Localization and Colocalization Theory

Resolution/Super-resolution and Overlapping/Co-localization

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Outline

Problem Formulation

Description and Motivation

- *Richard Feynman* $(1918 - 1988)$: It is very easy to answer many of these fundamental biological questions. You just look at the thing!. Make microscopes a hundred times more powerful and many problems of biology would be made very much easier.

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Problem

- *Quote*: Feynman Said *Just Look At The Thing!*.
  - What is the meaning of *look?*.
  - How could we *look?*.
  - How much can we *look?*.

Resolution

Optical Resolution

- *Formulation*: The term *optical resolution* refers to the power of an instrument to *separate* two objects in an image.
- *Astronomy*: The issue is the minimum angular distance between two stars may have, so that they can be distinguished separately. Stars are so far away they are always point sources.
- *Human Vision*: Angular Resolution.
- *Photography*: In general, lighting sources are external and uncontrolled. Images are based on how objects may reflect and refract light from these sources.
- *Microscopy*: Is possible to manipulate lighting sources. Furthermore, it is possible to manipulate how objects respond to these sources.

Resolution

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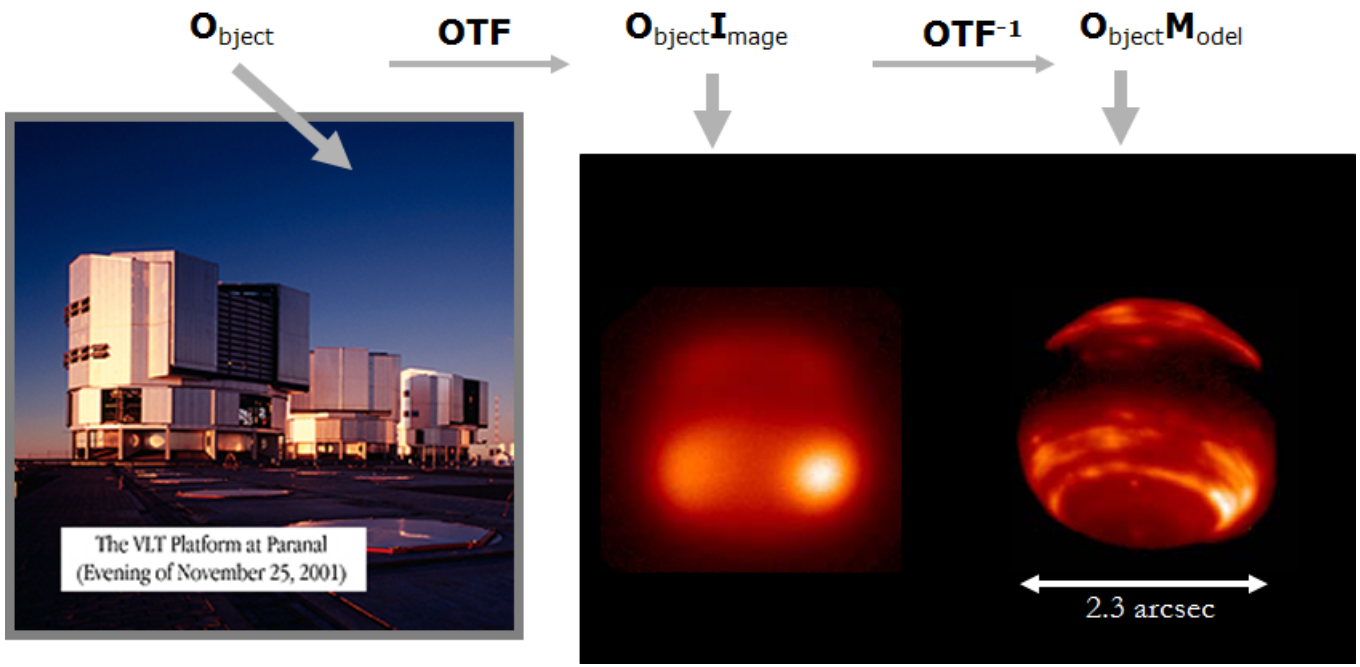
Problems

- The optical limit due to diffraction.
- Living samples, mechanical vibrations, etc.

Resolution
Astronomic Imaging

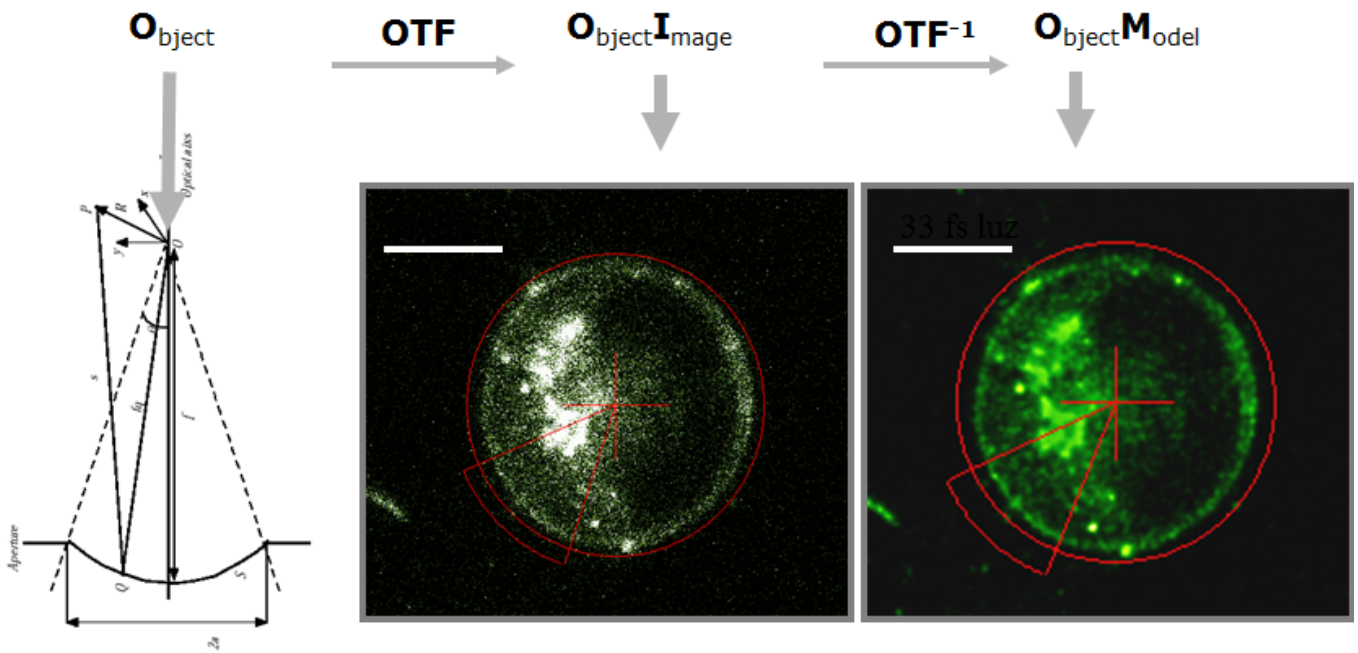
- Diffraction Limited Resolution for a $10m$ telescope $0{,}01arcsec$ is limited to $0{,}5arcsec$ by the turbulent atmosphere.



Resolution

Confocal Microscopy — From Geometric Optics to Diffraction Theory

- *Diffraction*: The deviation of an electromagnetic wavefront from the path predicted by geometric optics when the wavefront interacts with a physical object such as an opening or an edge.



Resolution

Generic Image Formation

PSF: *Point Spread Function*

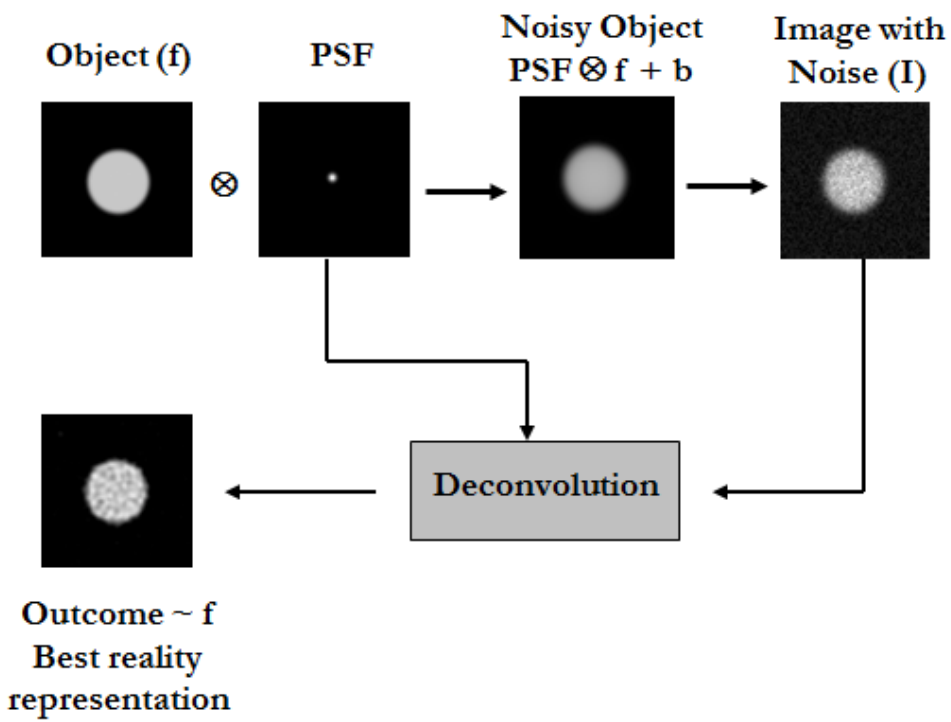
f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$



Object detection and localization

- In the field of cell biology, one of the most challenging issues is to non invasively investigate the motions of sub-cellular structures or biomolecular reactions in living cells with very high *spatial and temporal resolution*.
- Recently, several novel schemes have been proposed to visualize the sub-cellular structures with a spatial resolution breaking the diffraction limit.
- The main concepts of these approaches are either to switch off the fluorophores around the center area within the diffraction limited zone or to switch on one fluorophore at a time in the diffraction limited zone enabling the localization of the centroid of the activated fluorophore.
- However, ...

