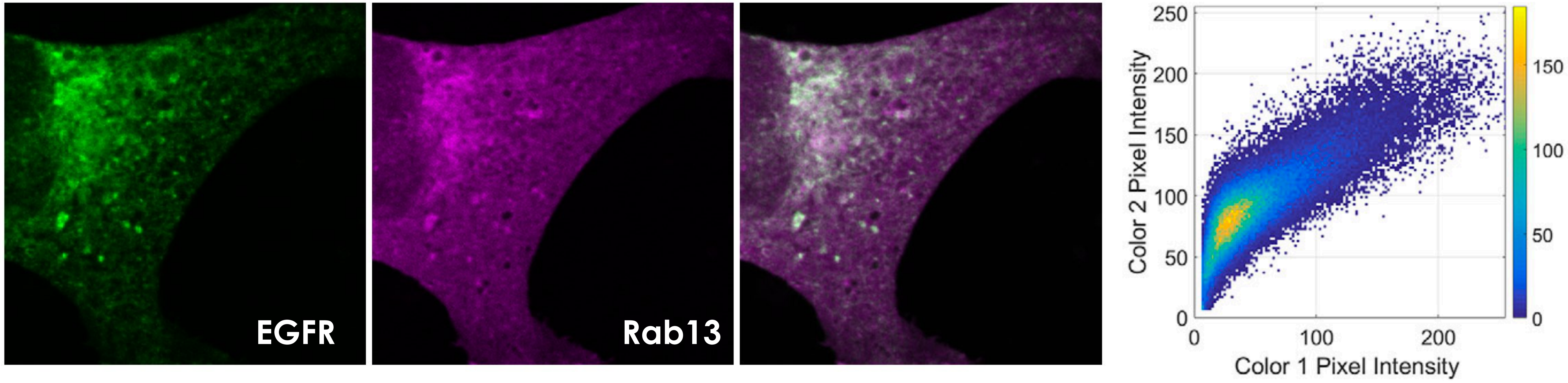
$$\sqrt{\sum_i (R_i - R_{avg})^2 \sum_i (G_i - G_{avg})^2} =$$

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

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



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

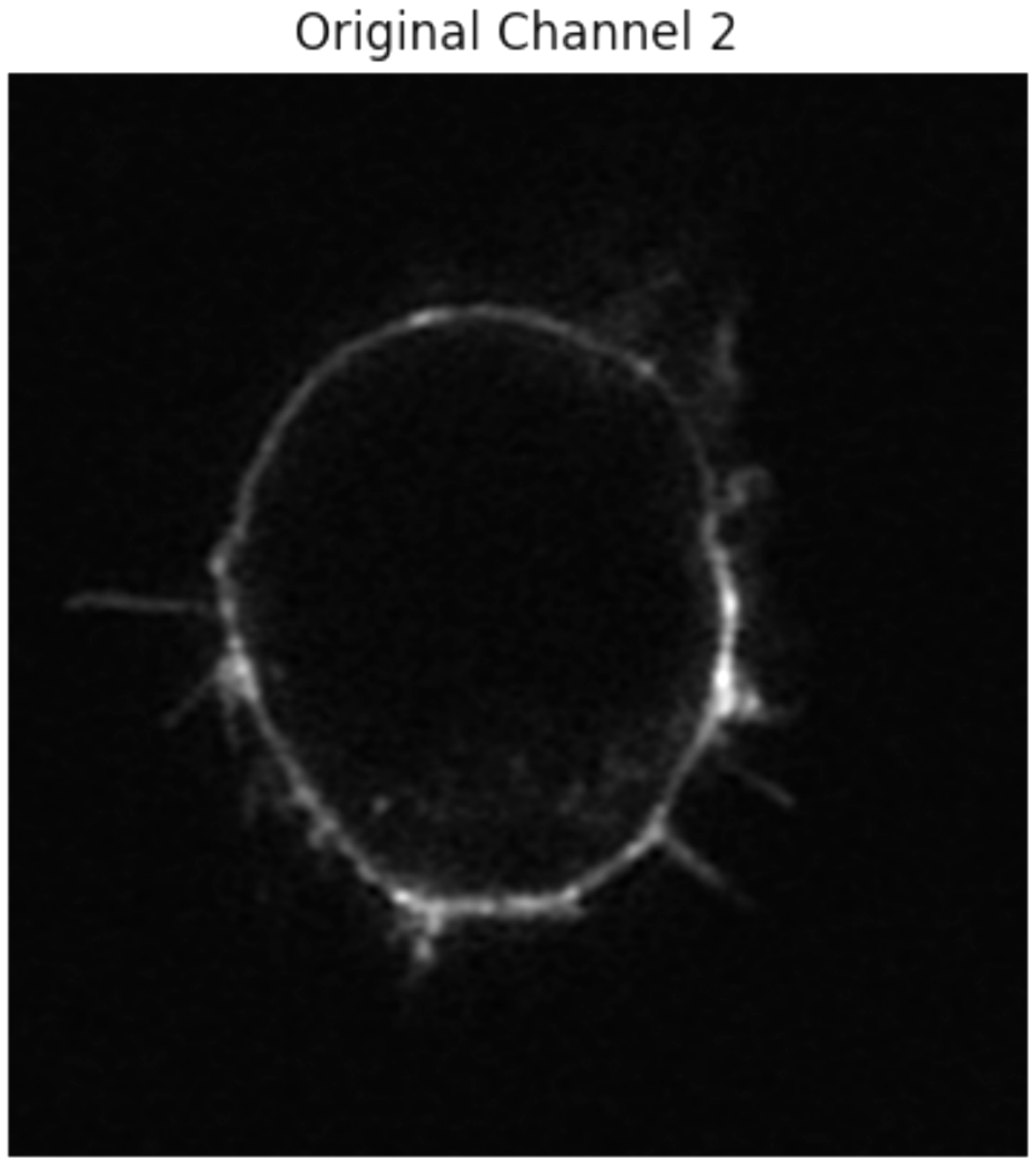


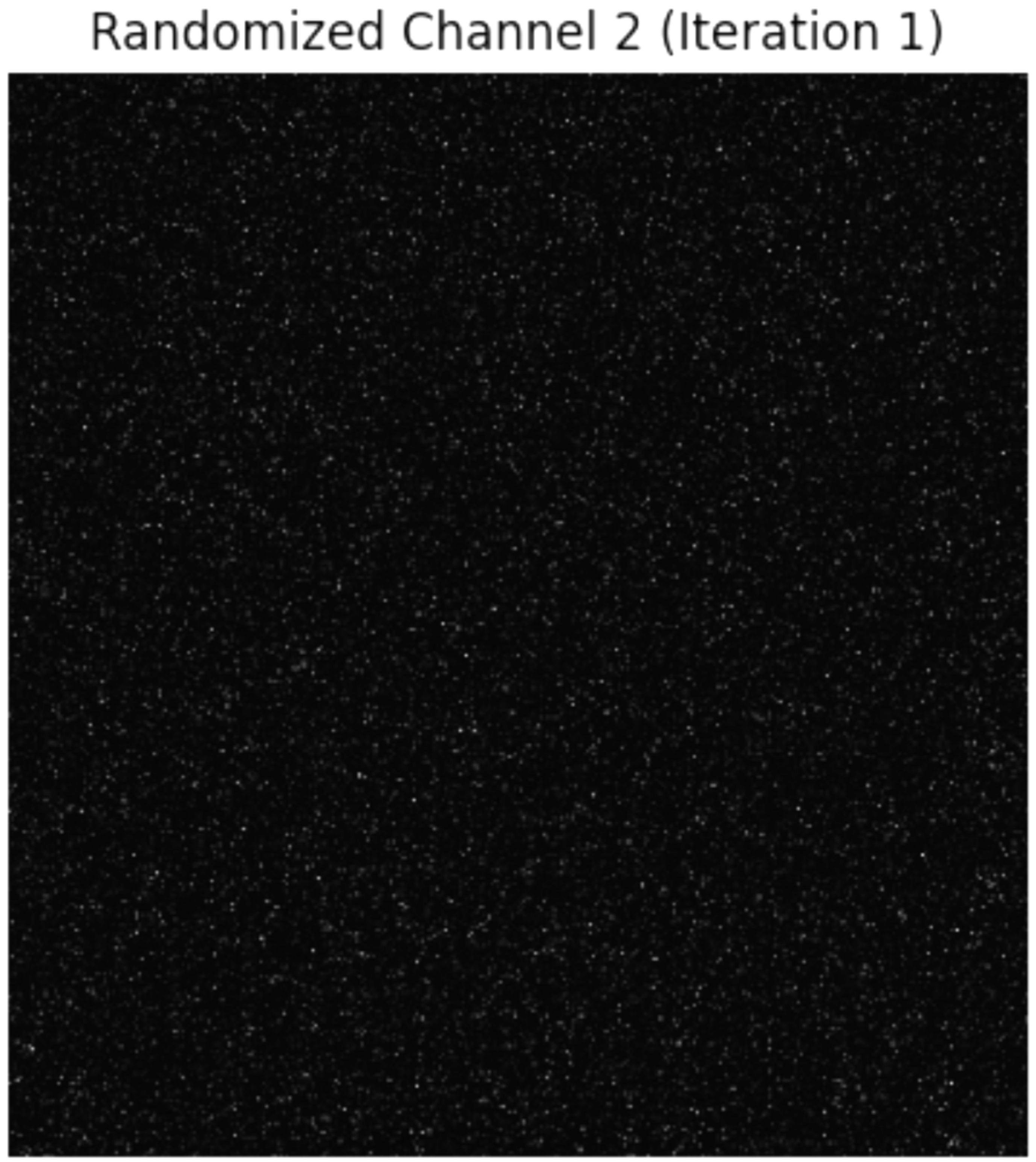
$r_P = 0.76$ EGFR and Rab13 concentrations predict each other relatively well, indicating a concentration-dependent relationship between these molecules.

