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Colocalisation microscopy: basics, experiment setup and analysis

"Seeing is believing"

REALLY?

Hong Yu, Cell Imaging Facility 4 July 2018

Outlines

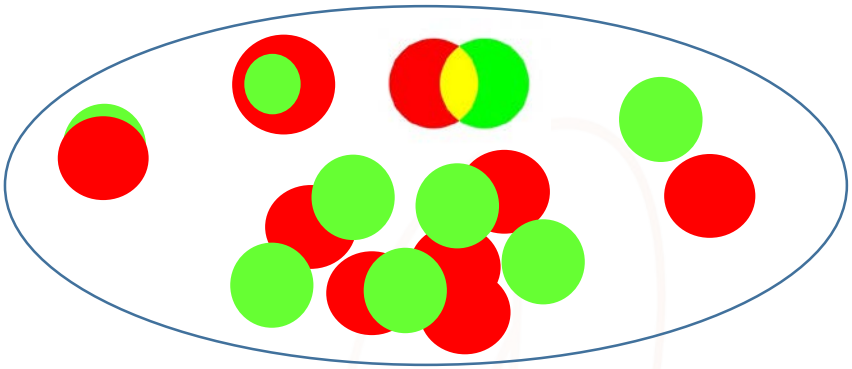
1. **Defining Coloc**
2. **Influence on Coloc**
3. **Experiment setup:** sample prep, acquisition, image processing
4. **Evaluating Coloc**
5. **Data analysis:** coefficients, available software
6. **Coloc microscopy workflow**

1. Defining colocalisation (Coloc)

Biology

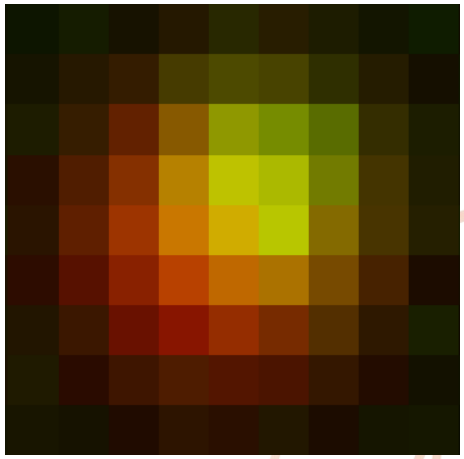
Co-occurrence (overlapping)

Correlation (closeness, association)