Autocorrelation as a measure of Fluorescence Fluctuations

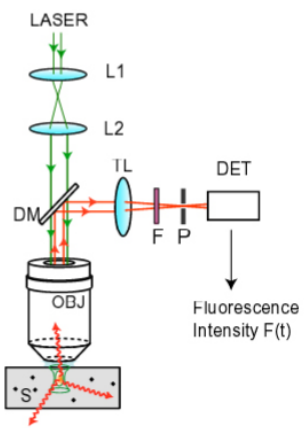
What Physical Processes Cause Fluorescence Fluctuations?

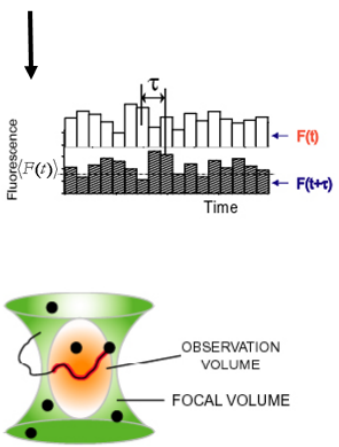
- Physical processes are typically in a state of dynamic equilibrium. For example, the dynamic process of diffusion causes a component's local concentration in a small section of a large sample solution to fluctuate about the average concentration.
- These fluctuations can arise from volume-dependent processes, such as local concentration fluctuations of fluorescent particles. They can also arise from volume-independent processes that act on the fluorescent dyes.
- Fluctuations in fluorescence intensity can result from the following volume-dependent and volume-independent processes:
  - Random diffusion
  - Directed flow (hydrodynamic and electrophoretic)
  - Chemical Equilibrium
  - Intersystem crossing between singlet and triplet states
  - Nonradiative fluorescence resonance energy transfer (FRET)

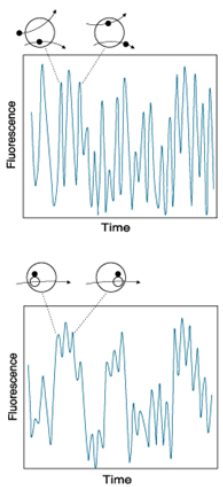
Autocorrelation as a measure of Localization

Fluorescence correlation spectroscopy *FCS* and Auto-Correlation

- FCS is a powerful single-molecule detection technique that measures and correlates fluctuations in fluorescence intensity within a very small detection volume (on the order of femtoliters).
- FCS measures fluctuations in fluorescence intensity in a small section of the total sample volume.
- Auto-correlation measures self-similarity of a time signal and highlights characteristic time constants of underlying processes.



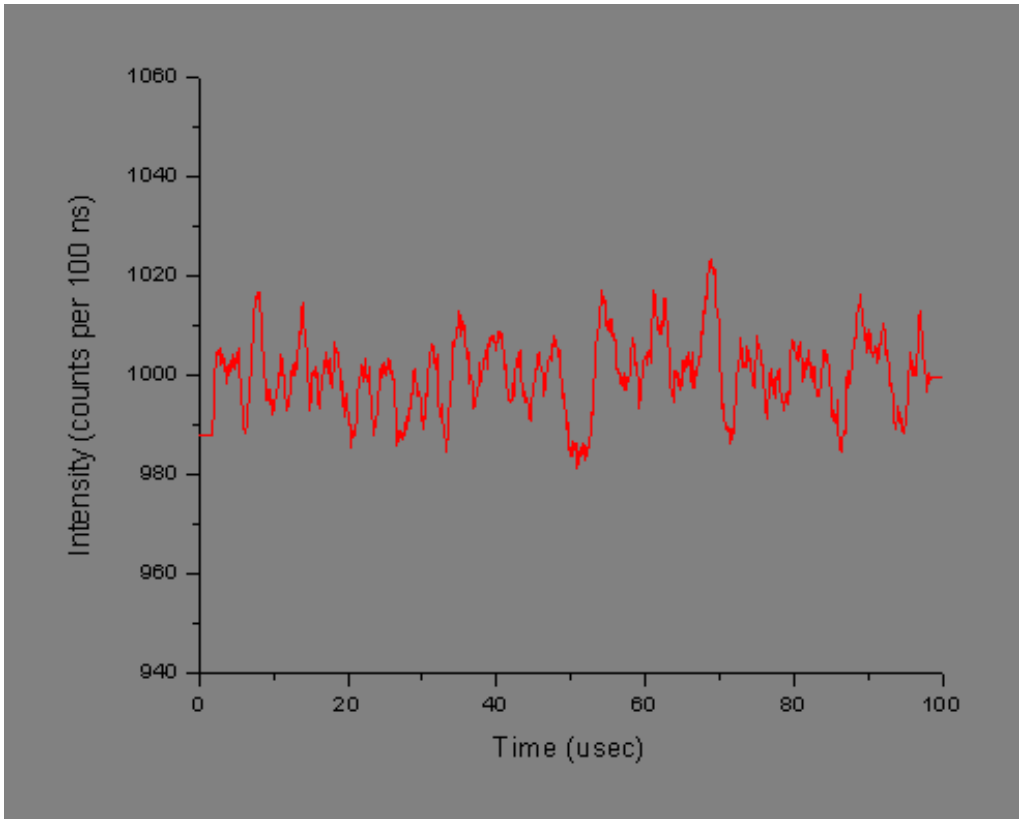




Autocorrelation on time

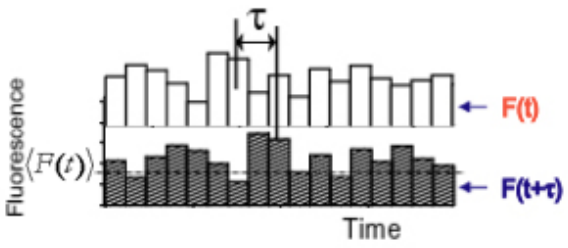
Fluorescence Intensity Measurements

- FCS measures fluctuations of the fluorescence signal intensity about the mean in a small detection volume.



Autocorrelation & FCS

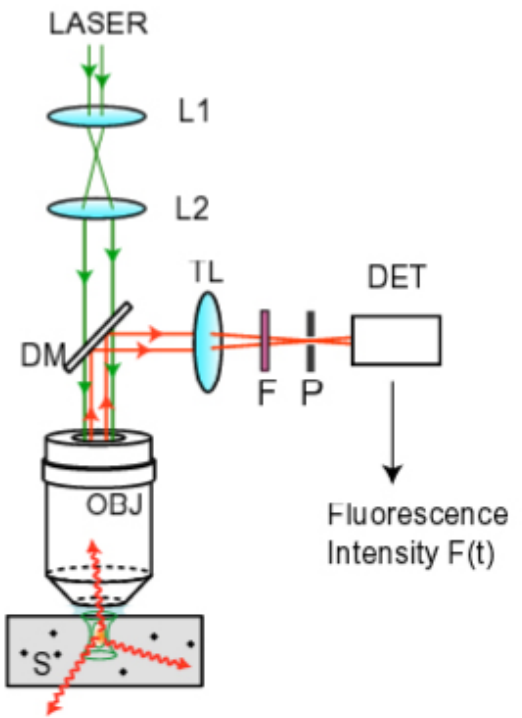
AutoCorrelation Theory

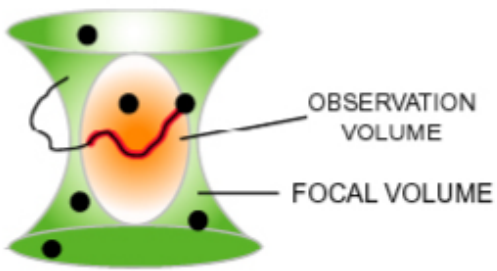


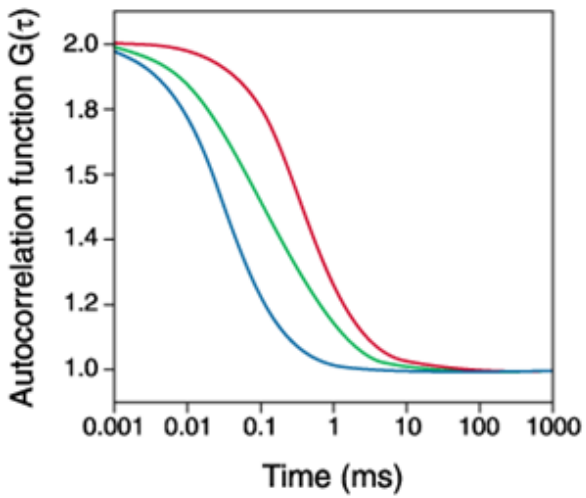
$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

$$\langle F(t) \rangle = 1/T \int_0^T F(t)dt, \quad \textit{mean}$$

$$\delta F(t) = F(t) - \langle F(t) \rangle, \quad \textit{deviation or fluctuation}$$