Adapted form Aaron et al, 2018

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

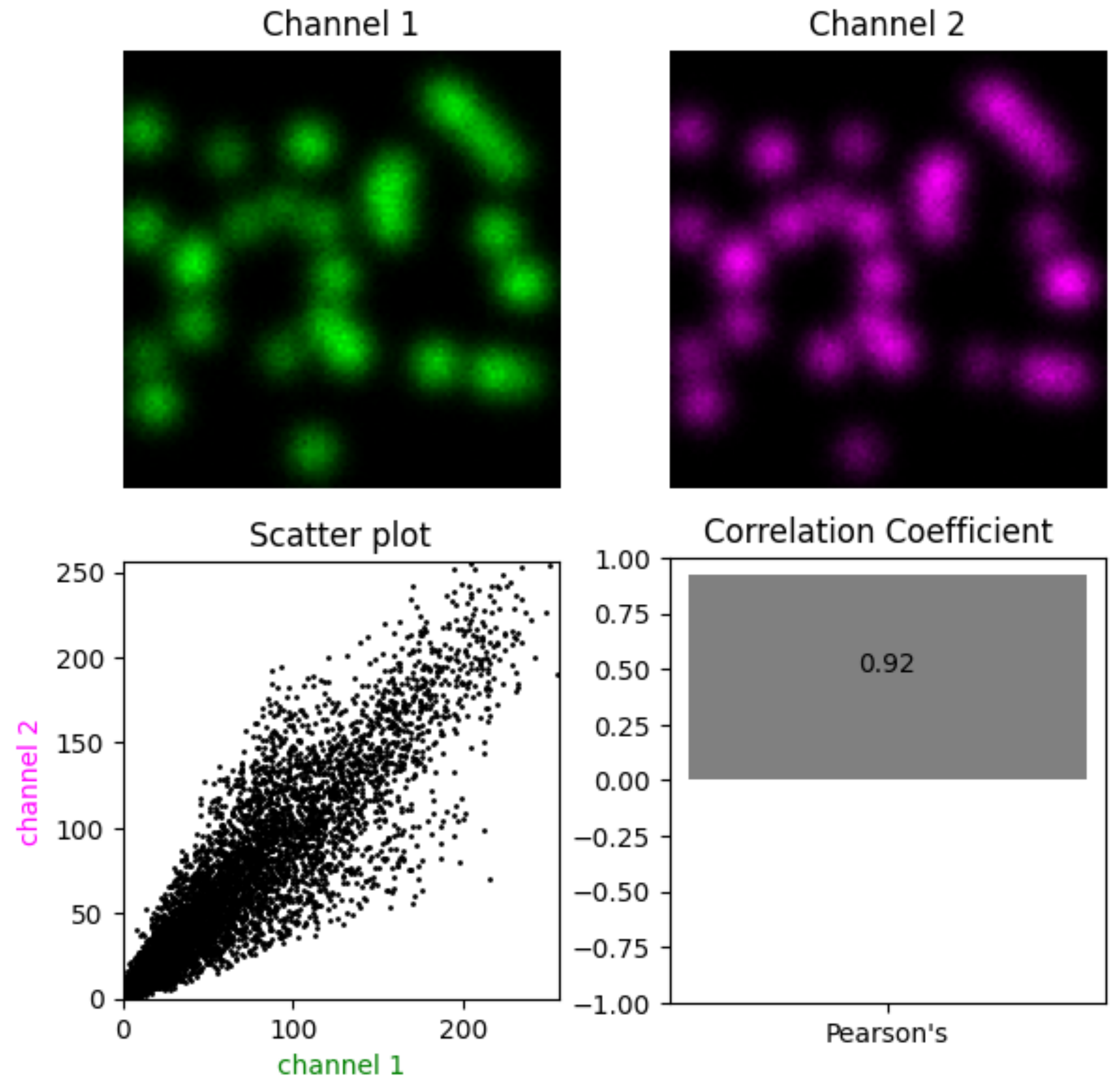
Pixel Randomization

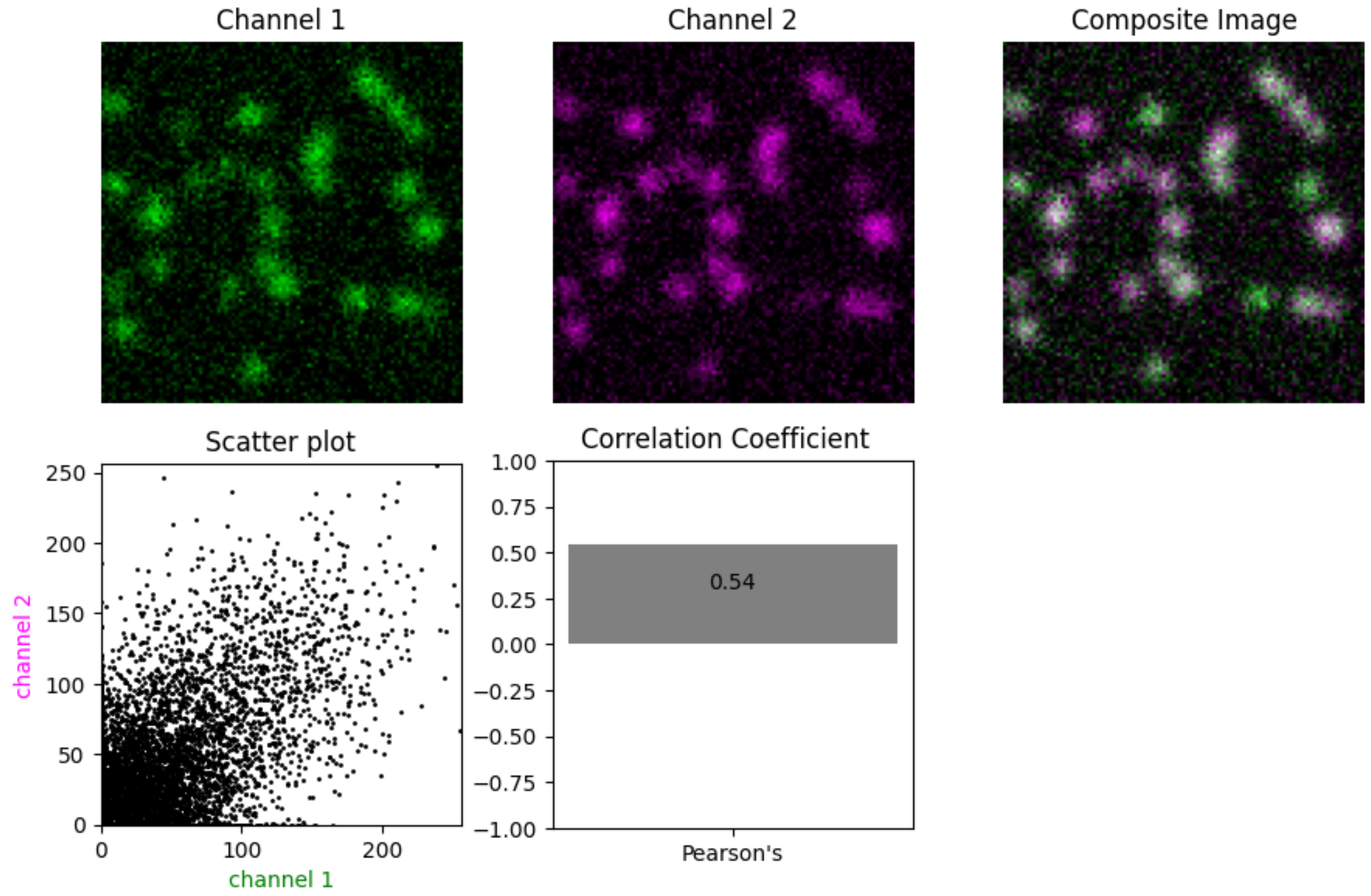




Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

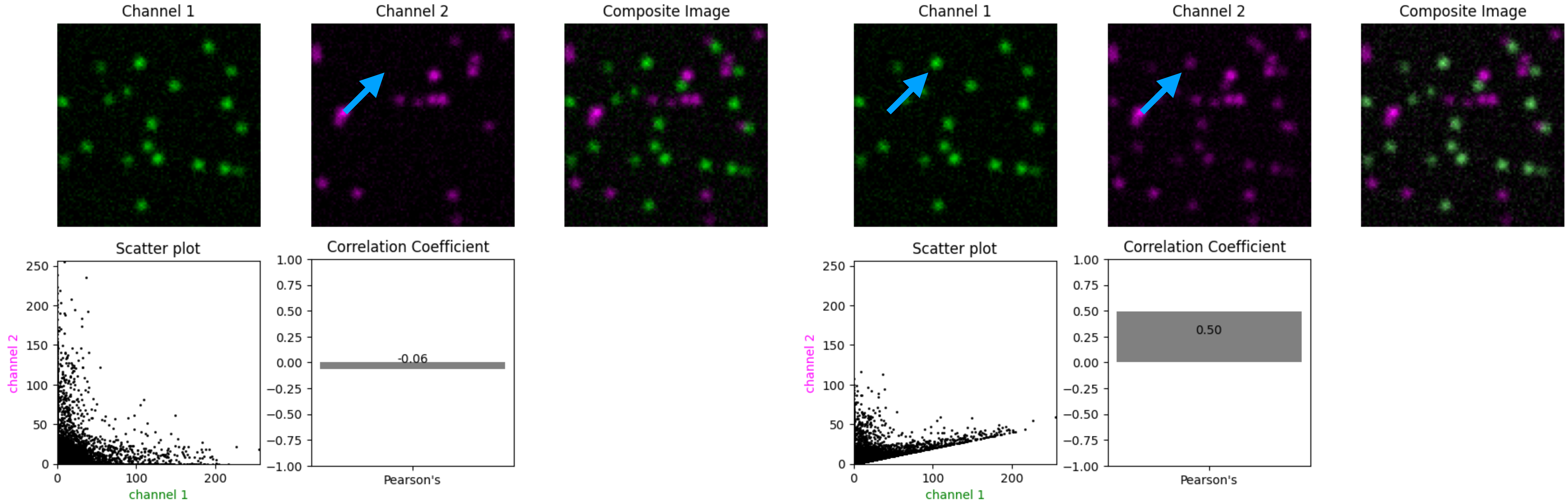
Noise





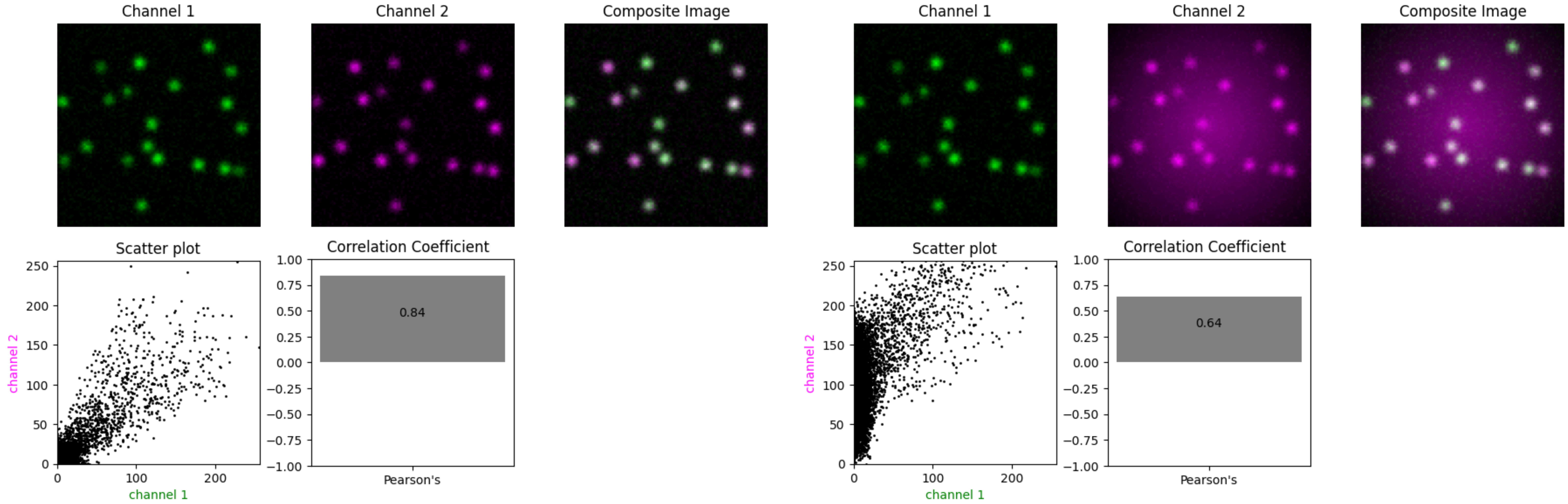
Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