Digital imaging

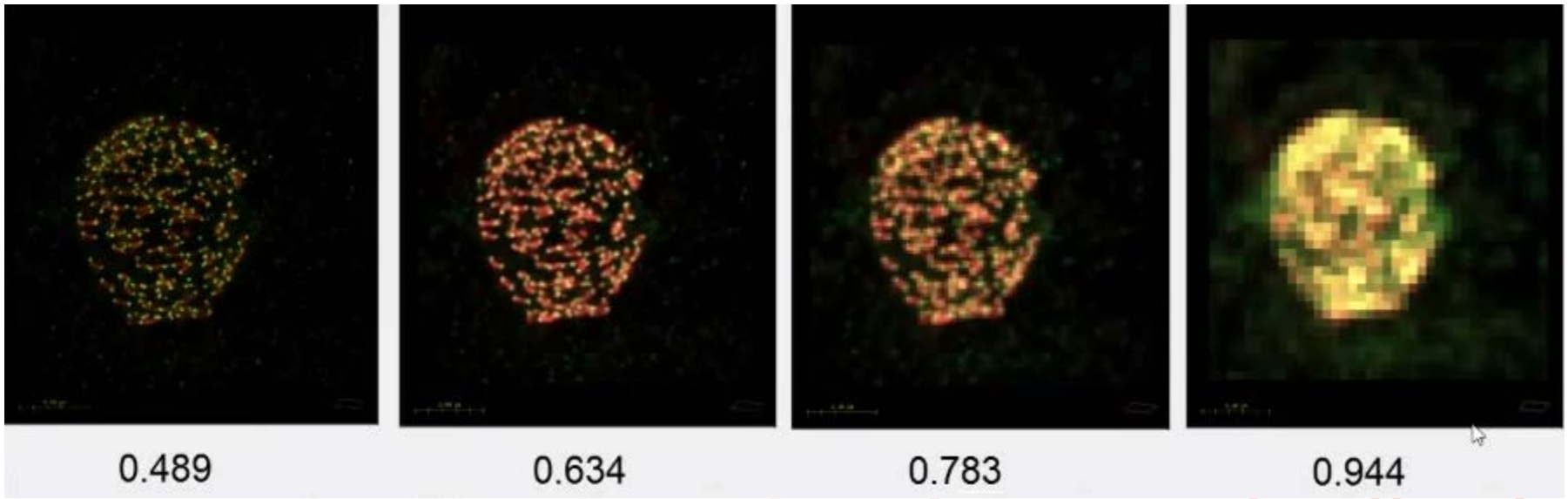
Sharing the same pixel location



- **Coloc never measures interactions**

2. Influence on Coloc—resolution

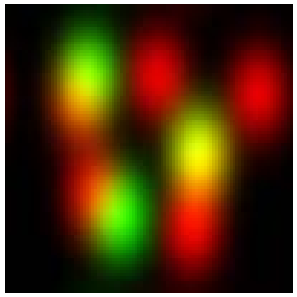
- **Resolution**



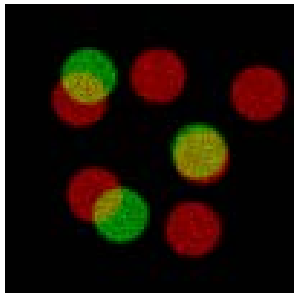
- **High resolution Coloc microscopy is more convincing**

2. Influence on Coloc—other difficulties

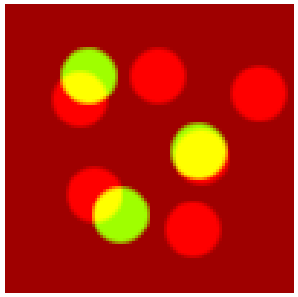
Resolution



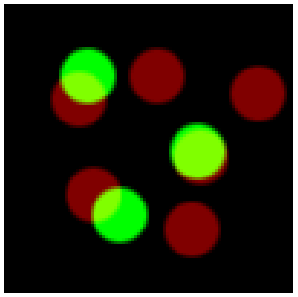
Noise



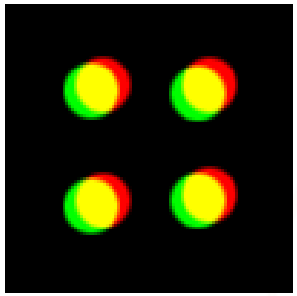
Background



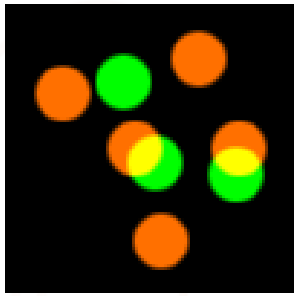
Intensity variations



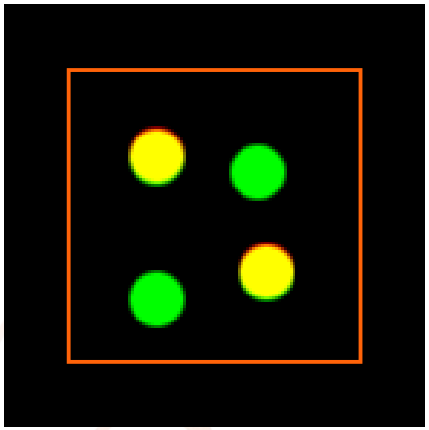
Cross-talk
Bleed-through



Chromatic
shift



Bkg margin



- Optimising experiment setup
- Image preparation helps: deconv, restoration etc

3. Experiment setup

Sample prep

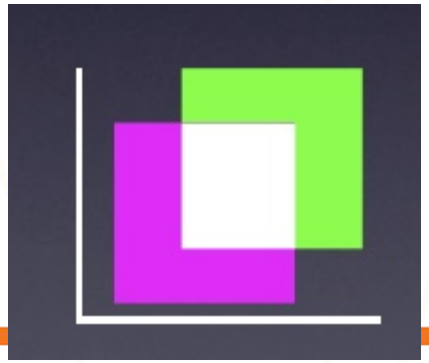
- Labelling optimisation
- AF
- Dye pair choice
- Controls