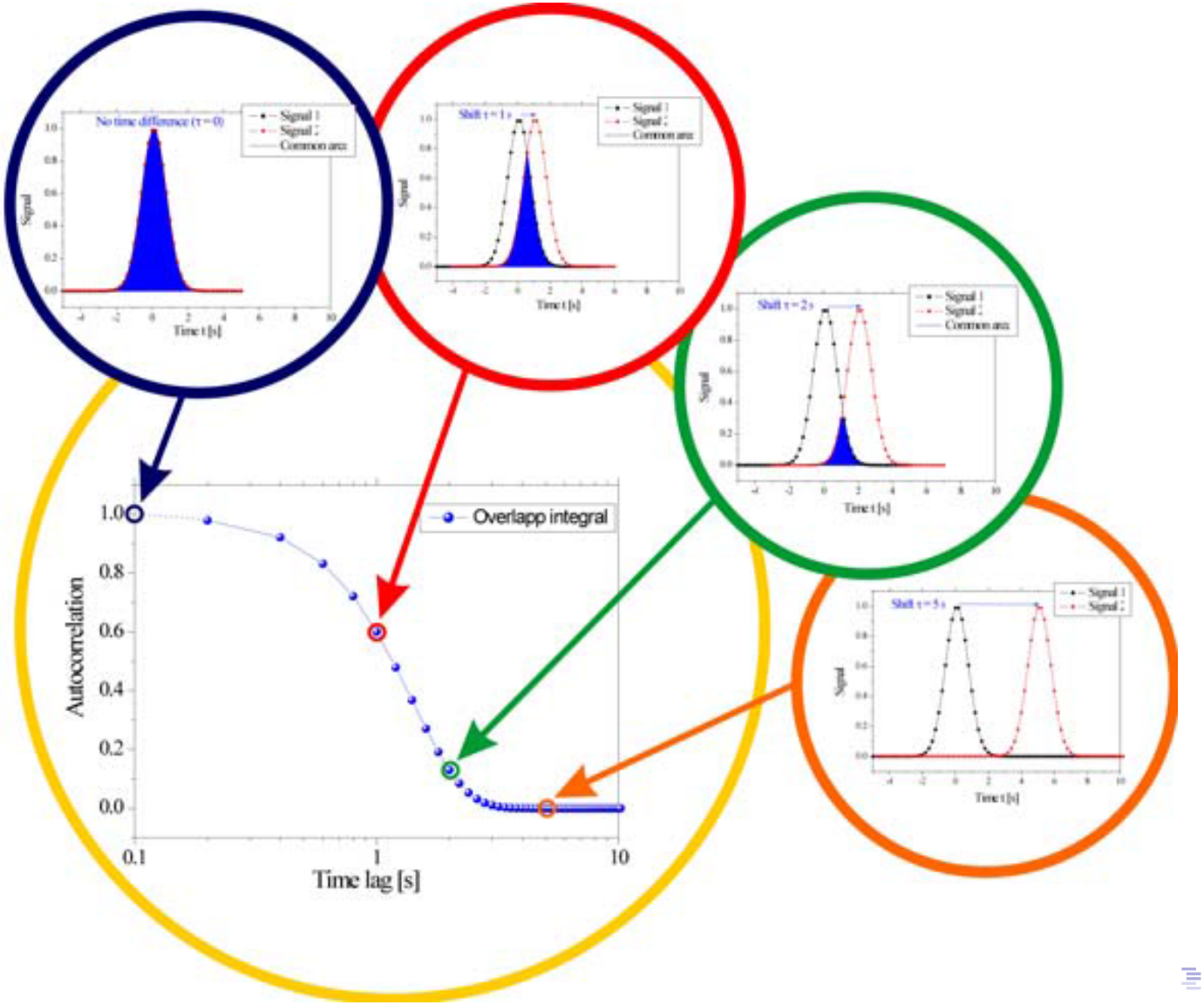






Autocorrelation & FCS

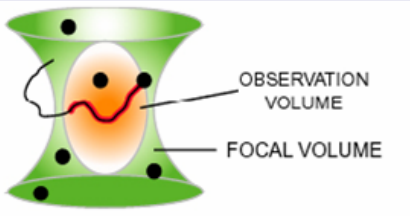
AutoCorrelation Curve: Example



FCS Theory
FCS Schemes

Static detection volume

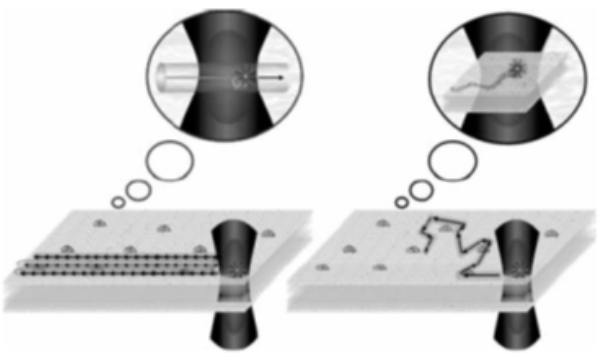
- Auto-correlation function for one freely diffusing species of molecules.



$$G(\tau)=\frac{\langle \delta F(t)\cdot \delta F(t+\tau)\rangle}{\langle F(t)\rangle^2}=\frac{1}{V_{eff}\langle C\rangle}\cdot\frac{1}{1+\tau/\tau_D}\cdot\frac{1}{\sqrt{1+\left(r_o/z_o\right)^2\tau/\tau_D}}$$

$$r_o, z_o: \quad I(r_o,z_o)=I_0 e^{-2}$$

Mobile detection volume



Amediek et al. 2002

$$G(\tau)=\frac{1}{V_{eff}\langle C\rangle}\cdot e^{-\left(\frac{\tau-v}{r_0}\right)^2}\approx\frac{1}{\pi^{3/2}r_0^{\,2}\langle C\rangle}\cdot e^{-\left(\frac{\tau-v}{r_0}\right)^2}$$

Different scanning patterns. Whereas the line scan is analogous to directed flow experiments, the random scan resembles rather 2D diffusion.

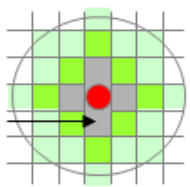
FCS Theory
AutoCorrelation Schemes

Spatial Autocorrelation

from time to space...

$$x(\tau) = \tau \cdot v = \tau \cdot \Delta x / \Delta \tau$$

$$v = \Delta x / \Delta \tau$$



from τ *to* x*:*

$$G(x) = \frac{1}{\pi^{3/2} r_0^2 \langle C \rangle} \cdot e^{-\left(\frac{x}{r_0}\right)^2} = \frac{\langle \delta F(x_0) \cdot \delta F(x_0 + x) \rangle}{\langle F(x_0) \rangle^2}$$

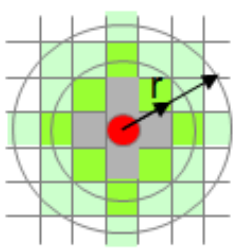
2D Autocorrelation

from $1D$ *to* $2D$ *...*

$$r(\tau) = \tau \cdot v = \tau \cdot \Delta r / \Delta \tau$$

$$v = \Delta r / \Delta \tau$$

$$r^2 = \Delta x^2 + \Delta y^2$$



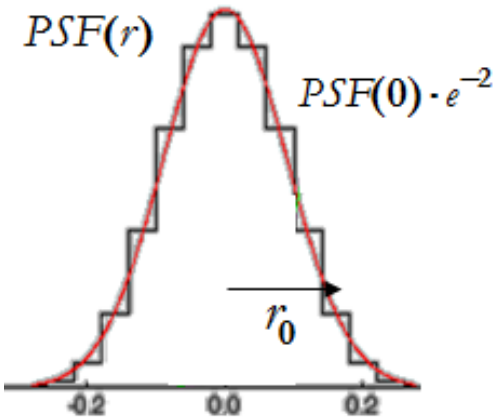
from x *to* r*:*

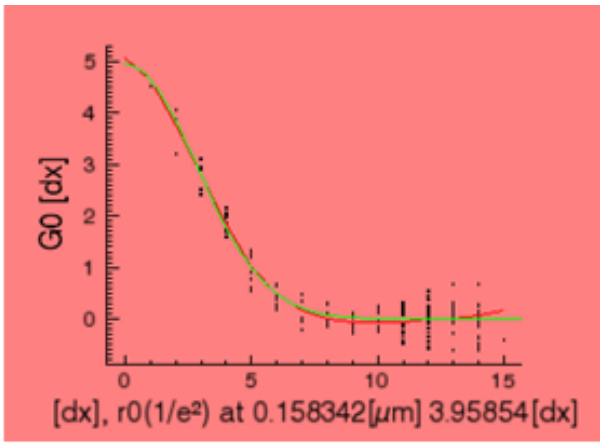
$$G(x) = \frac{\langle \delta F(x_0) \cdot \delta F(x_0 + x) \rangle}{\langle F(x_0) \rangle^2}$$

$$G(r) = \frac{\langle \delta F(r_0) \cdot \delta F(r_0 + r) \rangle}{\langle F(r_0) \rangle^2}$$

FCS Theory

Estimating Concentrations

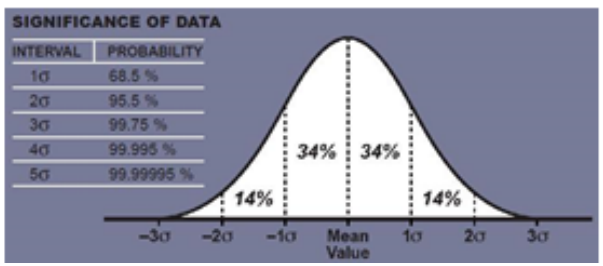




$$PSF(r) = PSF(0) \cdot e^{-\frac{1}{2}\left(\frac{r}{\sigma}\right)^2}$$

$$PSF(r') = PSF(0) \cdot e^{-2}$$

$$at\ r' = 2 \cdot \sigma$$



$$G(r) = \frac{\langle \delta F(r_0) \cdot \delta F(r_0 + r) \rangle}{\langle F(r_0) \rangle^2}$$

$$G(r) = \frac{1}{\pi^{3/2} r_0^{\,2} \langle C \rangle} \cdot e^{-\left(\frac{r}{r_0}\right)^2}$$

$$G(r'') = G(0) \cdot e^{-2}$$

$$at\ r'' = \sqrt{2} \cdot r_0$$

$$\boxed{\sqrt{2} \cdot \sigma = r_0}$$

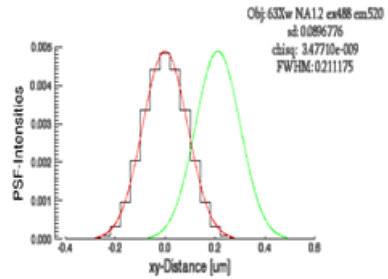
FCS Theory

Estimating Concentrations

$$G(0) = \frac{1}{\langle N \rangle} = \frac{1}{V_{eff} \cdot \langle C \rangle} \quad \Leftrightarrow \quad \langle C \rangle = \frac{1}{V_{eff} \cdot G(0)}$$