Bleedthrough



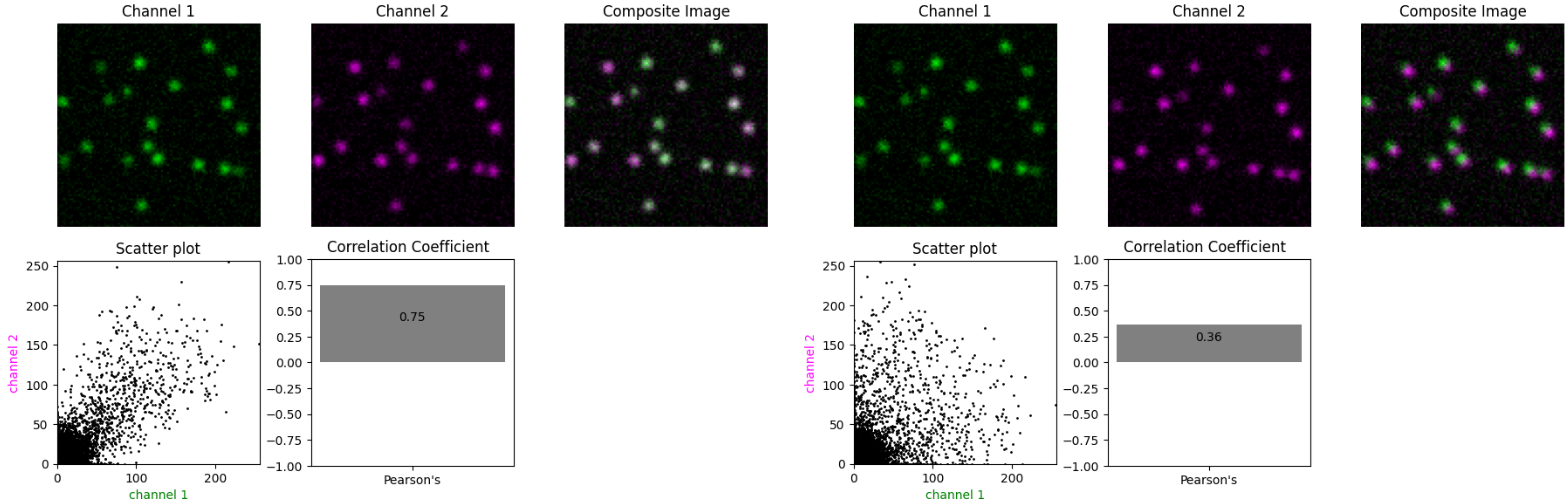
Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

Uneven Illumination



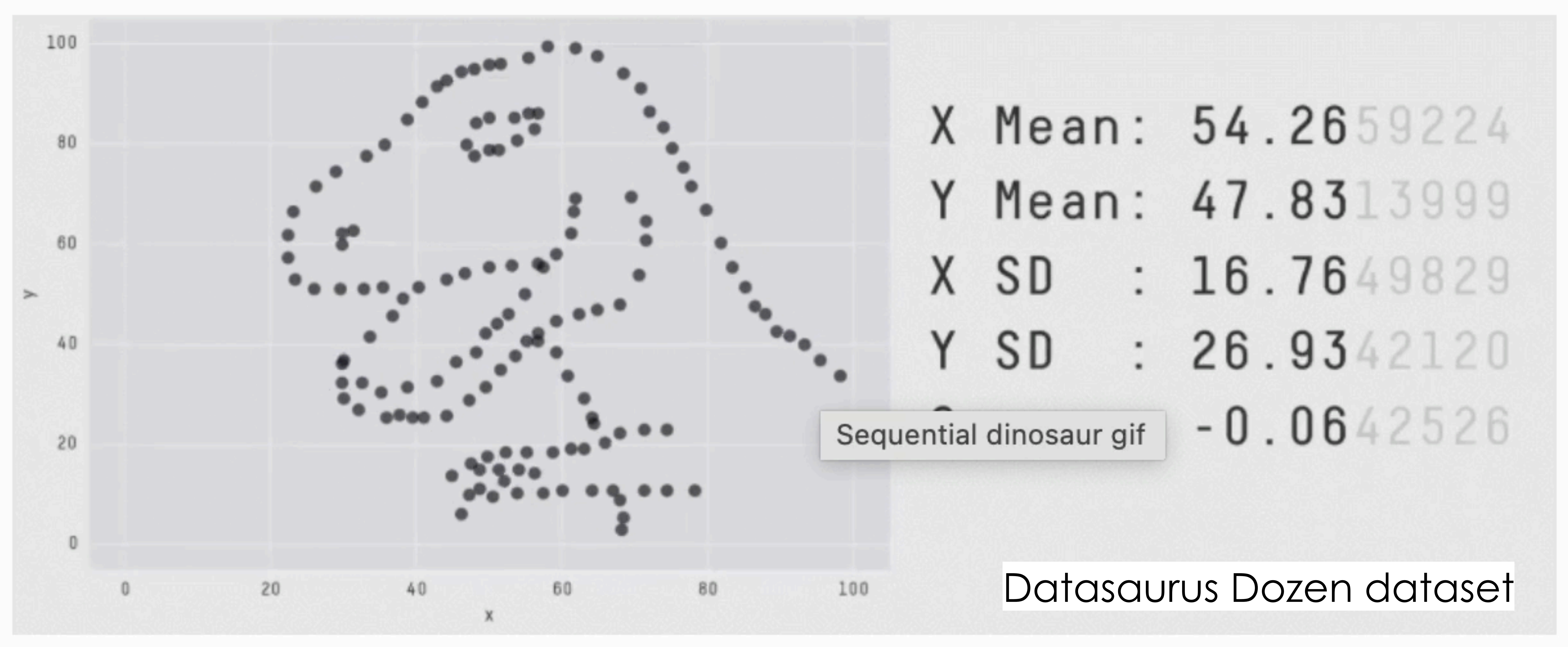
Intensity/Pixel-based: Pearson's correlation coefficient (correlation)

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.

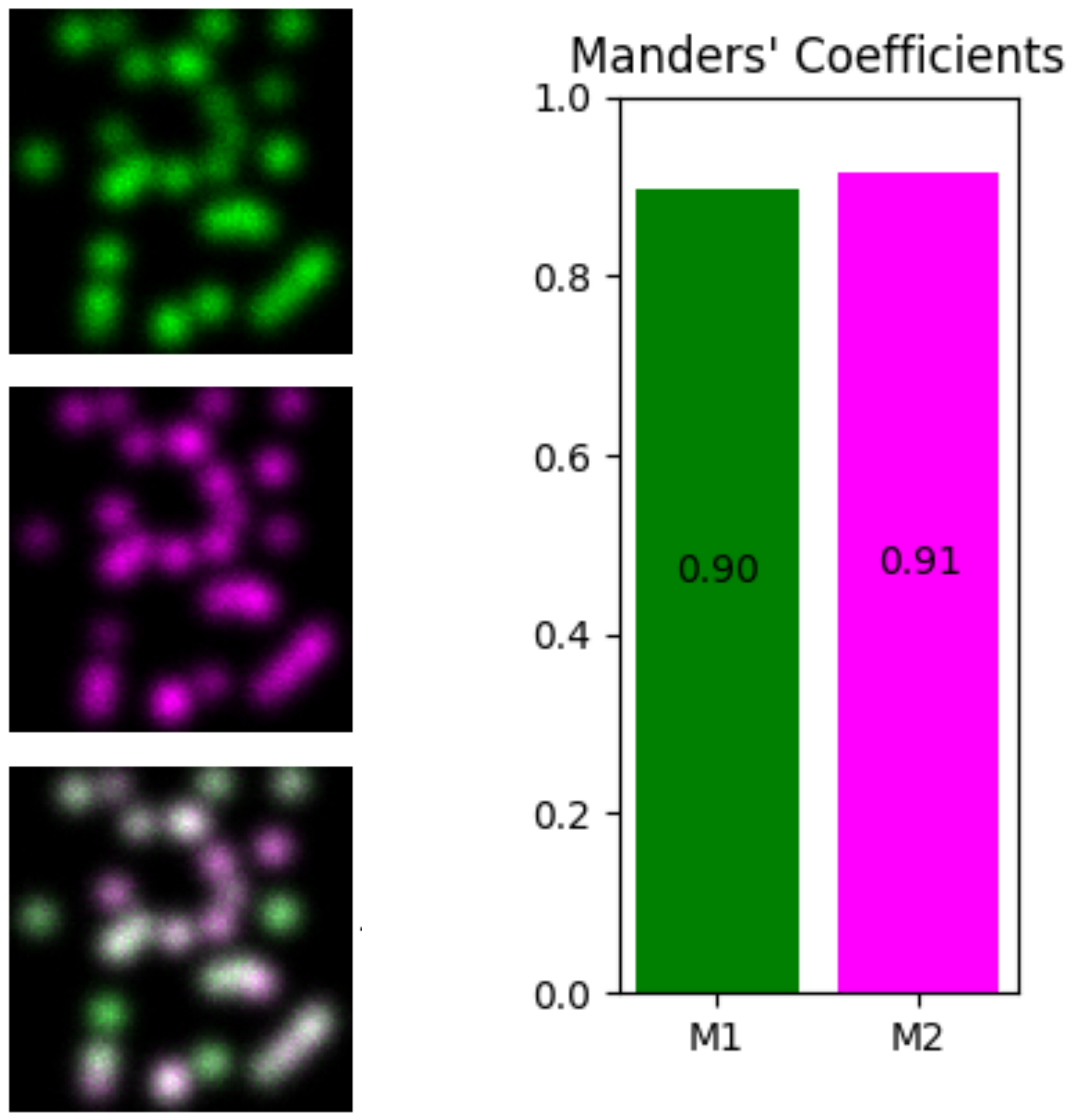
To measure the **proportion of pixel intensity** in one channel **that overlaps with pixel intensity** in the other channel.

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

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

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

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



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

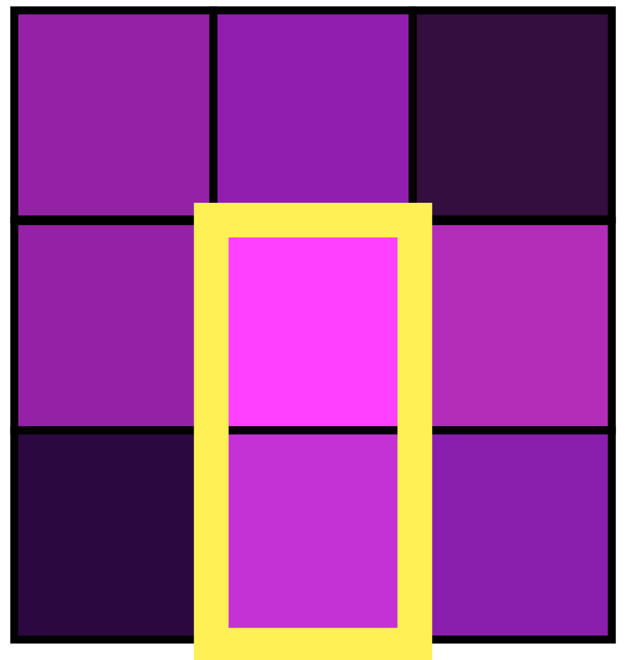
M2 = fraction of channel 2 that co-occurs with channel 1

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

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

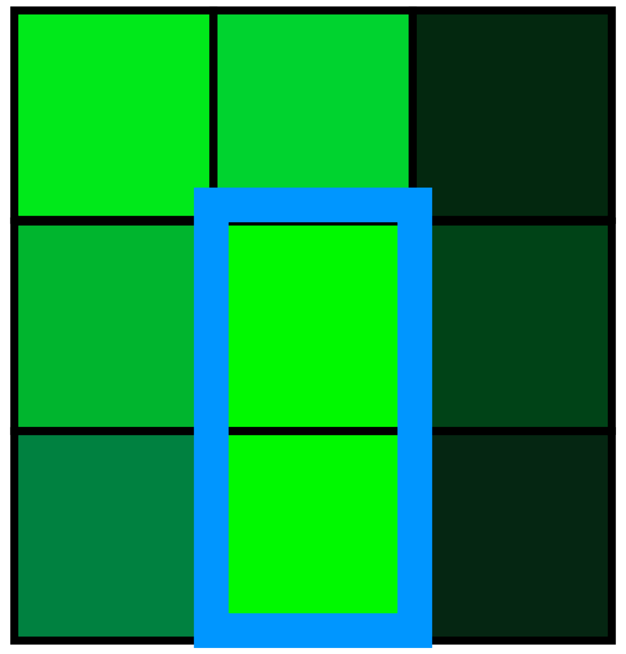
Set a threshold for channel 1:
consider only pixel with a **value > 75**