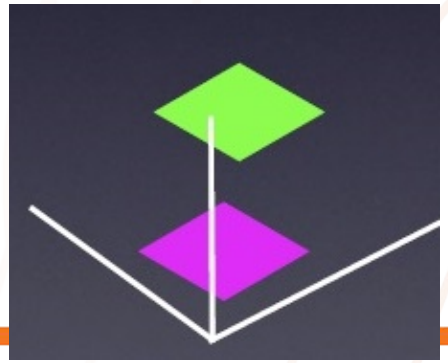
Mic & imaging

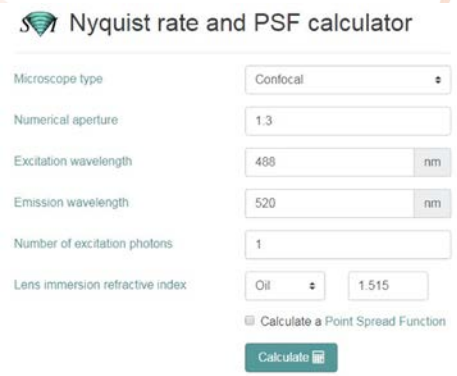
- Instrument choice
- Lens (NA, PlanApo, oil)
- Nyquist sampling
- **Z stacking**
- Low laser
- Seq, saturation
- Same conditions
- Beads imaging

Image processing

- Lossless format
- No change of Bitdepth
- No projection







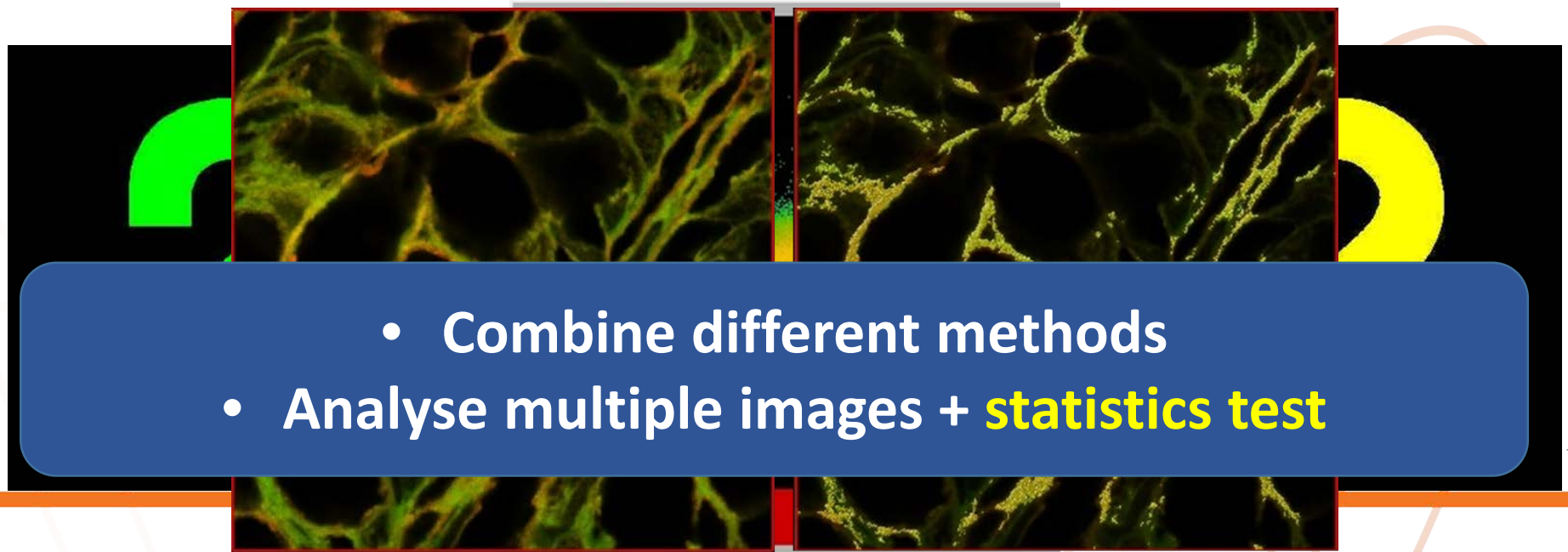
4. Evaluating Coloc

Qualitative/visual

- Color merge image
- Scatterplot
- Coloc map

Quantitative

- Object-based segmentation)
- Intensity-based (coefficients)