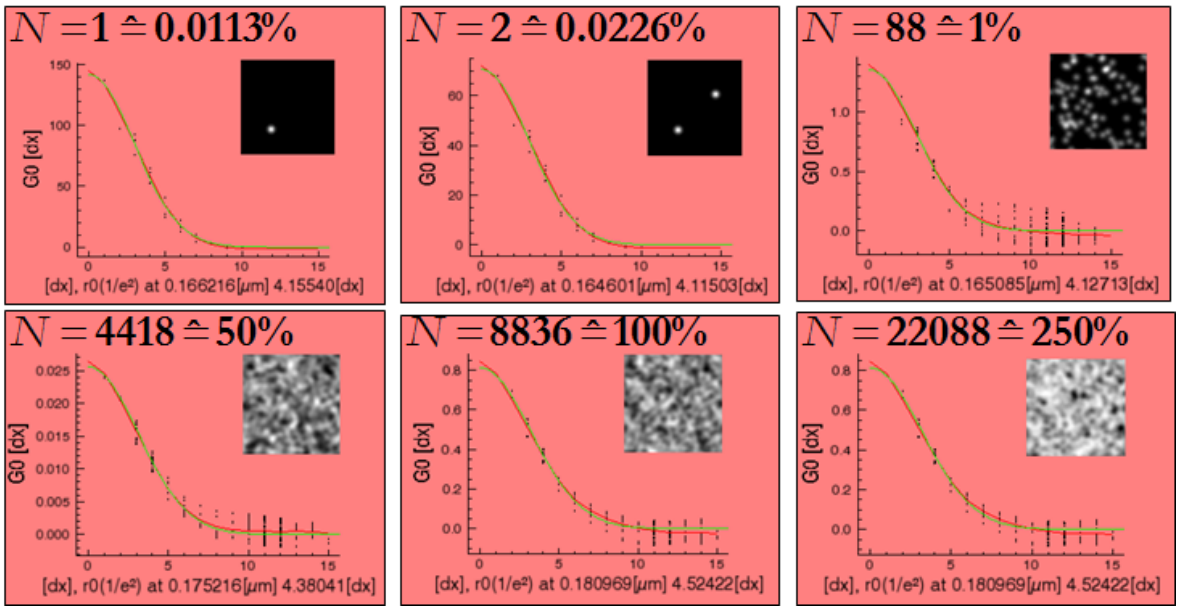
Autocorrelation

- The size of the underlying image structures can be determined by the decay-distance r of $G(r)$



$$G(r) = \frac{\langle \delta F(r_0) \cdot \delta F(r_0 + r) \rangle}{\langle F(r_0) \rangle^2}$$

$$G(r) = \frac{1}{\pi^{3/2} r_0^{\,2} \langle C \rangle} \cdot e^{-\left(\frac{r}{r_0}\right)^2}$$



FCS Equations for Various Physical Processes

I

1D Diffusion

$$G(t) = 1 + \frac{1}{N_p} \frac{1}{\sqrt{(1 + t/\tau_D \kappa^2)}}$$

2D Diffusion

$$G(t) = 1 + \frac{1}{N_p} \frac{1}{(1 + t/\tau_D)}$$

3D Diffusion

$$G(t) = 1 + \frac{1}{N_p} \frac{1}{(1 + t/\tau_D)} \frac{1}{\sqrt{(1 + t/\tau_D \kappa^2)}}$$

FCS Equations for Various Physical Processes II

Multicomponent 3D Diffusion

$$G(t) = 1 + \frac{f_1}{N_p} \frac{1}{(1 + t/\tau_{D1})} \frac{1}{\sqrt{(1 + t/\tau_{D1}\kappa^2)}} + \frac{1 - f_1}{N_p} \frac{1}{(1 + t/\tau_{D2})} \frac{1}{\sqrt{(1 + t/\tau_{D2}\kappa^2)}}$$

3D Diffusion with Directed Flow

$$G(t) = 1 + \frac{1}{N_p} \frac{1}{(1 + t/\tau_D)} \frac{1}{\sqrt{(1 + t/\tau_D\kappa^2)}} \times e^{\frac{-t/\tau_v}{1 + t/\tau_D}}$$

Chemical kinetics where binding produces a signal otherwise not present (e.g. FRET)

$$G(t) = 1 + \frac{1}{N_p} e^{-t/\tau_B}$$

where $\tau_B = (k_{on} + k_{off})^{-1}$

FCS Equations for Various Physical Processes III

3D Diffusion with Intersystem Crossing

$$G(t) = 1 + \left(1 + \frac{T}{(1-T)} e^{-t/\tau_T}\right) \frac{1}{N_M} \frac{1}{(1 + t/\tau_D)} \frac{1}{\sqrt{(1 + t/\tau_D K^2)}}$$

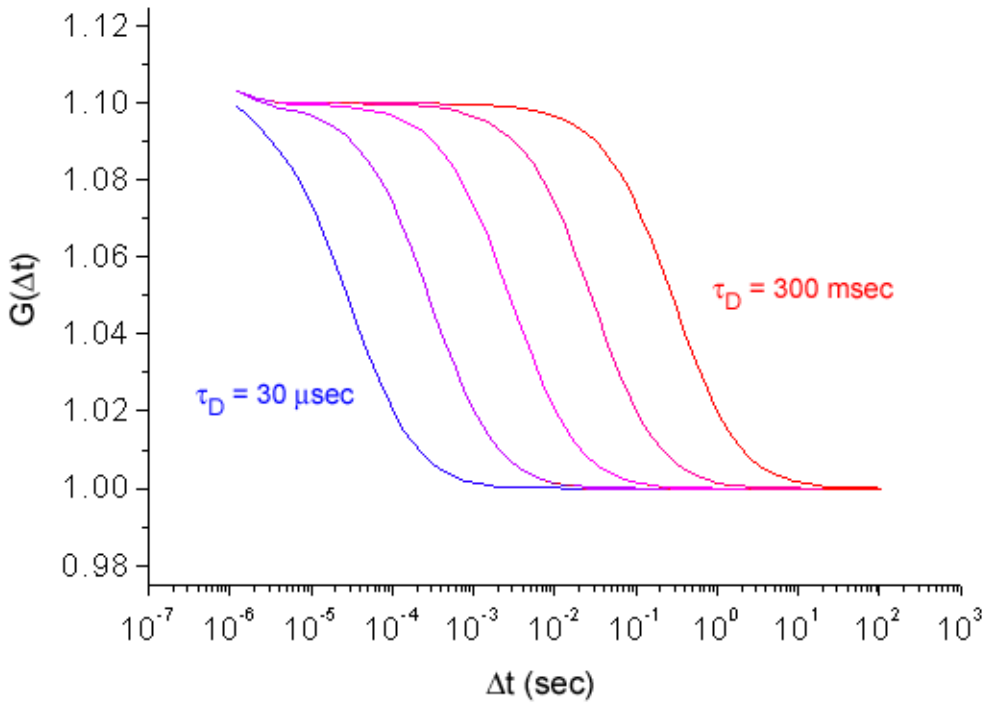
3D Diffusion with System Correction

$$G(t) = 1 + (1 + A e^{-t/\tau_C}) \frac{1}{N_P} \frac{1}{(1 + t/\tau_D)} \frac{1}{\sqrt{(1 + t/\tau_D K^2)}}$$

FCS & Autocorrelation

Extracting Parameters from Correlation Time

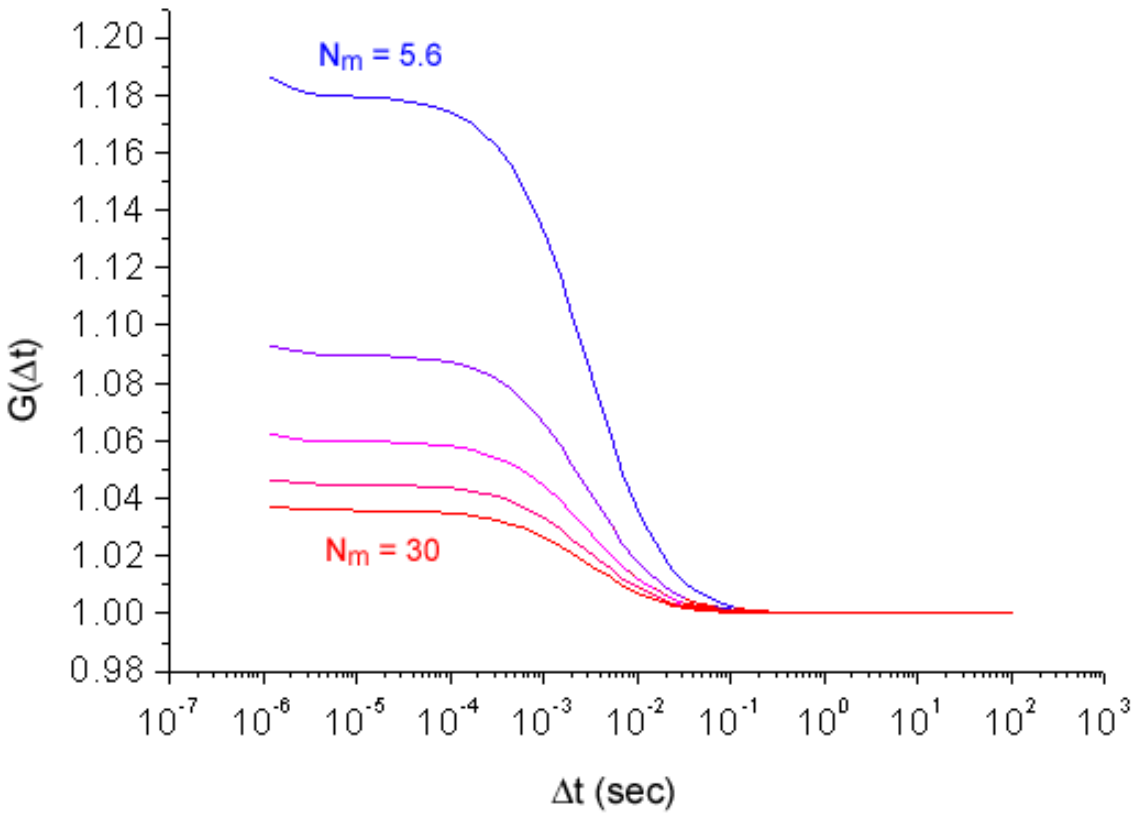
- The rate of decay of the correlation over time, the so-called correlation time, τ_D, describes the physical phenomenon, such as diffusion, that is causing the correlation. The longer the correlation persists, the slower the diffusion. Correlation persists longer for slowly diffusing particles and decays quickly for rapidly diffusing particles.



FCS & Autocorrelation

Extracting Parameters from the Intercept

- The intercept of the correlation function is inversely related to the number of fluorescent particles detected. As particle number decreases, the intercept increases.



Colocalization

Colocalization

- *Formulation*: Overlap full or partial of the physical distribution of molecular populations within a three dimensional volume.

Colocalization

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- *Formulation*: Overlap full or partial of the physical distribution of molecular populations within a three dimensional volume.

Problems

- Existing methods usually are considered ambiguous and inconsistent.
- Overlapping vs Colocalization.

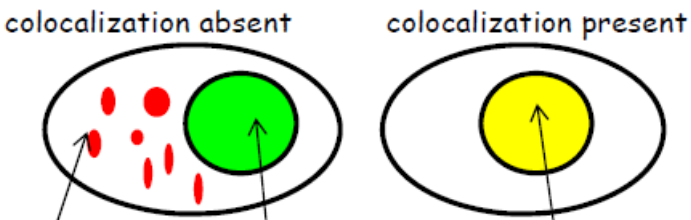
Colocalization

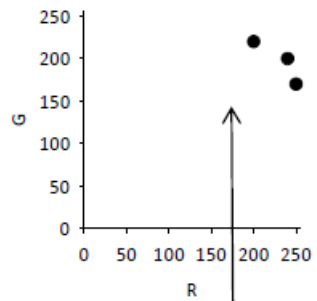
Main Question

- *Formulation*: Are 2 or more objects located in the same structure in a 3D volume?. Considering:
  - fluorophores.
  - Distribution of fluorophores in nm range.
  - Resolution of the microscope in hundreds of nm.

