MRC | Laboratory of Molecular Biology

Image Analysis Tools

Biophysical Techniques Lecture Series 2018

Jérôme Boulanger
Light Microscopy

MRC-LMB
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Outline

1. Image formation
2. Signal-to-noise enhancement
3. Deconvolution with Huygens
4. Visualization & analysis with Imaris
5. Access & training

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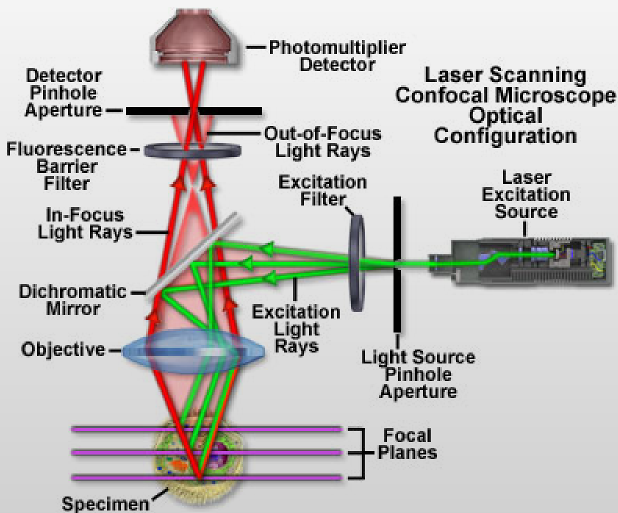
Image formation in fluoresence microscopy



Rayleigh criterion

- The image of a point through a circular aperture is an Airy function

$$I(\theta) = \left(2\frac{J_1(x)}{x}\right)^2$$

with $x = ka\sin\theta = k\mathrm{NA}r$, $k = 2\pi/\lambda$, a the aperture radius.

- Rayleigh criterion: Two points are separables if the maximum corresponds to the first minima of the other spot ($x \approx 3.83$):

$$\Delta_{xy} = \frac{0.61\lambda}{\mathrm{NA}}$$

Ex: $\mathrm{NA} = 1.42$, $\lambda = 520nm$, $\Delta = 208.68\mathrm{nm}$

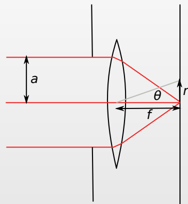


Fig. 1: Diffration by a circular aperture

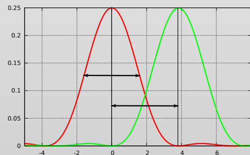


Fig. 2: FWHM and Rayleigh

Rayleigh criterion

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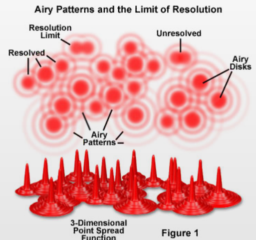

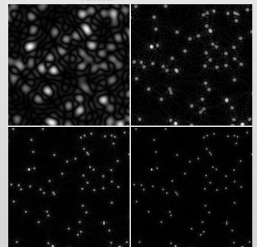

Fig. 1: Simulation of a field of point source.

Optical sectioning

- Slice in 3D by defocusing the sample
- Depth of field of the microscope:

$$\Delta_z \approx \frac{\lambda n}{\mathrm{NA}^2}$$

  with n the index of the medium between the coverslip and the objective
- Many techniques exists to achieve optical sectioning (Confocal, TIRF, Structured Illumination)

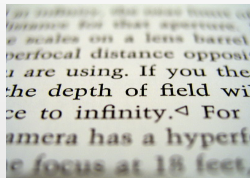

Fig. 2: Depth of field in photography

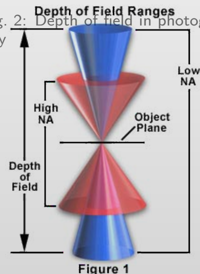

Fig. 3: Depth of field vs numerical aperture

Point Spread Function

Definition

- Image of a point source
- Impulse response of the optical system

Properties

- Characterize the aberration of the microscope
- The image of an object is the convolution of this object by the point spread function of the microscope