- Combine different methods
- Analyse multiple images + statistics test

5. Coloc analysis—image preparation

- **Image restorations (i.e. Huygens)**

  cross talk

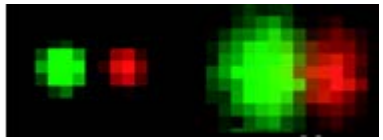
  chromatic aberration

  focal plane drift

  others

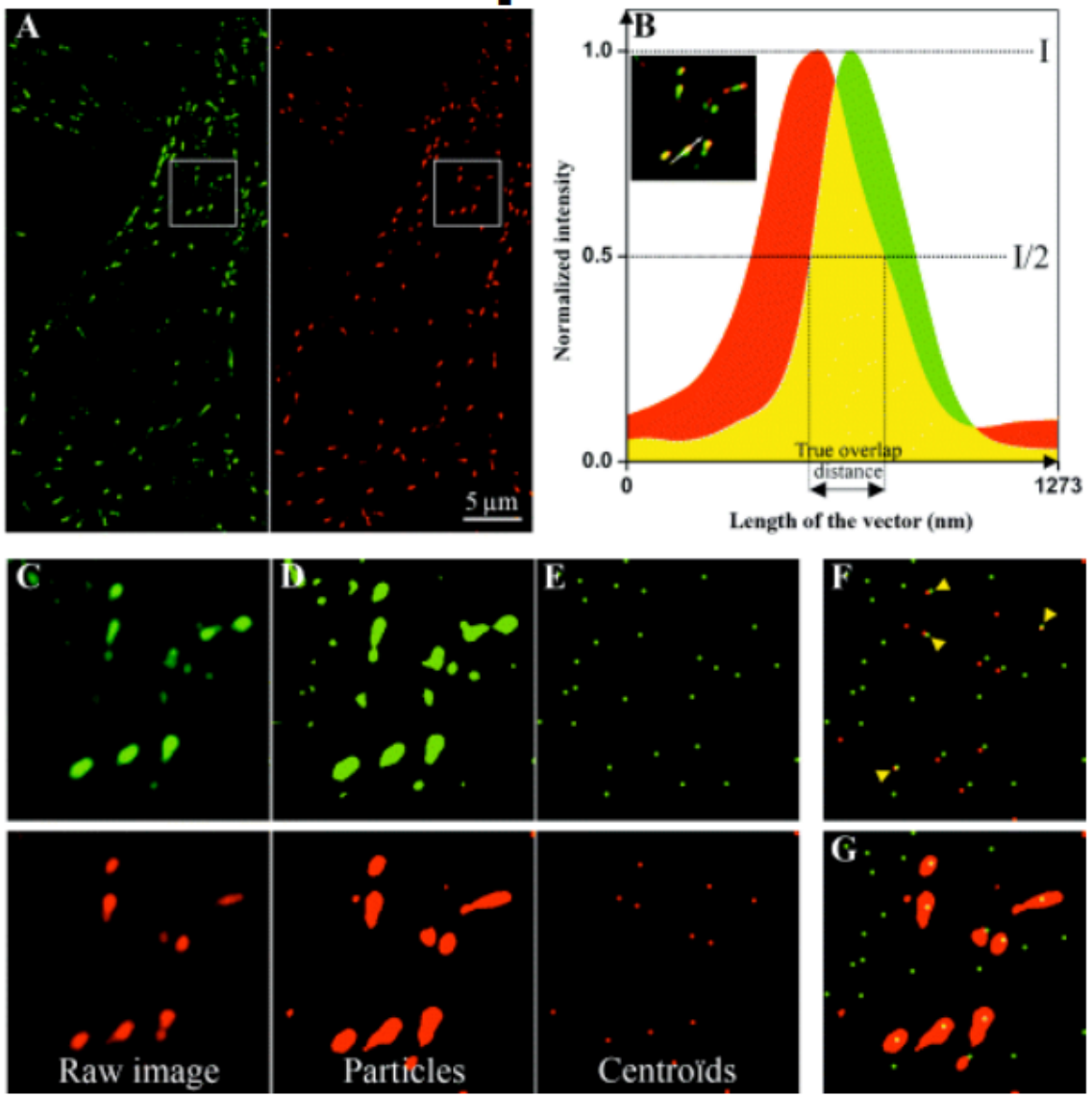
- **Deconvolution**

- **Background correction**



5. Coloc analy

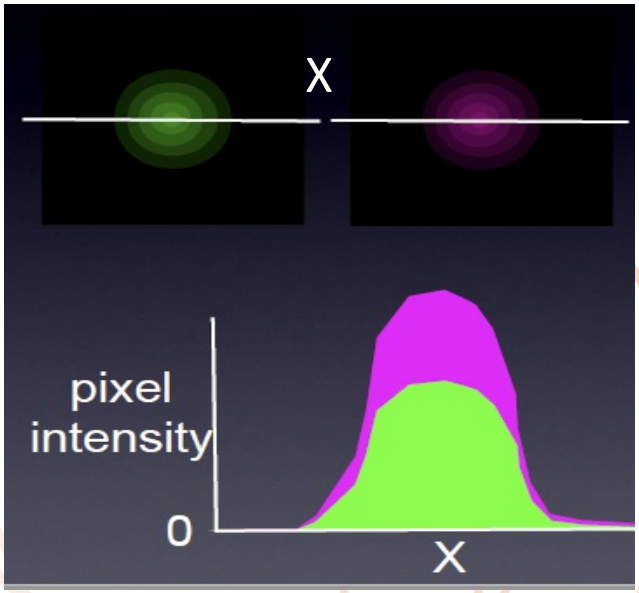
- Large objects, se
- Less intensity de
- Measurements: c
- Co-occurrence/ov