Colocalization
Definitions

Ordinary definition	Scientific definition
if the signals of the two fluorescent labels are at the same place	if the **correlation** between the distributions of the two fluorescent labels is larger than expected for random distributions
E.g. in a sample labeled by green and red fluorophores yellow implies colocalization, but does it?	





RGB: 250,170,0

RGB: 0,255,0 RGB: 255,255,0

RGB: 240,200,0

RGB: 200,220,0

RGB (red, green, blue) code: 255,0,0

Although based on the presence of yellowish color colocalization is assumed, analysis of correlation does not support this assumption.

Colocalization

Issues

- *Colocalization measurement depends on*:
  - Understanding the 3D organization of the structures of interest.
  - Labeling techniques.
  - Dimensions defined by the optical system.
  - Imaging procedure.
  - Processing and Analysis.

Colocalization
Limitations of Optical Microscopy

Motivation

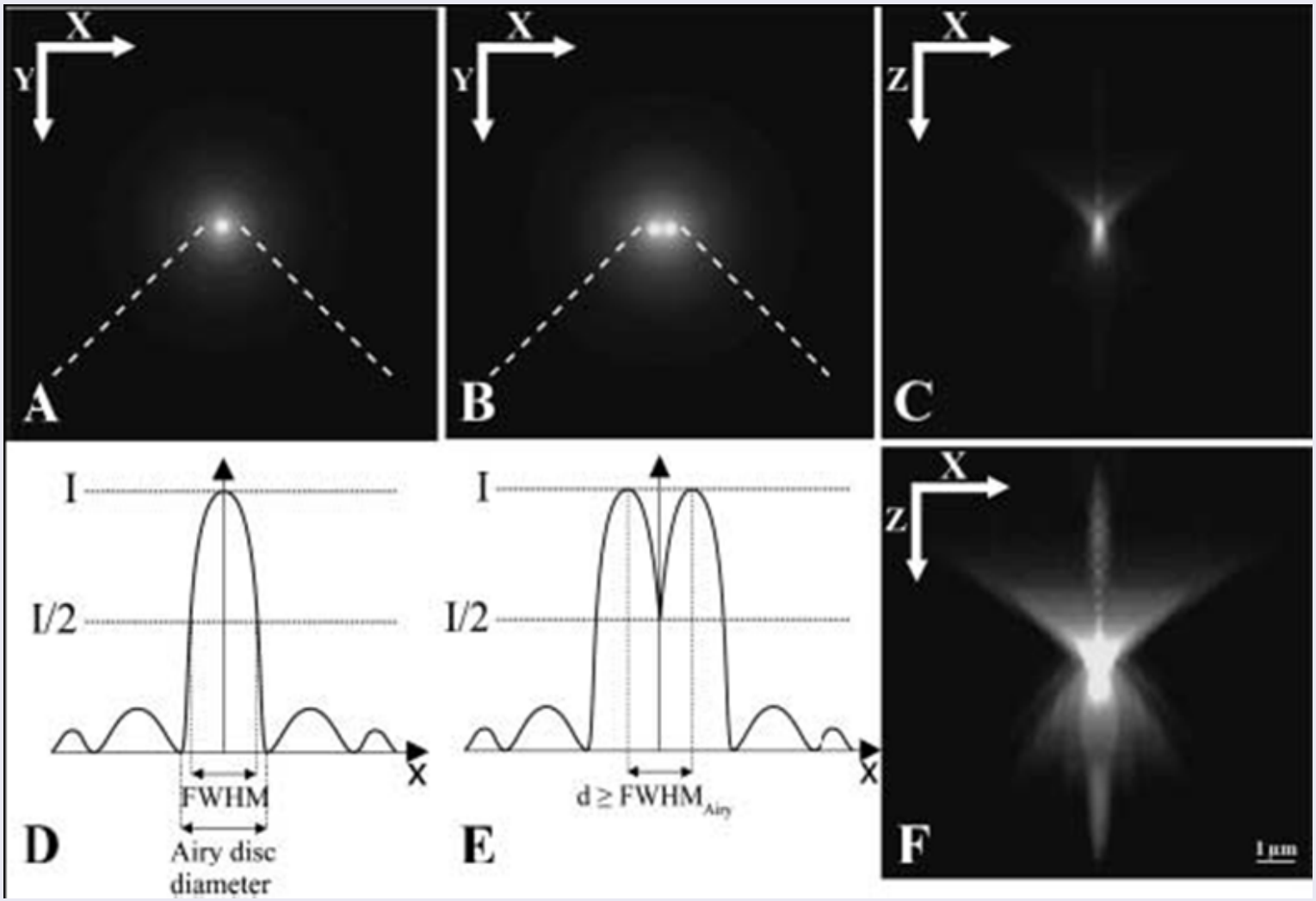
- Dual nature of light.
  - Wave.
  - Particle.

Partial light collection by the objective lens

- Quantified by the Numerical Aperture (NA):
  - Related to the angle of light collection provided by the object.
  - Determines the ability to distinguish two adjacent point light sources.
  - *Each point of light exiting the lens can be regarded as a single light source emitting a circular wave front (Huygens principle).*