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



60	65	10
60	200	100
5	90	76

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



100	90	5
60	150	10
50	150	6

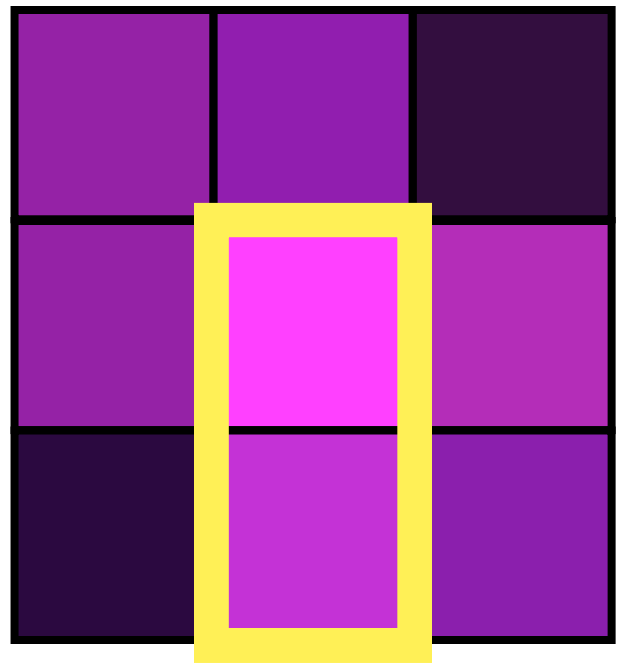
$$\mathbf{M2} = \frac{150+150=\mathbf{300}}{100+90+60+150+50+150=\mathbf{600}} = \mathbf{0.5}$$

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

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

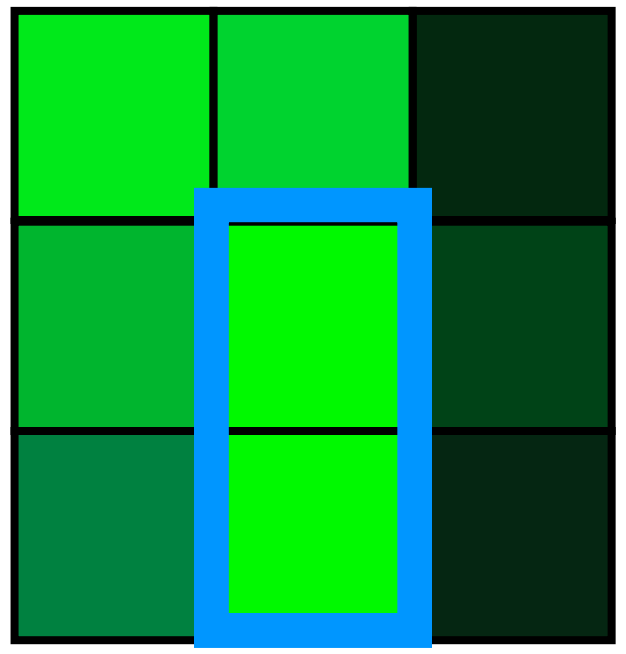
Set a threshold for channel 1:
consider only pixel with a value > 75

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



60	65	10
60	200	100
5	90	76

M1 = 0.62



100	90	5
60	150	10
50	150	6

M2 = 0.5

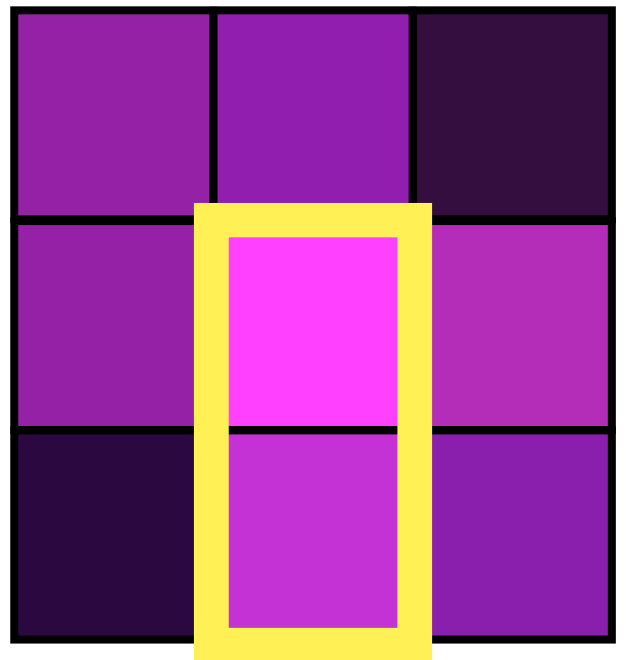
- Mander’s **M1** and **M2 can be different** from each other

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

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

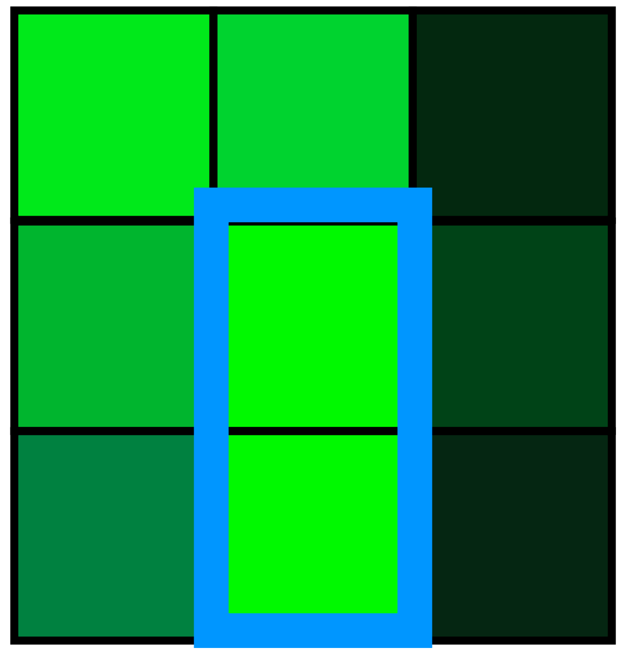
Set a threshold for channel 1:
consider only pixel with a value > 75

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



60	65	10
60	200	100
5	90	76

M1 = 0.62



100	90	5
60	150	10
50	150	6

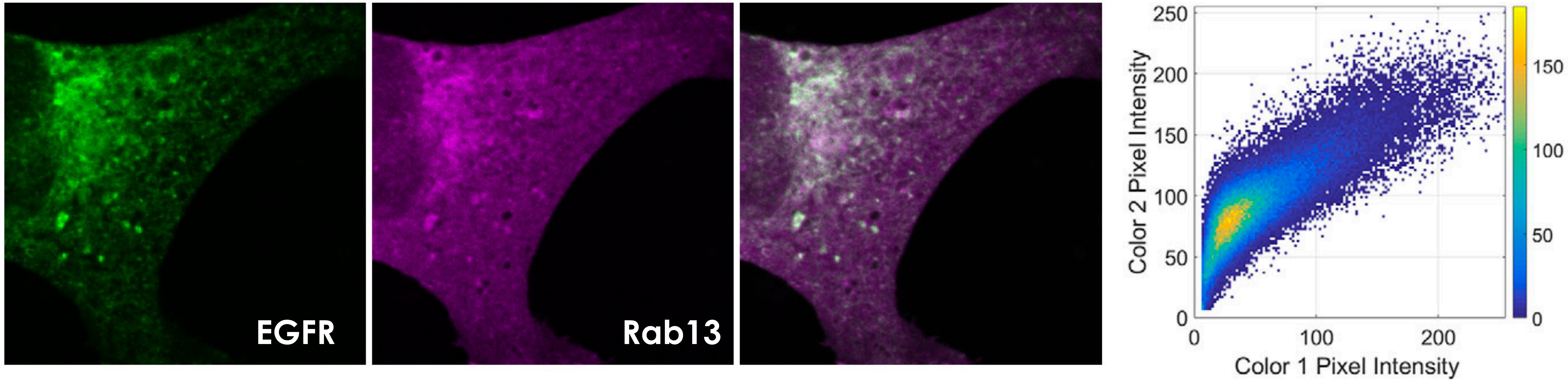
M2 = 0.5

- 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.

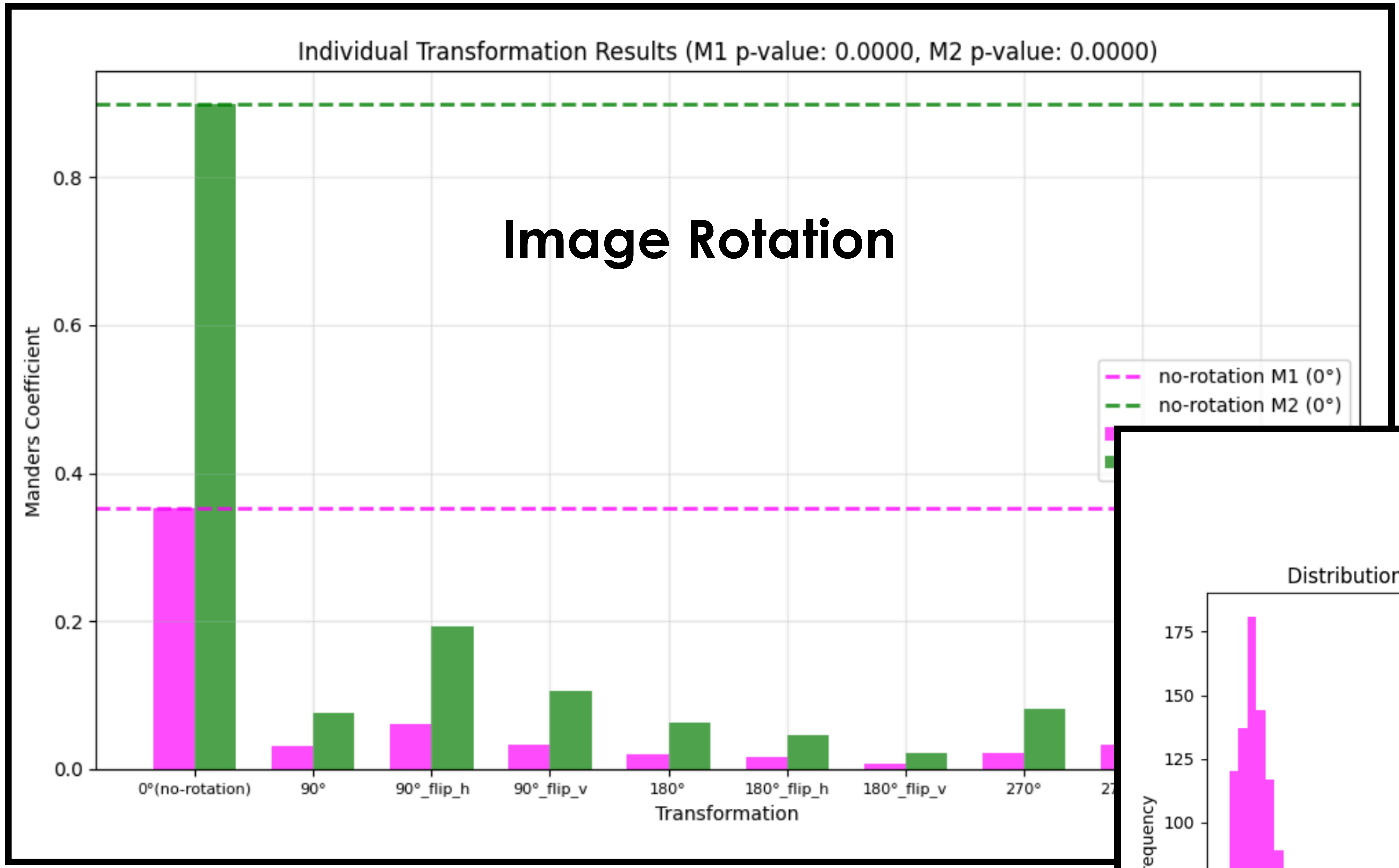
M1 = 0.99

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.