5. Coloc analysis---Intensity based analysis_1

- **Relationship between channel intensities**
- **Intensity profiles overlap**
- **Measurements**

  coefficients



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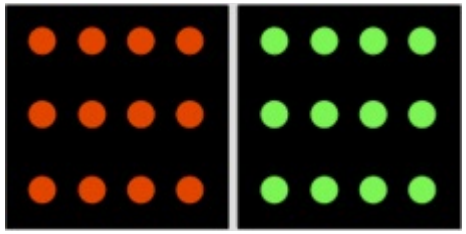
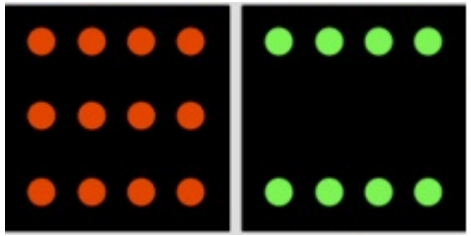
5. Coloc analysis---Intensity based analysis_2

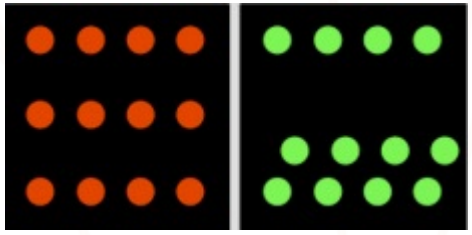
100%



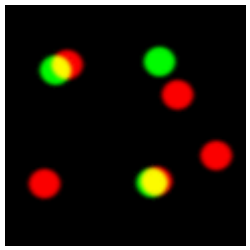
66% or 100?



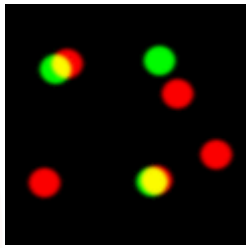
66%?



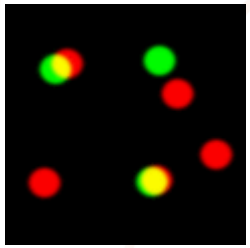
We need C are INSENSATIVE to…



the addition of extra non-coloc



relative variations of intensities



the presence of background

5. Coloc analysis---major coefficients for intensity based analysis

Which coefficient(s)? questions to ask…

- Correlation or co-occurrence?
- Random or true?

Correlation	Co-occurrence
1. Pearson's Correlation Coefficient (PCC)	**1. Manders' Overlap Coefficients (MOC)**
• Object PCC	• K1, k2
2. Spearman's Rank Correlation Coefficient (SRCC)	**2. Manders' Coloc Coefficients**
• Object SRCC	• m1, m2; M1, M2
Costes' Randomisation Analysis	

5. Coloc analysis---Pearson's Correlation C (PCC)

$$R_r = \frac{\sum_i (Ch1_i - Ch1_{mean}) \cdot (Ch2_i - Ch2_{mean})}{\sqrt{\sum_i (Ch1_i - Ch1_{mean})^2 \cdot \sum_i (Ch2_i - Ch2_{mean})^2}}$$

Pearson's Correlation Coefficient (PCC)	
Meaning	the linear correlation of the intensity distribution between two channels. the similarity/correlation between shapes/pattern/variation of two channels, while ignoring signal intensities
Standard Values	-1~+1. 0: no correlation; >0: G is higher than Avg when R is higher than Avg (correlation); <0: G is lower than Avg when R is higher than Avg
Values indicating Colc	0.5-1
Values indicating absence of coloc	-1-0.5
Example	PC=0.8. 80% of pixels in both ch are positively correlated
Advantage	Not sensitive to the intensity of constant background Not sensitive to each channel/overlapping pixels (brightness)
Disadvantage	• Difficult to interpret • sensitive to non-coloc signals • No information about the individual channels. • Affected by noise
Application	Any coloc. Co-correlation. Sub-domains.