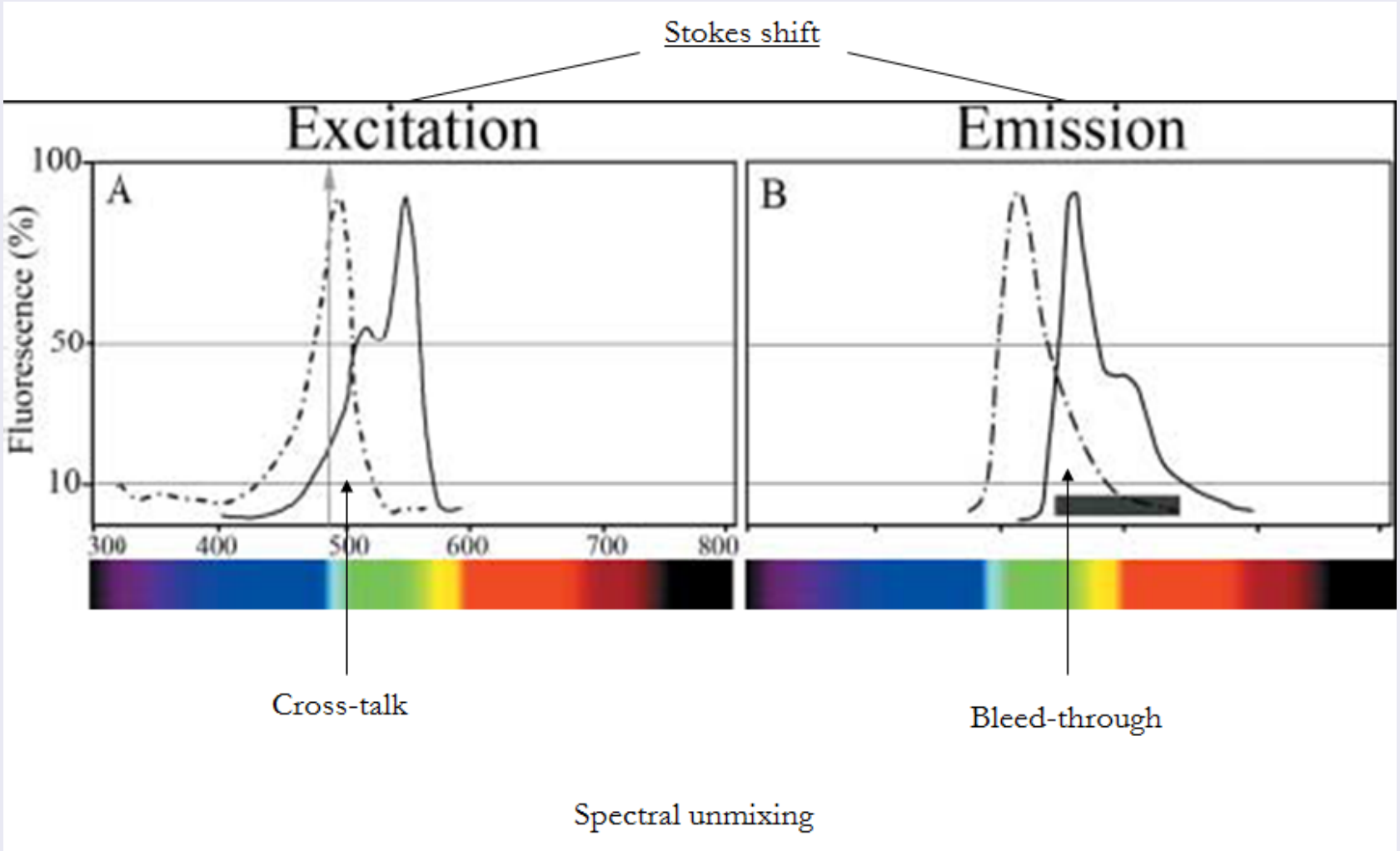
Colocalization

Issues: I



Colocalization

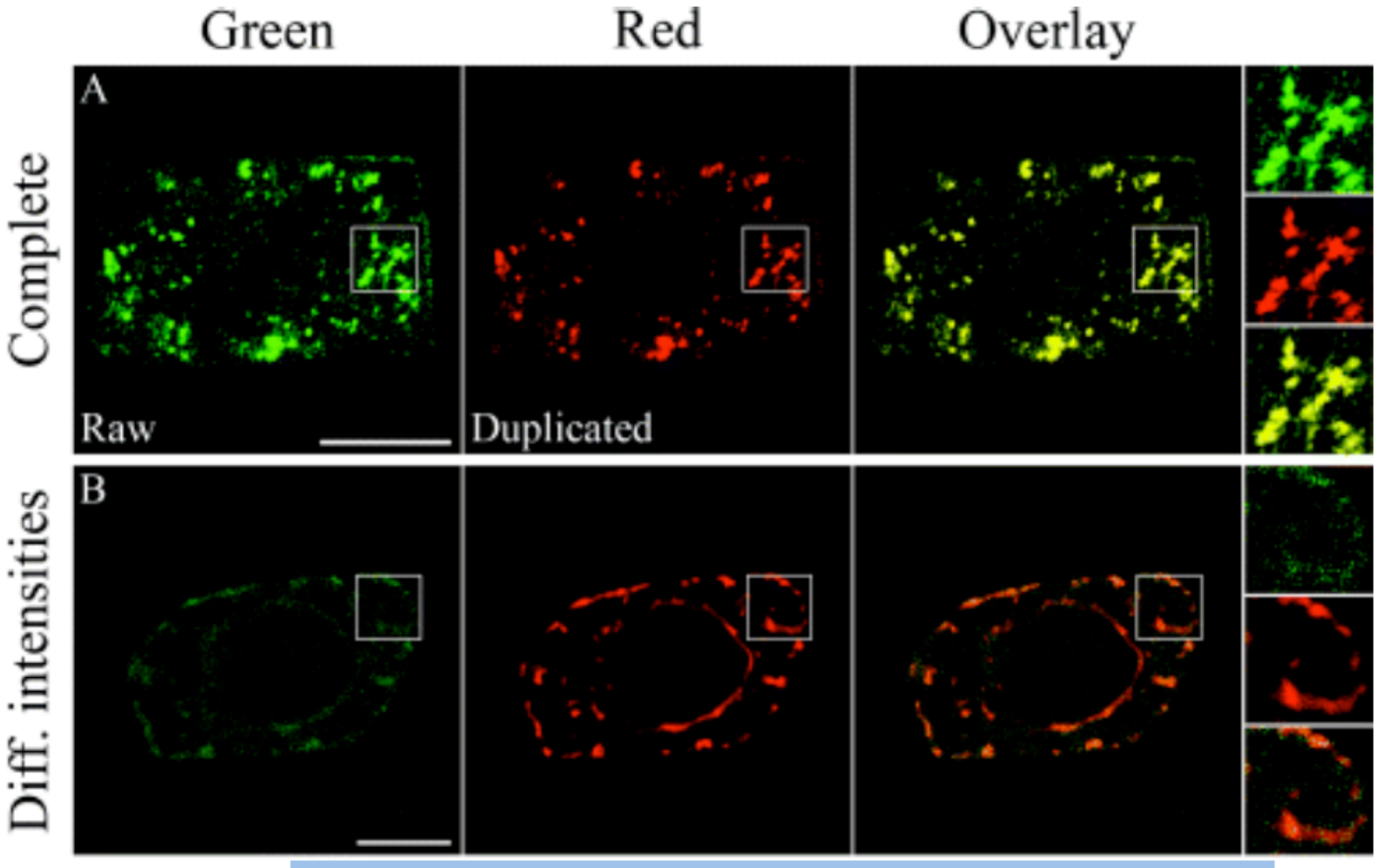
Issues: II



Spectral unmixing

Colocalization

Visual detection of colocalization



Is green + red always = yellow?

Colocalization
Methods

ICCB (intensity correlation coefficient-based)	Object-based analysis
• Pearson correlation coefficient • Manders coefficient • Costes' method • van Steensel's method • Li's method	Difficult to classify, not that wide-spread, usually involves relatively complex image analysis

A guided tour into subcellular colocalization analysis in light microscopy
S. Bolte, F.P. Cordelières
J. Microscopy, 224: 213-232 (2006)

ICCB Methods

Pearson Correlation Coefficient *PC*

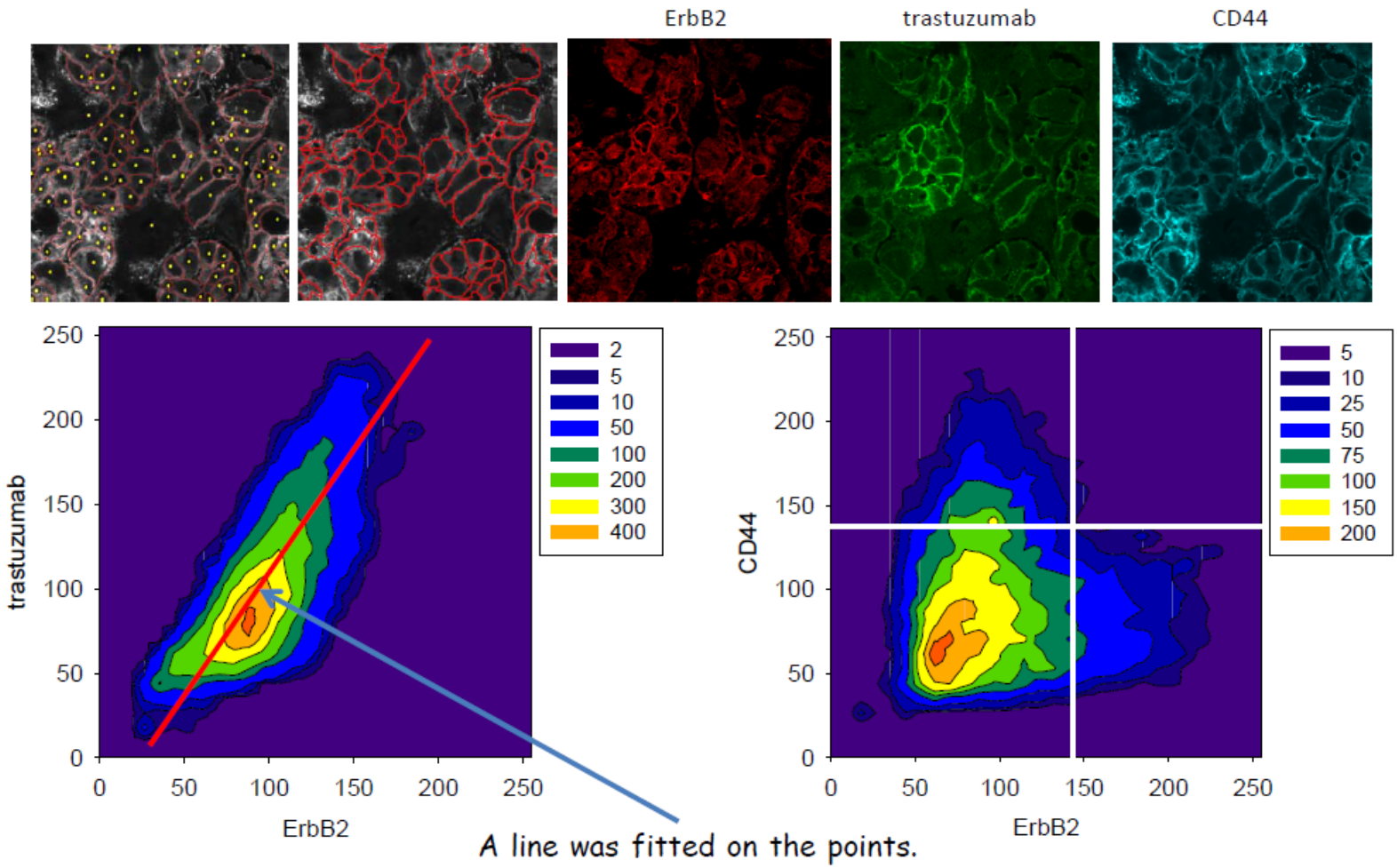
- Measure of What percentage of variability in one channel is caused by the variability in the other channel (Squaring Rr and making it a percentage).
- It measures the LINEAR relationship between the variables, i.e. how well a STRAIGHT LINE can be fitted to the x-y points

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

- Interpretation:
  - $R_r = 1$: perfect colocalization/correlation.
  - $R_r = 0$: random (no) colocalization.
  - $R_r = -1$: perfect exclusion/anti correlation.