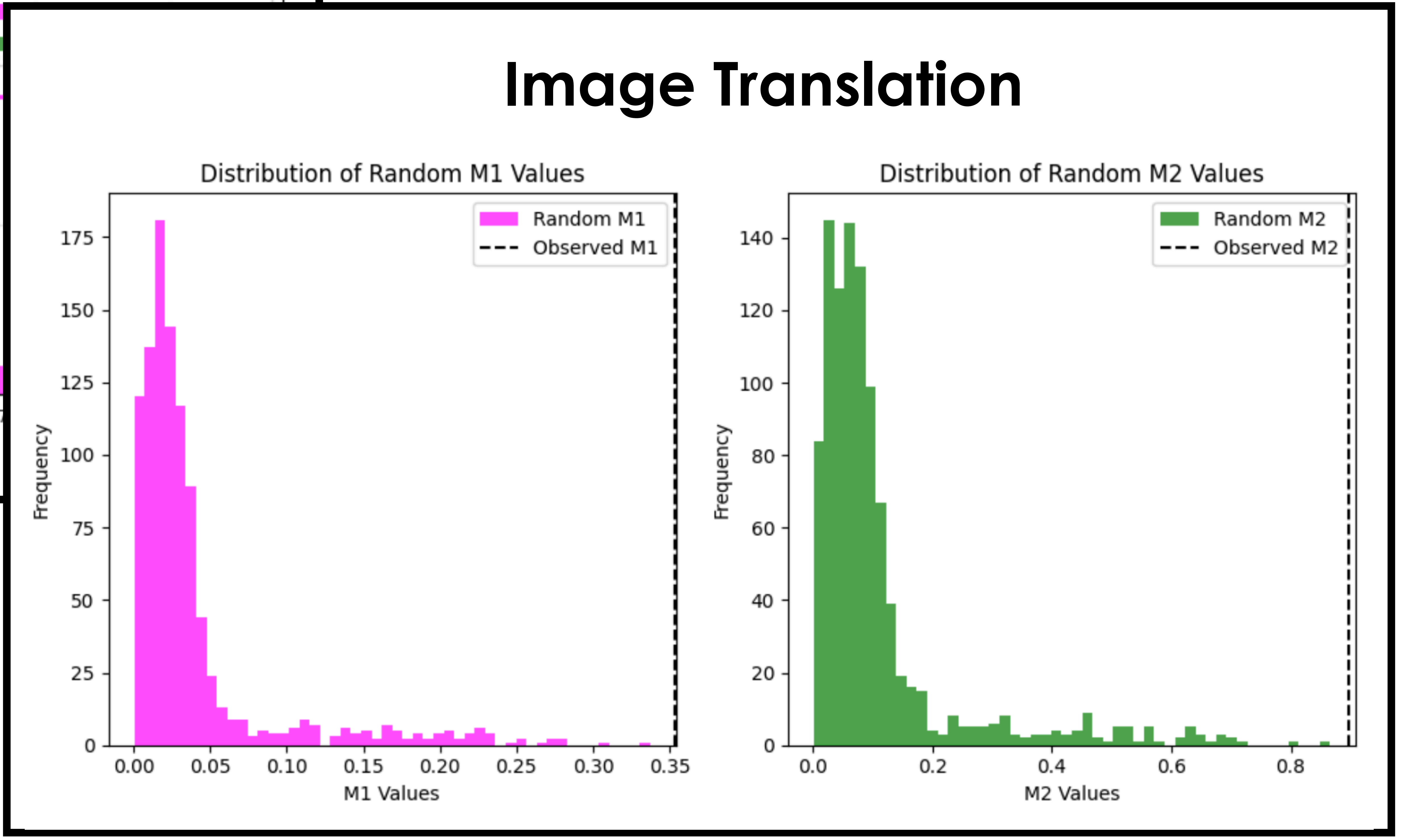
Adapted form Aaron et al, 2018

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



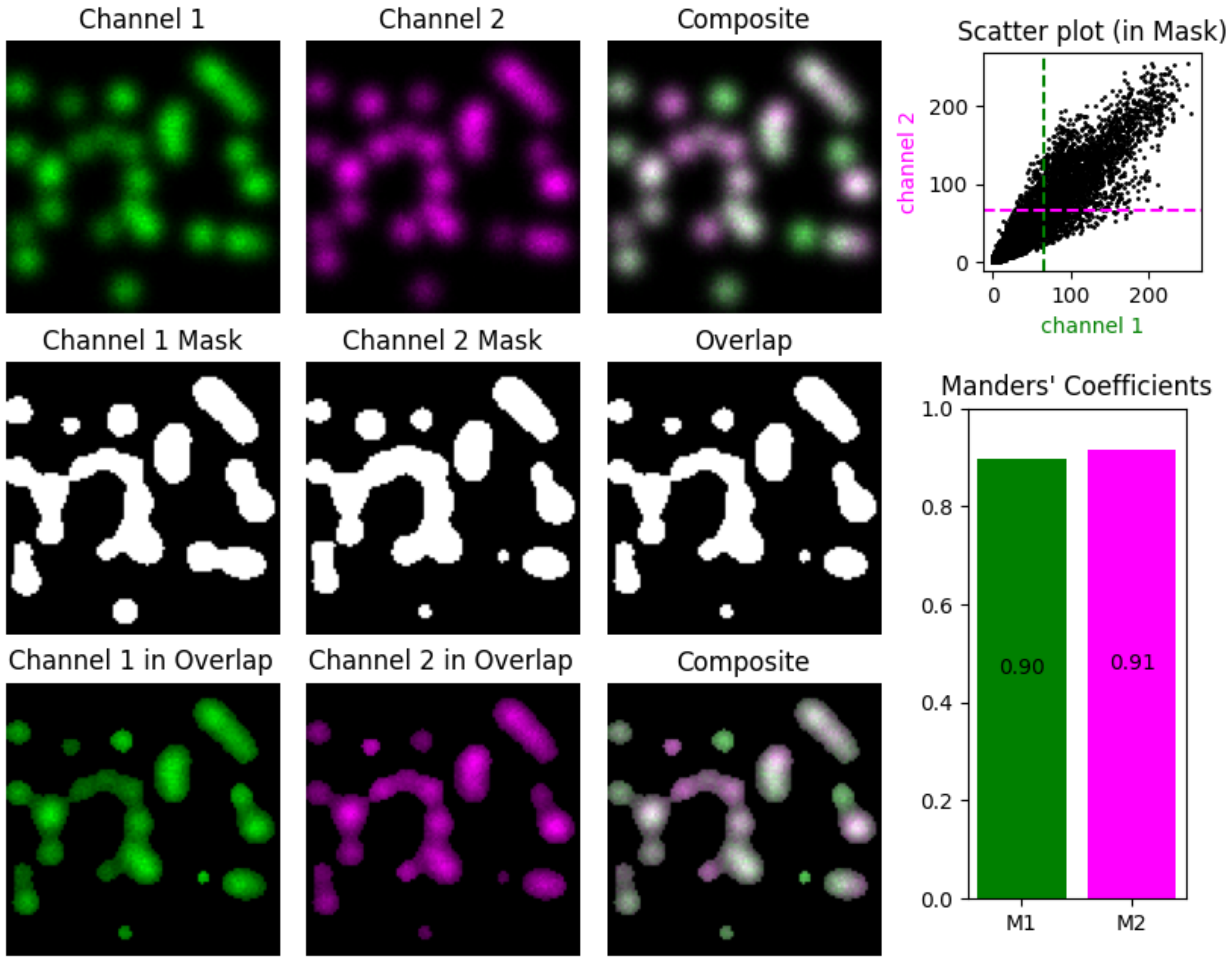
Block Scrambling

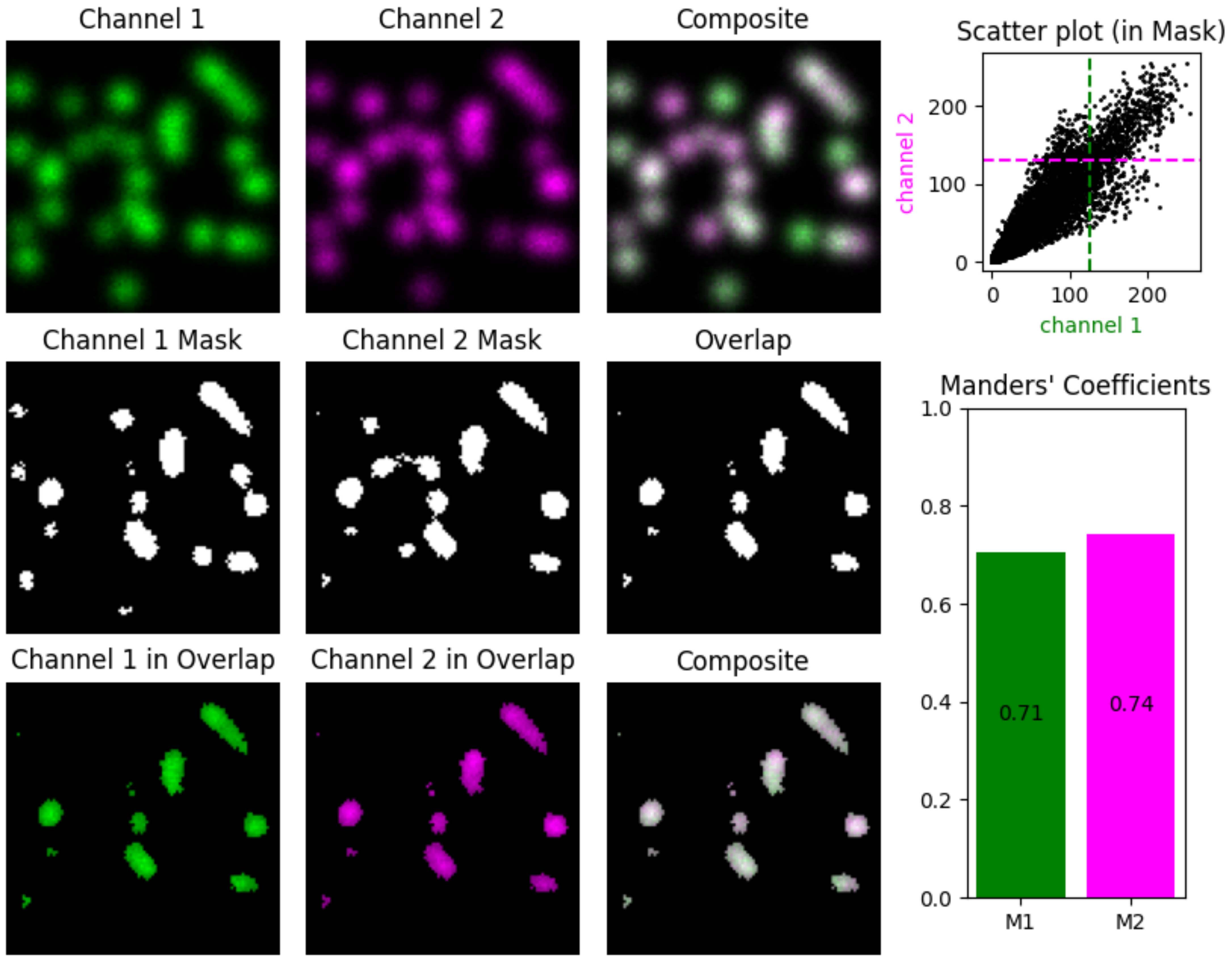
…



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

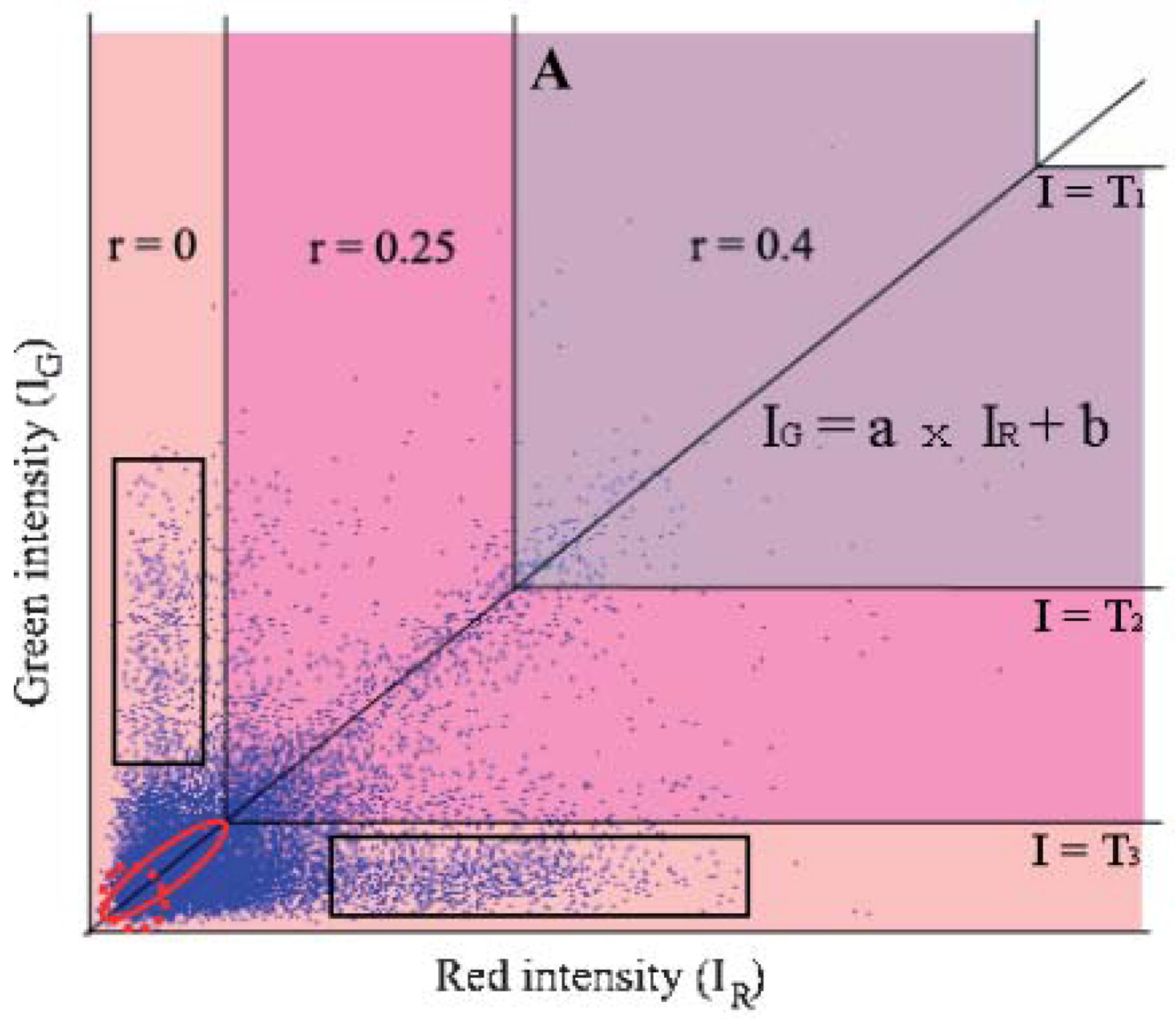