5. Coloc analysis---Spearman's Rank C (SRCC)

	Spearman's Rank Correlation Coefficient (SRCC)
Meaning	SRCC is equivalent to the PCC, just to apply pixel intensity ranks (rank number 1, 2,3…) rather than to the intensities themselves
Standard Values	
Values indicating Colc	
Values indicating absence of coloc	
Example	
Advantage	None linear correlation
Disadvantage	
Application	Non-linear correlation or codependance

5. Coloc analysis---Manders' Overlap C (MOC)

$R = \frac{\sum_i Ch1_i \cdot Ch2_i}{\sqrt{\sum_i (Ch1_i)^2 \cdot \sum_i (Ch2_i)^2}}$	**Overall Overlap Coefficient (R) also Manders' Overlap Coefficient (MOC)**
Meaning	Indicates an actual overlap of the signals. the ratio of intersecting volume (ch1&2) to total object volume (Ch1&2)
Standard Values	0~+1. • R = 1 : perfect coloc/correlation • R= 0 : random (no) coloc
Values indicating Colc	>0.6
Values indicating absence of coloc	<0.6
Example	R= 0.5 – 50% of both chan (objects) overlap
Advantage	Rough but easy to interpret • Not sensitive to the intensity of the overlapping pixels
Disadvantage	Sensitive to background & noise• No information about the individual ch
Application	Volume in each ch is more or less the same and intensities are constant inside the objects and the image can be considered as binary (B/W).

5. Coloc analysis---Manders' K overlap C (k1, k2)

$$k_1 = \frac{\sum_i Ch1_i \cdot Ch2_i}{\sum_i (Ch1_i)^2} \qquad k_2 = \frac{\sum_i Ch1_i \cdot Ch2_i}{\sum_i (Ch2_i)^2}$$

$$R^2 = k_1 \cdot k_2$$

The k overlap coefficients (k1, k2)	
Meaning	R2=k1• k2 . Split the value of coloc into two separate parameters, allows to determine the contribution of each ch/Ag to the areas with coloc
Standard Values	vary
Values indicating Colc	>0.5
Values indicating absence of coloc	Close values (0.5 and 0.6; 0.8 an d0.9)
Example	Distant values (0.5 and 0.9; 0.2 and 0.7)
Advantage	The 2 channels are analyzed separately • Addition of a not colocalized signal will affect only one of the channels
Disadvantage	depend strongly on the ratio of total intensities in both channels
Application	When two ch intensities are at a similar level

5. Coloc analysis---Manders' Coloc C (m1, m2)

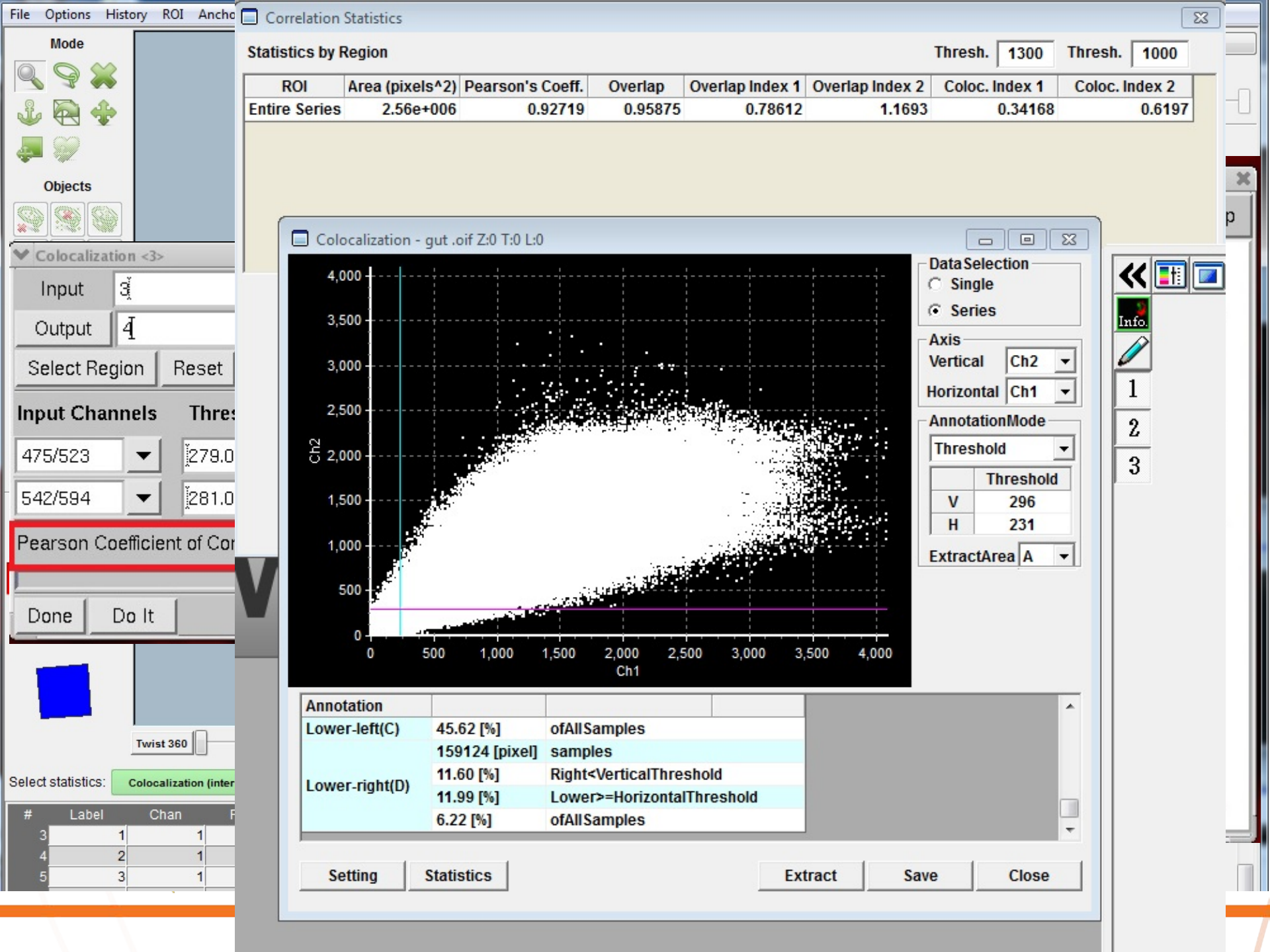
$$m_1 = \frac{\sum_i Ch1_{i,coloc}}{\sum_i Ch1_i} \quad m_2 = \frac{\sum_i Ch2_{i,coloc}}{\sum_i Ch2_i}$$