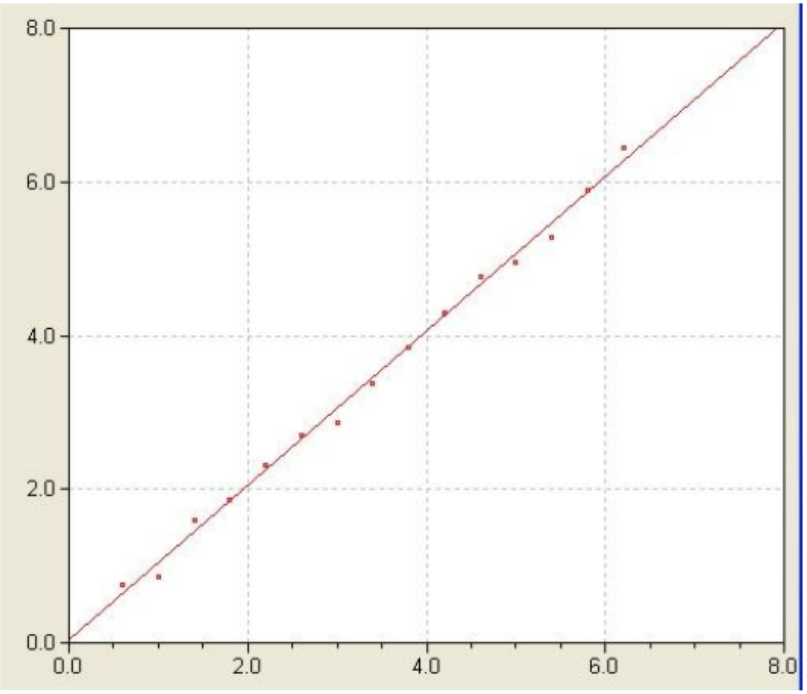
ICCB Methods

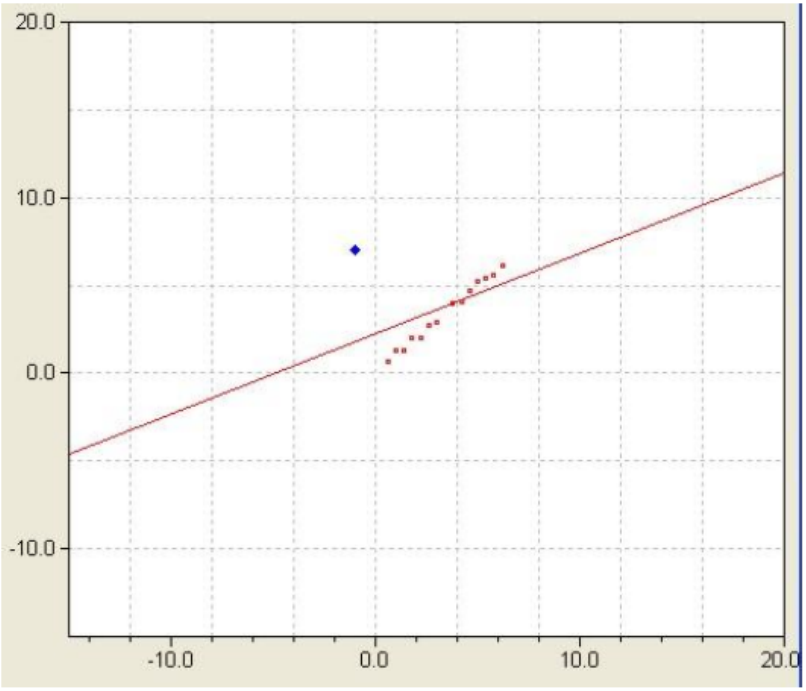
PC: Example



ICCB Methods

PC: Outliers significantly deteriorates the correlation





ICCB Methods

PC: Features

Advantages

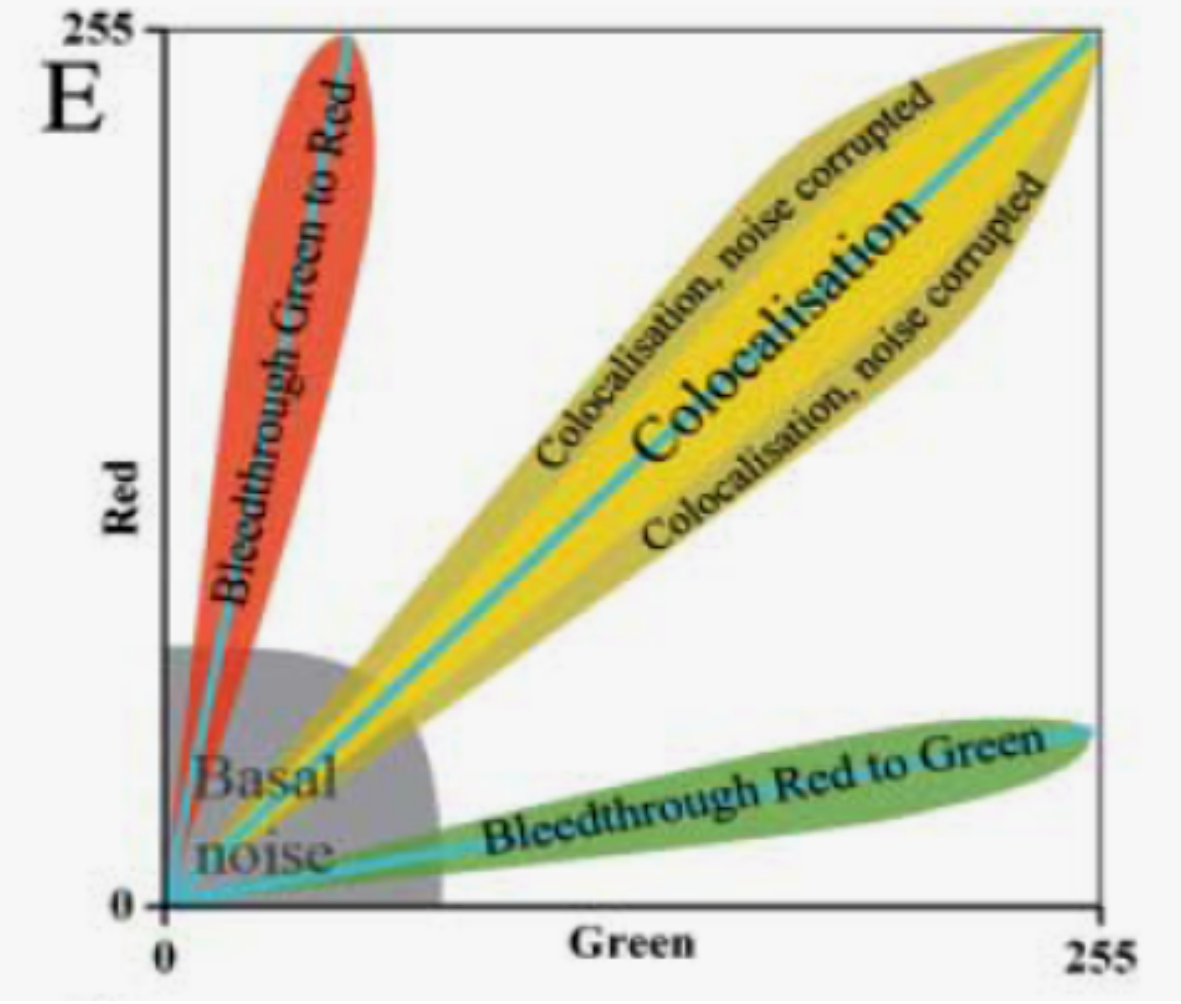
- Not sensitive to the intensity of a background (e.g. a constant value).
- Not sensitive to the intensity of the overlapping pixels.

Disadvantages

- Difficult to interpret.
- Affected by the addition "presence" of non-colocalizing signals
- No information about the individual channels
- Affected by noise.

ICCB Methods

PC: small bleed-through Problems



ICCB Methods

The overlap coefficient

- Same as the Pearson's but the mean is not subtracted.

$$R = \frac{\sum_i Ch1_i \cdot Ch2_i}{\sqrt{\sum_i (Ch1_i)^2 \cdot \sum_i (Ch2_i)^2}}$$

- Interpretation:
  - $R = 1$: perfect colocalization/correlation.
  - $R = 0$: random (no) colocalization.
  - R: pixels (objects) overlap.
- Advantages:
  - Easier to interpret
  - Not sensitive to the intensity of the overlapping pixels.
- Disadvantages:
  - Sensitive to background
  - No information about the individual channels
  - Affected by noise.

ICCB Methods

The k overlap coefficients

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

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

- Advantages:
  - The 2 channels are analyzed separately
  - Addition of a not colocalized signal will affect only one of the channels.
- Disadvantages:
  - The parameters scale with the signal increase in the other channel.

ICCB Methods

Manders (original) coefficients

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

- m_1 comes from k_1 by replacing $Ch2_i$ with 0 if $Ch2_i = 0$ and with 1 otherwise. (Similarly for m_2)
- Alternatively: $Ch1_{i,coloc} = Ch1_i$ if $Ch2_i > 0$
- Values: 0 to 1; m_1=1 and m_2=0.4 for a dye pair means that 100% of Ch1 pixel intensities colocalize with Ch2, but only 40% of Ch2 pixel intensities colocalize with Ch1

- Advantages:
  - Solves the previous scaling problem.
- Disadvantages:
  - The parameters scale with the signal increase in the other channel.

ICCB Methods

Manders (tresholded) coefficients

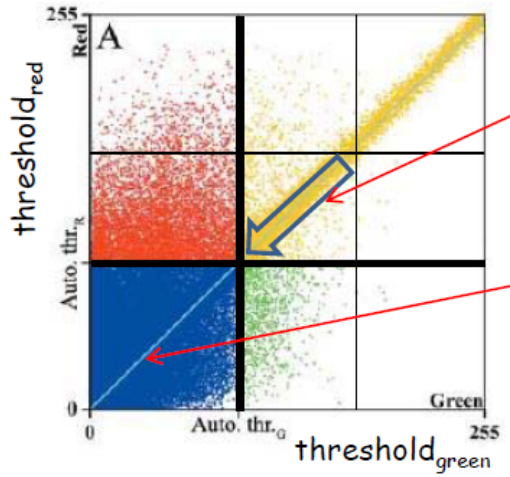
$$M_1 = \frac{\sum_i Ch1_{i,coloc}}{\sum_i Ch1_i} \qquad M_2 = \frac{\sum_i Ch2_{i,coloc}}{\sum_i Ch2_i}$$

- $Ch1_{i,coloc} = Ch1_i$ if $Ch2_i >$ Treshold
- Values: 0 to 1; $m_1=1$ and $m_2=0.4$ for a dye pair means that 100% of Ch1 pixel intensities colocalize with Ch2, but only 40% of Ch2 pixel intensities colocalize with Ch1

- Advantages:
  - Less sensitive to background problems.

ICCB Methods

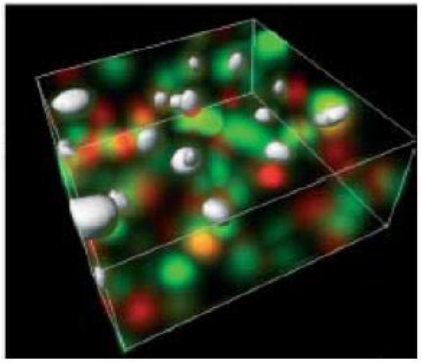
Costes' method

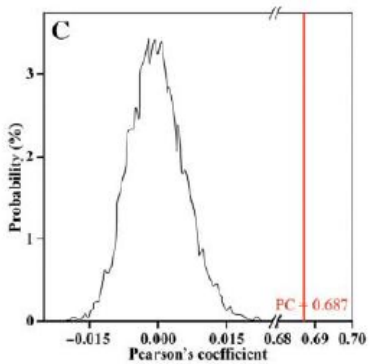


- The threshold is decreased until the correlation coefficient calculated for the under-threshold values (blue area) is zero.
- The yellow area corresponds to pixels exhibiting cololcalization.

$$I_{RED} = a\, I_{GREEN} + b$$

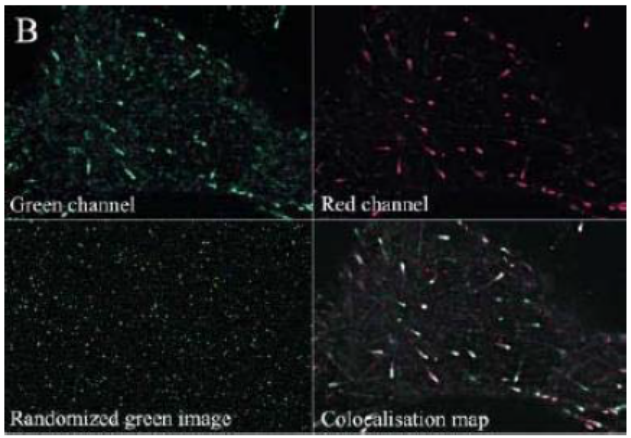
$$thr_{RED} = a\, thr_{GREEN} + b$$





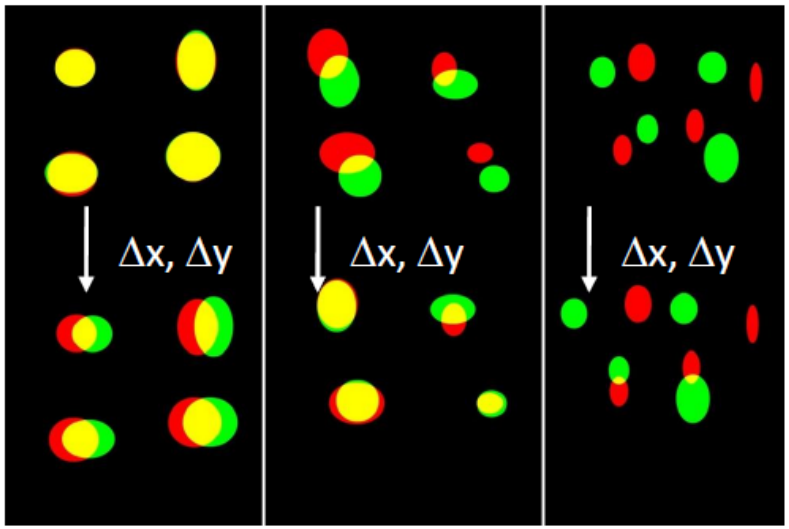
Determining significance of correlation:

- The pixels of the two images are randomly reshuffled, and the correlation coefficient is determined for the resultant images.
- The above procedure is repeated several hundred-times yielding the distribution of r for random images.
- If the correlation coefficient for the original images is outside the 95% confidence interval, the correlation is significant.