Highly **depends** on **threshold**





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

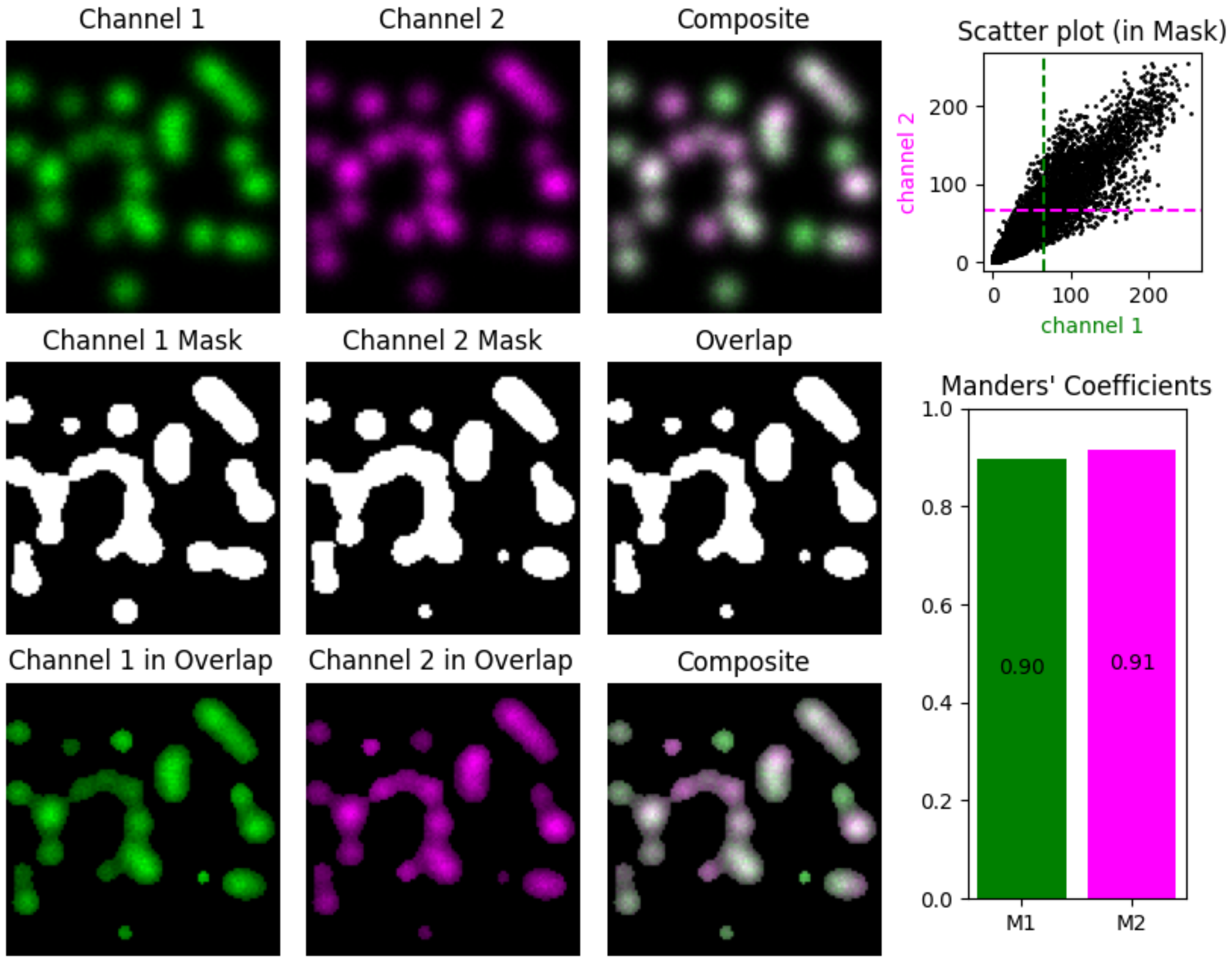
Costes Auto-Threshold

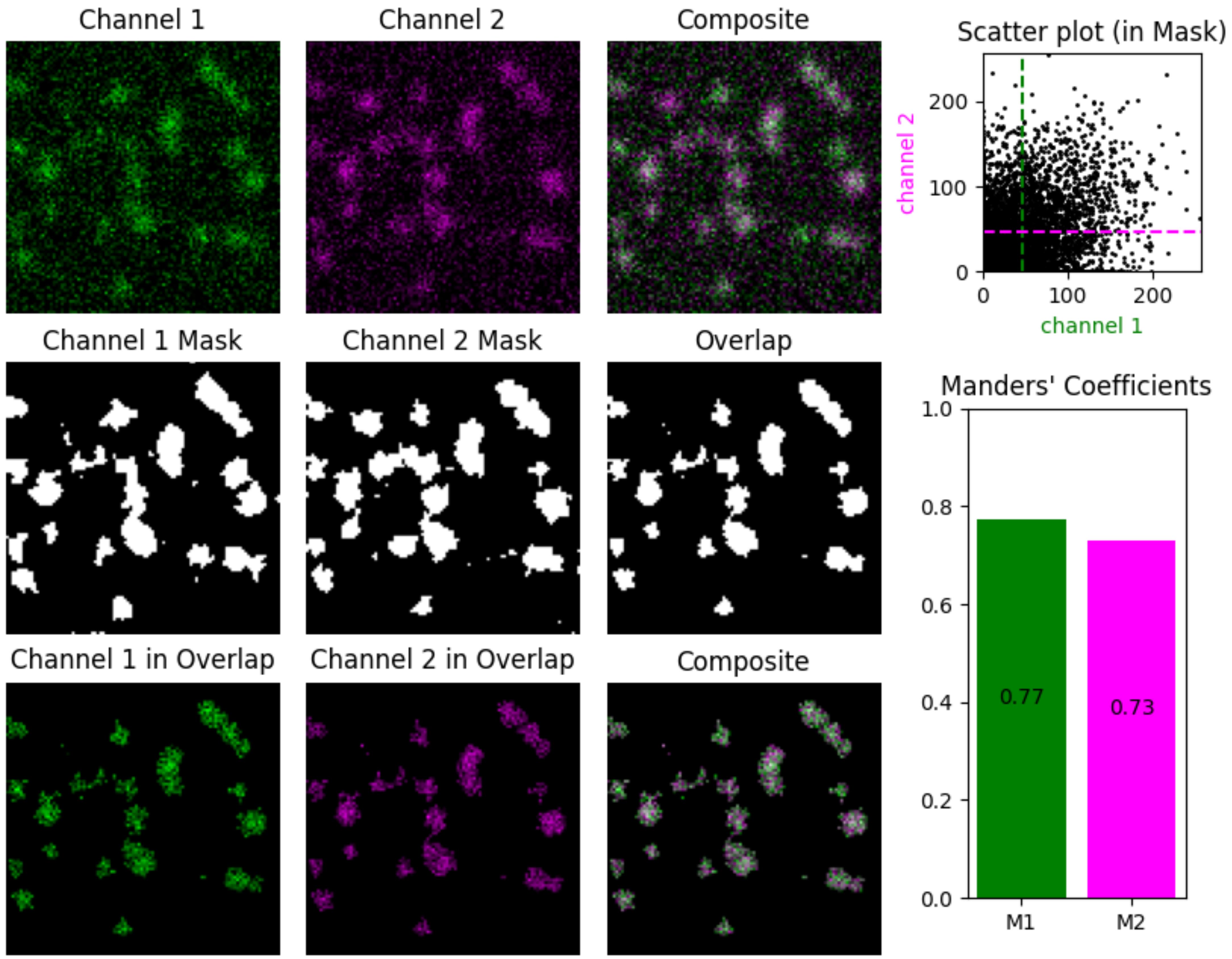


Costes et all., Automatic and Quantitative Measurement of Protein-Protein Colocalization in Live Cells