Manders' original coefficients (m1, m2)	
Meaning	Describes portion of each ch coloc with the other. % of Ch1 pix intensities coloc with Ch2 (m1).
Standard Values	0~+1.
Values indicating Colc	0.5-1
Values indicating absence of coloc	<0.5
Example	m1=1, m2=0.4: 100% of Ch1 pixel intensities coloc/overlap with Ch2, but only 40% of Ch2 pixel intensities coloc/overlap with Ch1.
Advantage	independent of signal proportionality (good for non linear); provides two components
Disadvantage	Sensitive to background
Application	Any coloc.

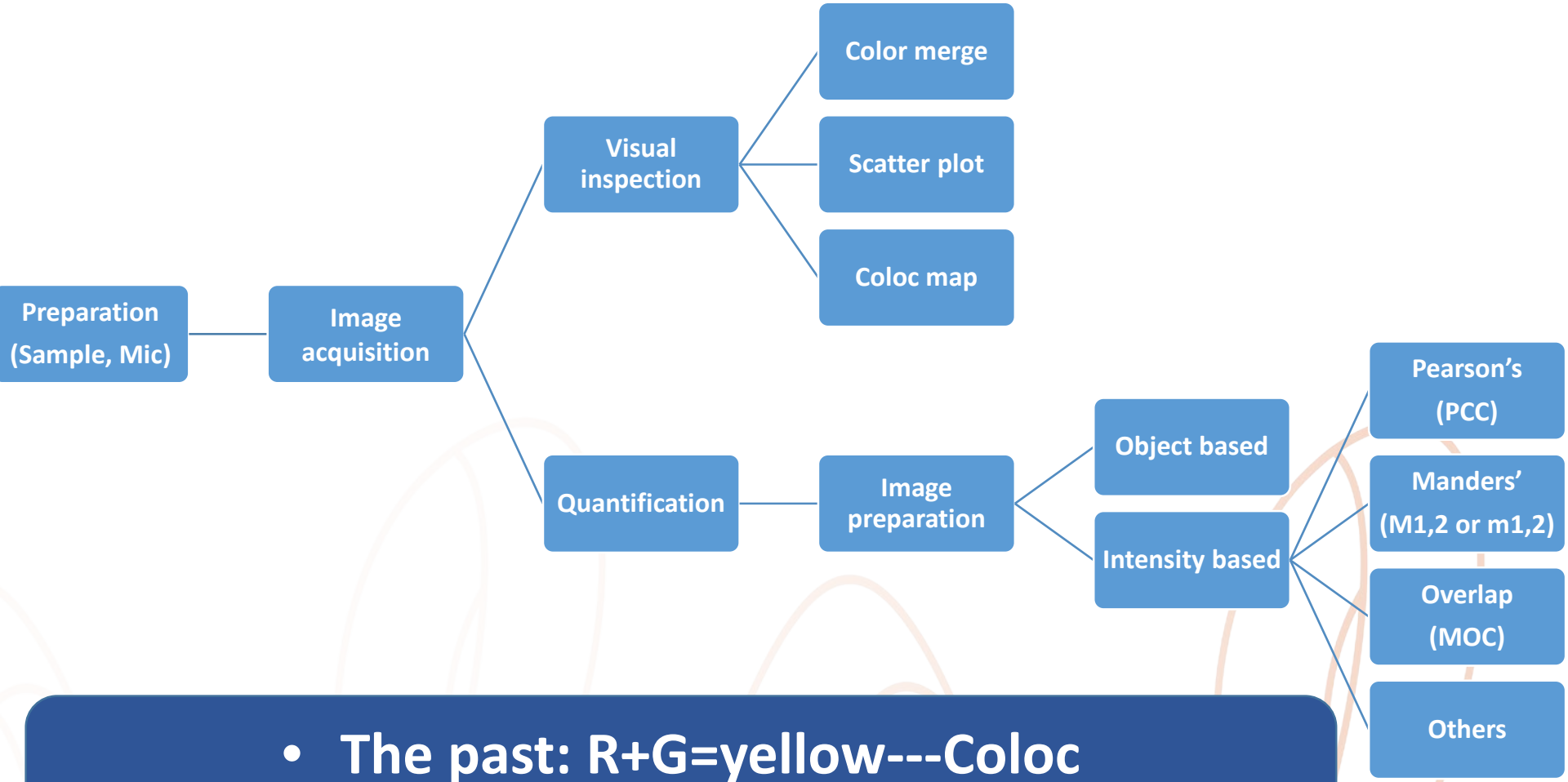
5. Coloc analysis---Manders' Coloc C (M1, M2)