ICCB Methods

van Steensel's method

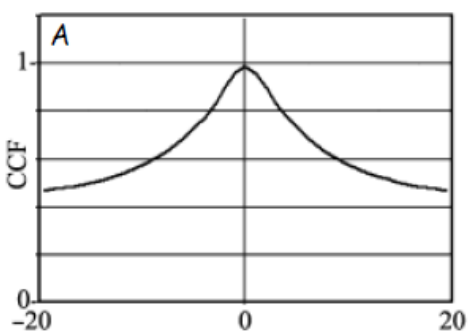


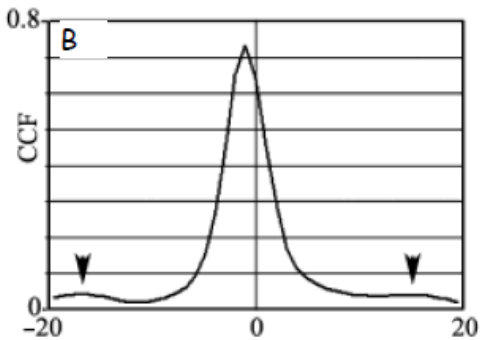
A. complete colocalization

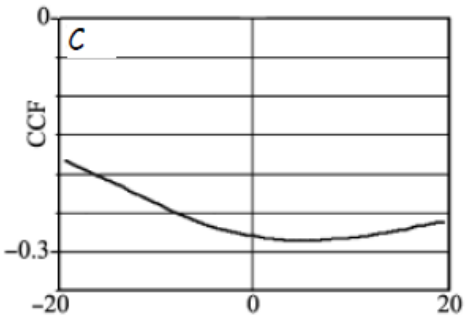
B. partial colocalization

C. exclusion

- The green image is shifted in x and y directions relative to the red one.
- The correlation coefficient (CCR – cross-correlation coefficient) is determined after each step.
- In the case of colocalization correlation (the area of the yellow regions) is expected to decrease.

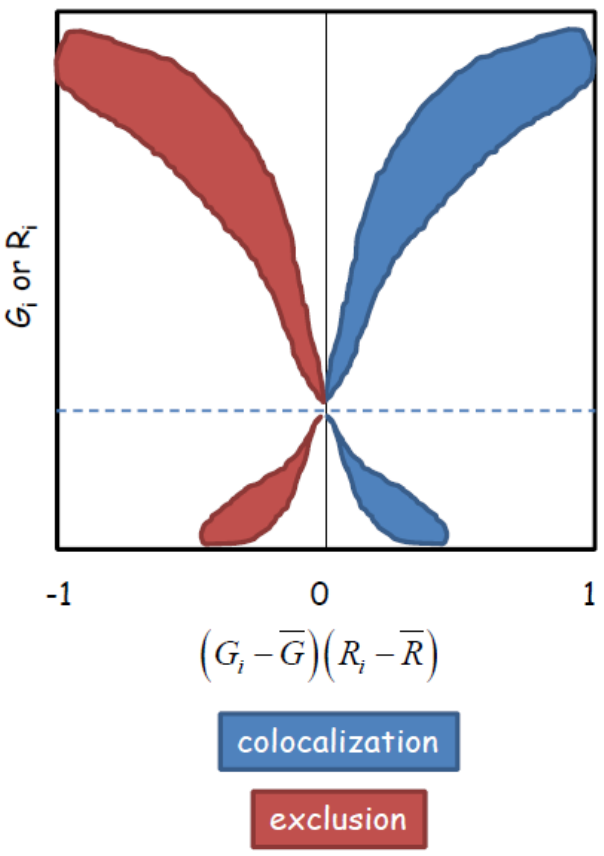






ICCB Methods

Li's method



$$\sum_i (G_i - \overline{G}) = 0$$

$$\sum_i (R_i - \overline{R}) = 0$$

If G_i deviates from $\overline{G}$ where R_i is different from $\overline{R}$ (correlation), then the value of $\sum_i (G_i - \overline{G})(R_i - \overline{R})$ will be positive.

- It provides an easy-to-interpret graphical representation of colocalization.
- Quantitative evaluation: ICQ - intensity correlation quotient (the fraction of pixels in the positive region of the horizontal axis).

ICCB Methods

Rules for methods

Coefficient	Values indicating colocalization	Values indicating absence of colocalization
Pearson's correlation coefficient (R_r)	From 0.5 to 1.0	From –1.0 to 0.5
Overlap coefficient according to Manders (R)	From 0.6 to 1.0	From 0 to 0.6
Overlap coefficients k1 and k2	Any close values, like 0.5 and 0.6 or 0.8 and 0.9	Any distant values, like 0.5 and 0.9 or 0.2 and 0.7
Colocalization coefficients m1 and m2	More than 0.5	Less than 0.5
Colocalization coefficients M1 and M2	More than 0.5	Less than 0.5

ROIs based Methods

- The ICCB numerical indicators suffer from being based on the composite nature of the images, which is actually a mosaic of structures (the actual regions of interest) and, although minimized, some background.

Advantages

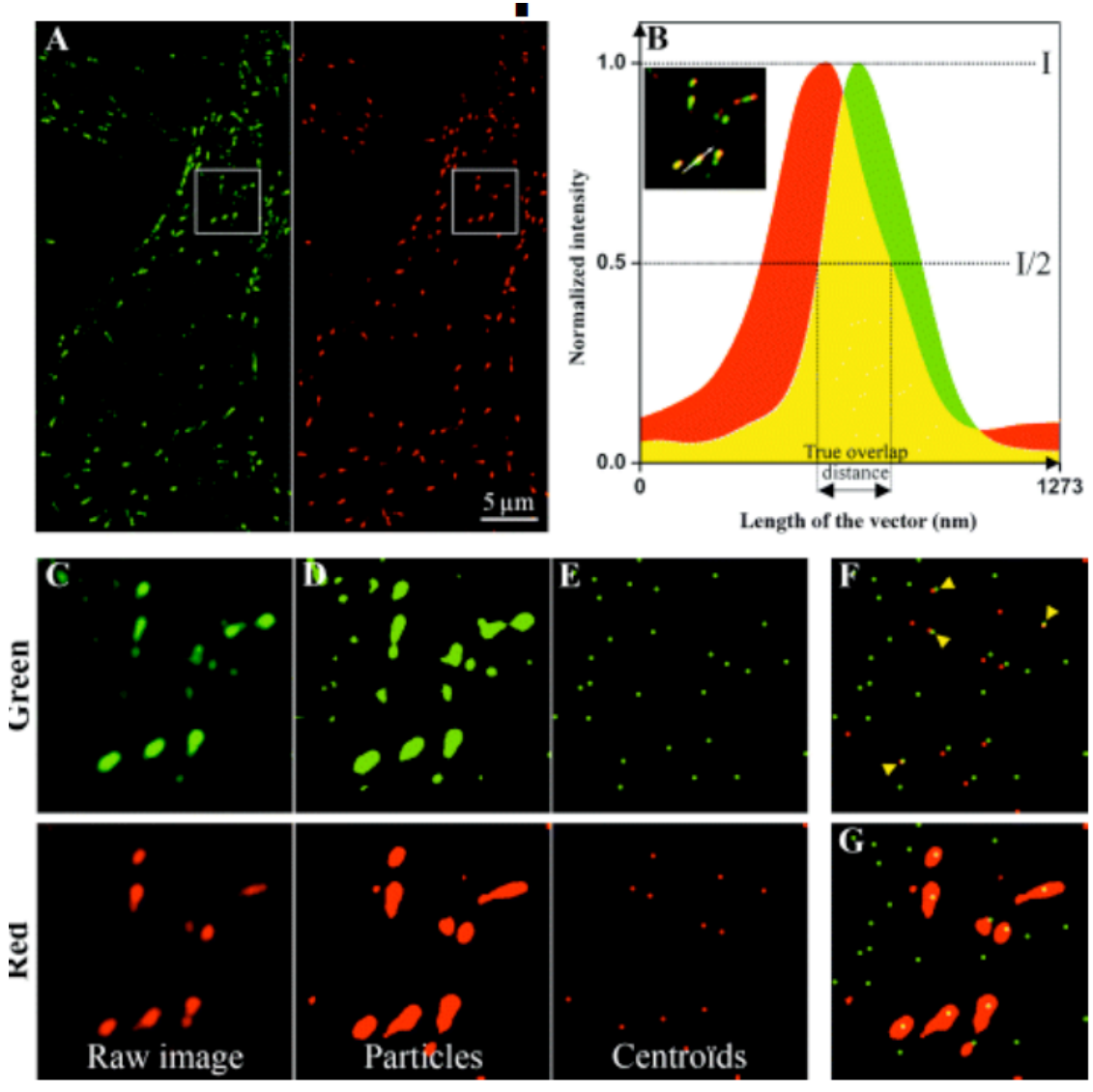
- Less dependent on intensities (diffuse labeling)
- Can be automated.

Disadvantages

- Segmentation needed (difficult)
- Doesn't work for diffuse labeling.

ROIs based Methods

Example



Thanks

Questions?