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

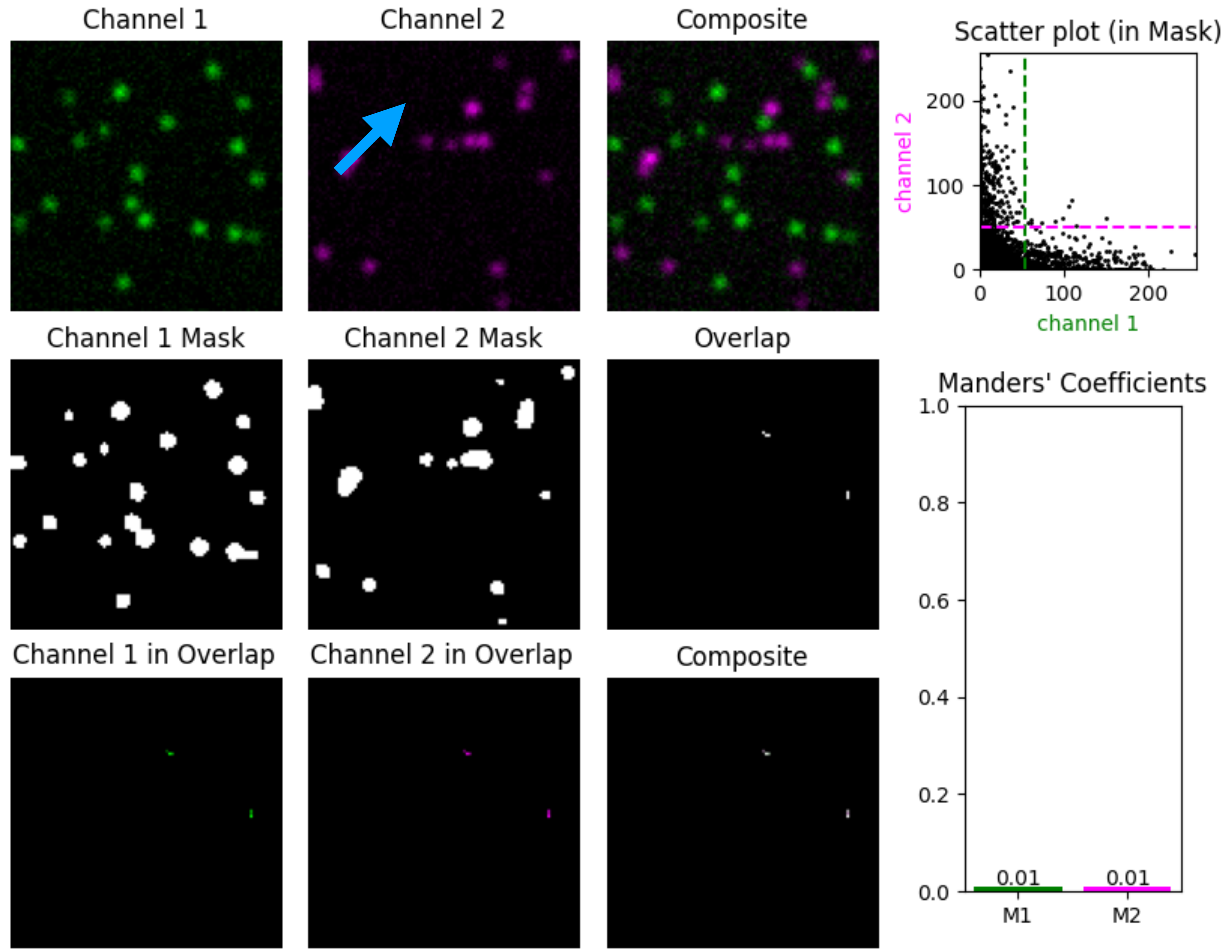
Noise

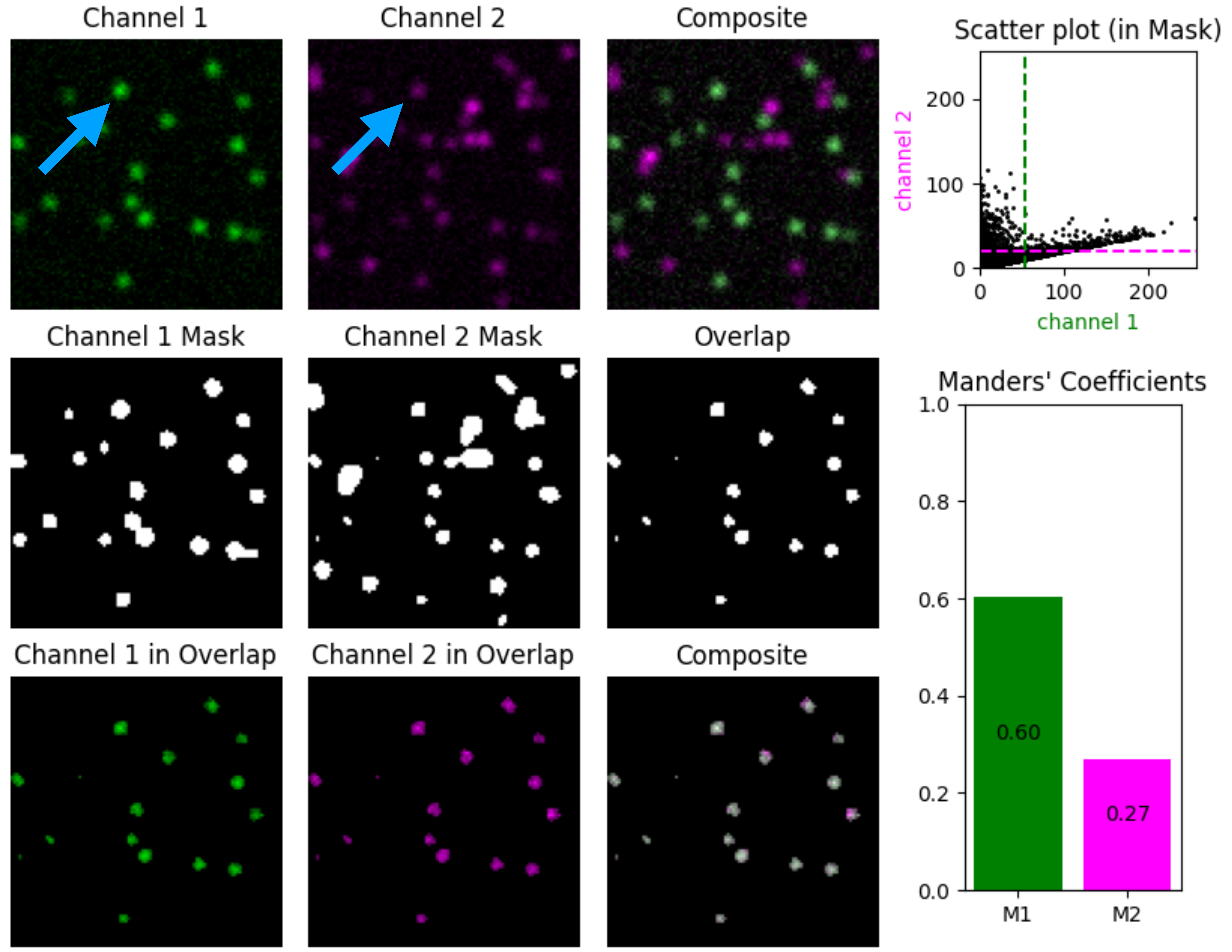




Intensity/Pixel-based: Mander's colocalization coefficients (co-occurrence)