$$M_1 = \frac{\sum_i Ch1_{i,coloc}}{\sum_i Ch1_i} \quad M_2 = \frac{\sum_i Ch2_{i,coloc}}{\sum_i Ch2_i}$$

Manders' Thresholed Coefficients (M1, M2)	
Meaning	% of Ch1 pix intensities coloc with Ch2 (m1). Same as m1 and m2 but applied to analyze scatter gram ROI. Thus, M1 can be defined as the co-occurrence fraction of color 1 with color 2. Likewise, M2 is the co-occurrence fraction of color 2 with color 1.
Standard Values	0~+1.
Values indicating Colc	0.5-1
Values indicating absence of coloc	<0.5
Example	M1=1, M2=0.4: 100% of Ch1 pixel intensities coloc/overlap with Ch2, but only 40% of Ch2 pixel intensities coloc/overlap with Ch1.
Advantage	Less sensitive to background problems. less dependent on the actual intensity ratios between channels
Disadvantage	Difficult to interpret • No information about the individual channels • Affected by noise
Application	Any coloc.


File Options History ROI Ancho
Mode
Objects
Colocalization <3>
Input 3
Output 4
Select Region Reset
Input Channels Thres
475/523 279.0
542/594 281.0
Pearson Coefficient of Cor
Done Do It
Twist 360
Select statistics: Colocalization (inter
Label Chan
3 1 1
4 2 1
5 3 1
Correlation Statistics
Statistics by Region
Thresh. 1300 Thresh. 1000
ROI Area (pixels^2) Pearson's Coeff. Overlap Overlap Index 1 Overlap Index 2 Coloc. Index 1 Coloc. Index 2
Entire Series 2.56e+006 0.92719 0.95875 0.78612 1.1693 0.34168 0.6197
Colocalization - gut .oif Z:0 T:0 L:0
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
Ch2
0 500 1,000 1,500 2,000 2,500 3,000 3,500 4,000
Ch1
Data Selection
Single
Series
Axis
Vertical Ch2
Horizontal Ch1
AnnotationMode
Threshold
Threshold
V 296
H 231
ExtractArea A
Annotation
Lower-left(C) 45.62 [%] ofAllSamples
159124 [pixel] samples
Lower-right(D) 11.60 [%] Right<VerticalThreshold
11.99 [%] Lower>=HorizontalThreshold
6.22 [%] ofAllSamples
Setting Statistics Extract Save Close
Info.
1
2
3

6. Coloc microscopy workflow



- The past: R+G=yellow---Coloc
- From now on: 3D, Decon, def, quantif, stat…

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Acknowledgement & References

https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1365-2818.2006.01706.x

https://svi.nl/ColocalizationCoefficients

http://jcs.biologists.org/content/joces/131/3/jcs211847.full.pdf

And many others…

Thank you!