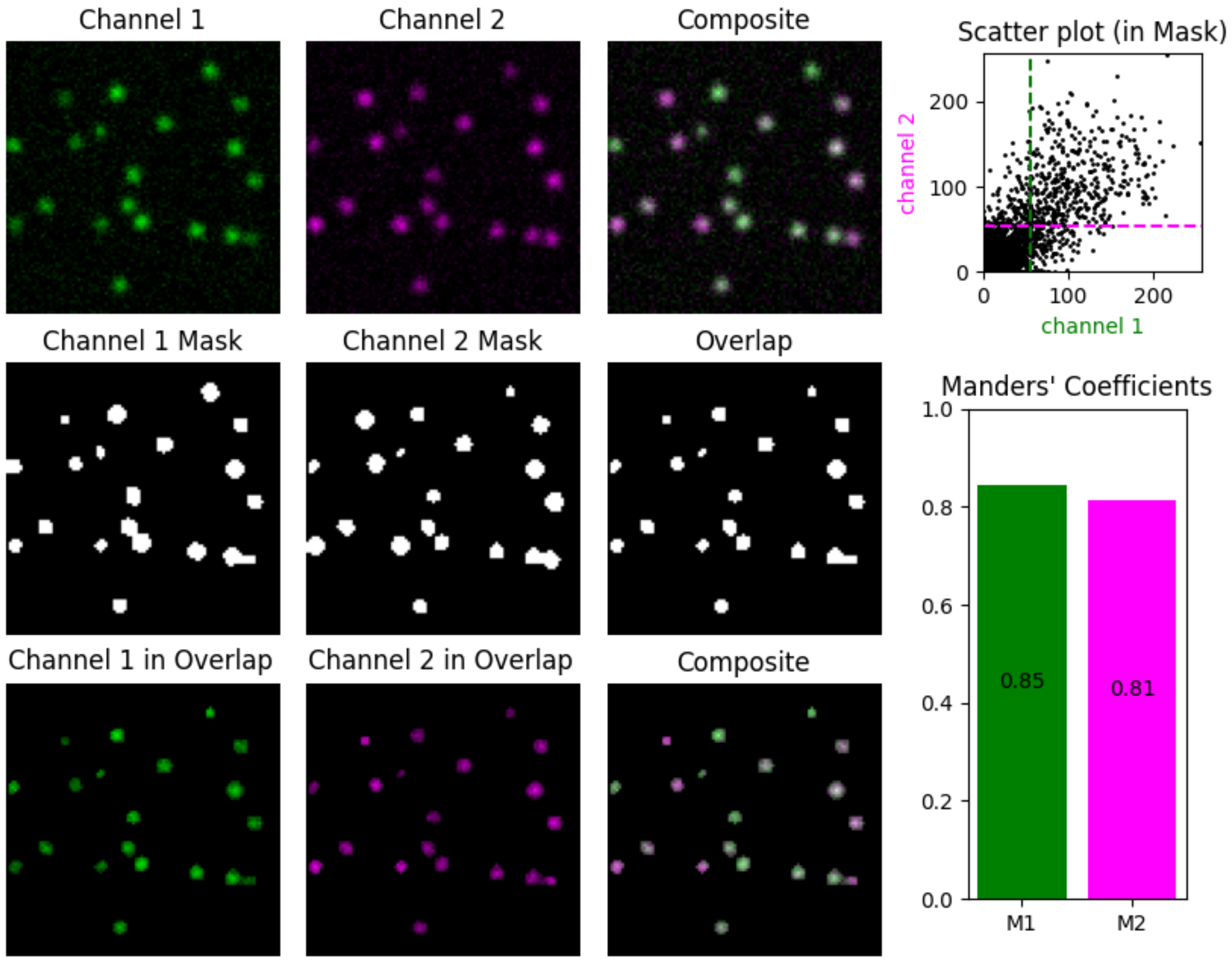
Bleedthrough

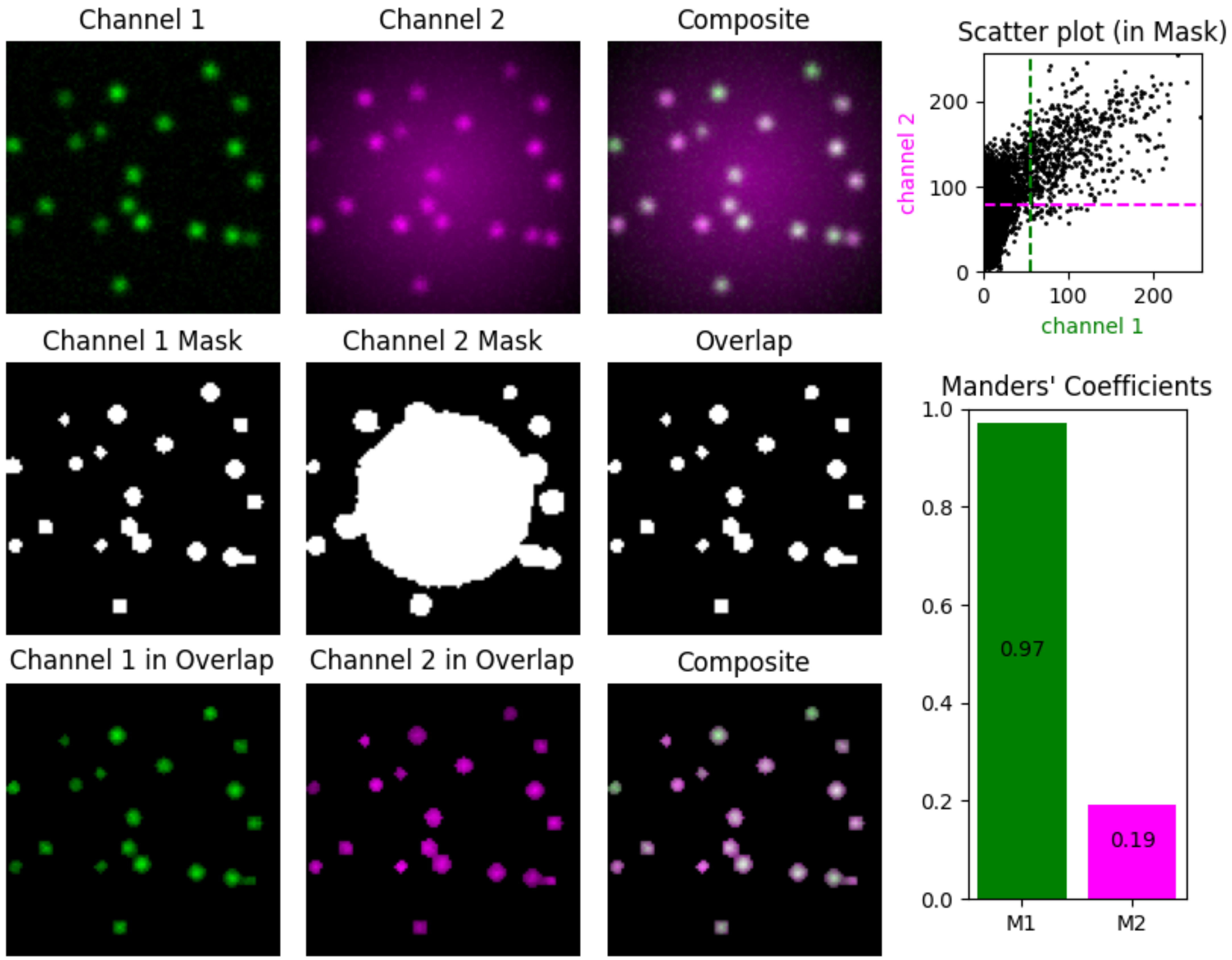




Intensity/Pixel-based: Mander's colocalization coefficients (co-occurrence)

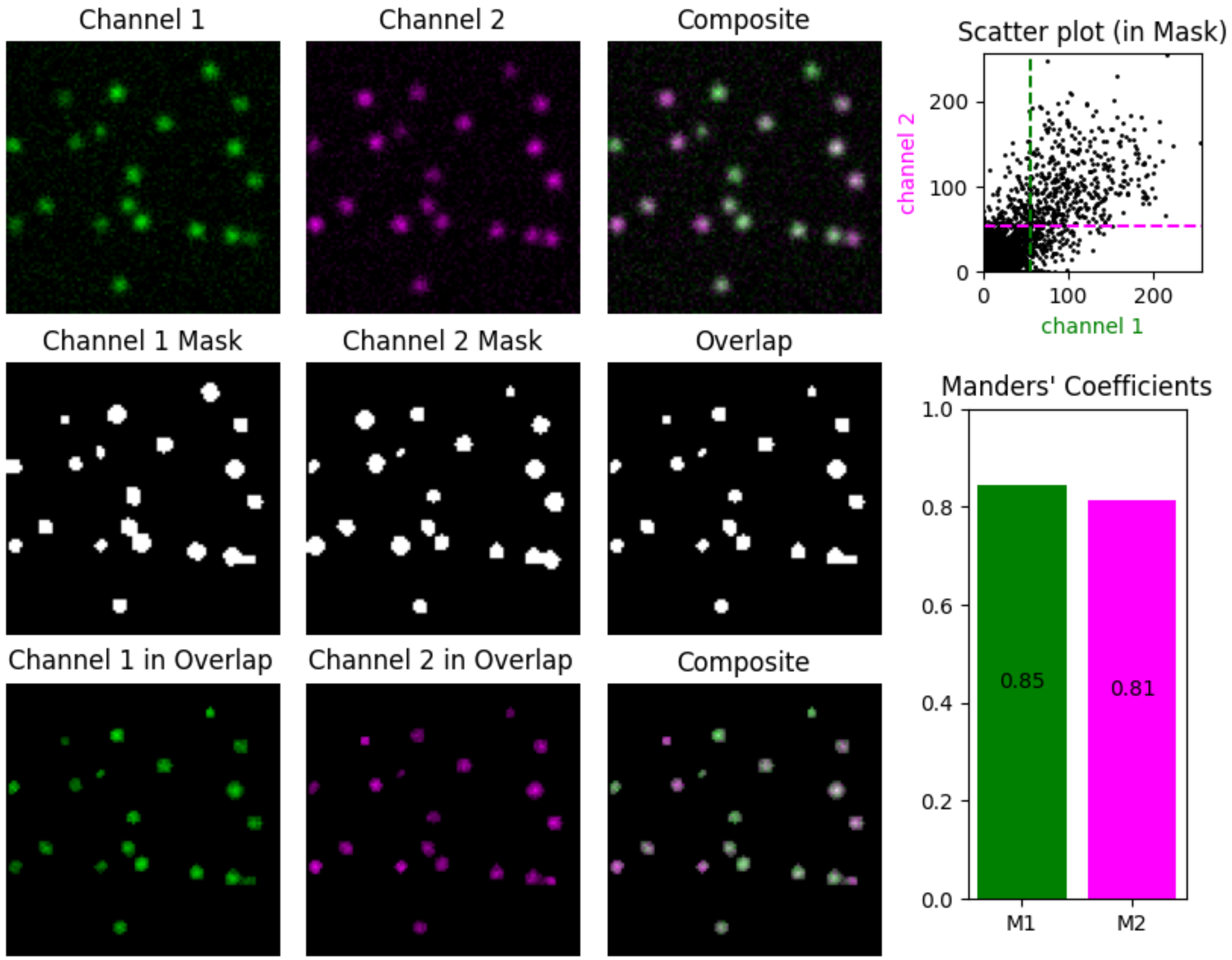
Uneven Illumination

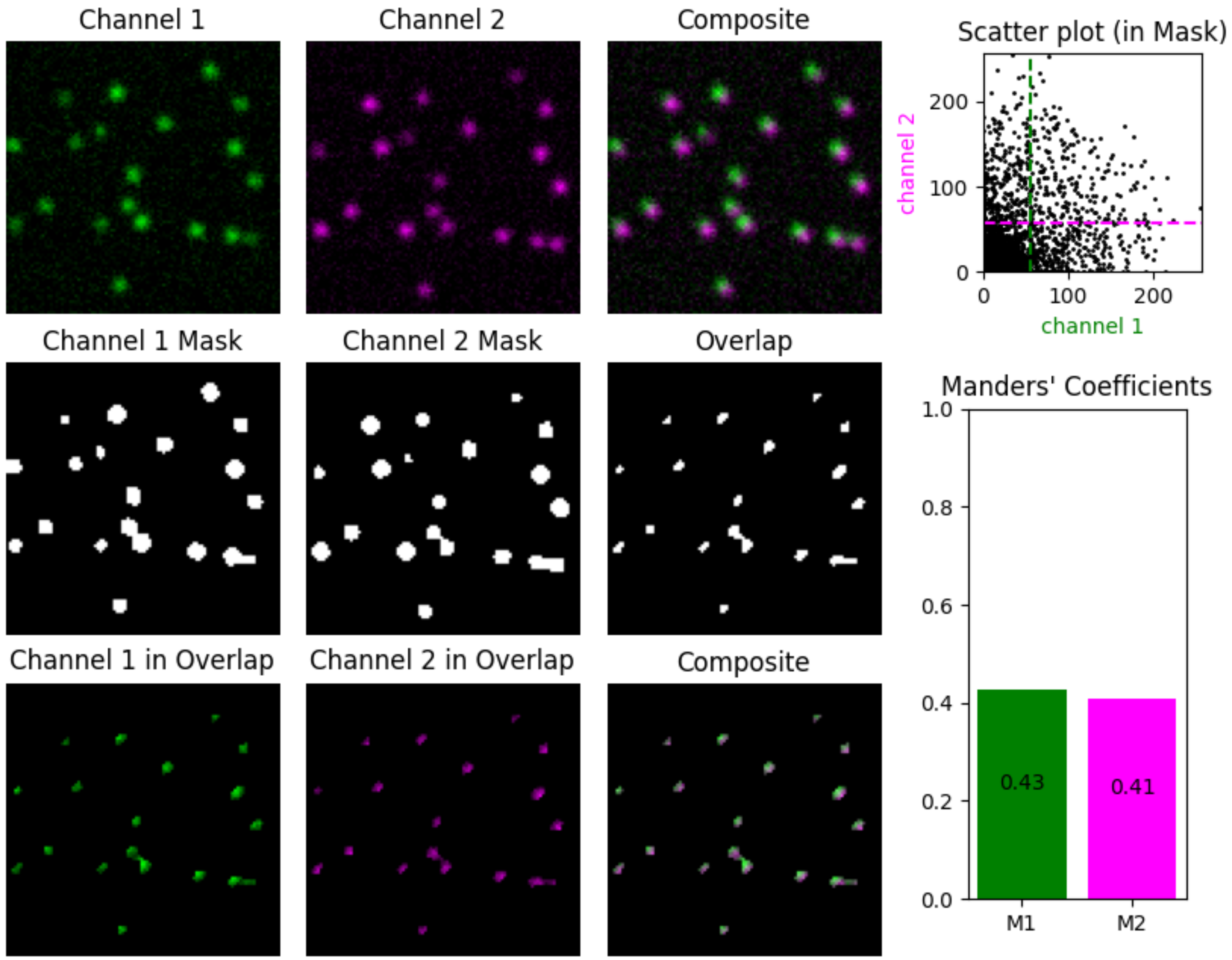




Intensity/Pixel-based: Mander's colocalization coefficients (co-occurrence)

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)