Gibson and Lanni theoretical model

- By computing the difference of optical path:

$$h(r,z) = \left| \frac{C}{d} \int_0^1 J_0\left(\frac{ka\rho r}{z}\right) e^{\imath k \delta(\rho)} \rho \, d\rho \right|^2$$

  with k wave vector $(2\pi/\lambda)$,$\delta(\rho)$ the optical path difference, $a = d\,\mathrm{NA}/\sqrt{\mathrm{M}^2 - \mathrm{NA}^2}$, NA the numerical aperture et M the magnification.

- Need to know all the parameters of the system
- May be able to adapt the model within the sample (spherical aberrations)

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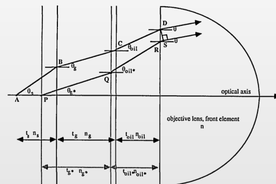
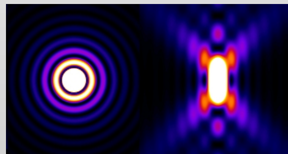


Fig. 1. Paths of rays from an on-axis point source to the front element of a high-magnification oil-immersion objective lens. *ABCD* is a ray from a point source in a nondesign system that enters the front lens element at an angle θ when the object lies at a depth t_s in a medium of refractive index n_s, the coverslip has a thickness t_g and refractive index n_g, and the immersion oil has a thickness t_{oil} and refractive index n_{oil}. *PQRS* is the corresponding ray in the design system and enters the front lens element at an angle θ. In the design system the point-source object is located immediately below the coverslip, the coverslip has a thickness t_{g*} and refractive index n_{g*}, and the oil-immersion layer has a thickness t_{oil*} and refractive index n_{oil*}.



Experimental measure

- A measure of the impulse response can be obtained by acquiring the image of an object smaller than the resolution
- We can use fluorescent beads or gold particle mounted in a medium similar to the one used to image the sample.

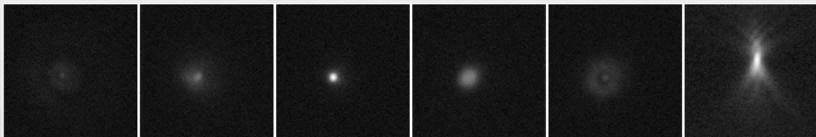


Fig. 4: Axial and lateral view of a fluorescent bead (100nm) using a widefield microscope.

- Drawbacks
  - Noisy measures
  - Need to have exactly the same condition than the sample (pixel size, binning, wavelength, mounting medium,...)
- Advantage:
  - Can capture complex aberrations due to the imperfection of the objective (but better hope the objective is in good state.)

Linear time invariant system

- The output of a **linear time invariant** system is the convolution of the input by the impulse response of the system

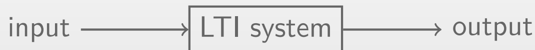


Fig. 5: Linear time invariant system

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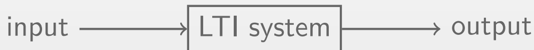

Fig. 5: Linear time invariant system

- If we approximate the microscope by a LTI, then the acquired image is the convolution of the PSF and the sample.

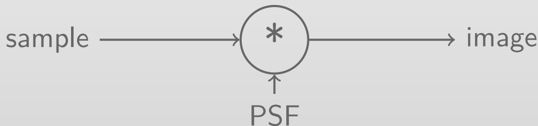

Fig. 6: The microscope approximated by a LTI

Linear time invariant system

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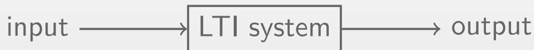

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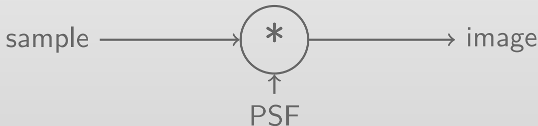


Fig. 6: The microscope approximated by a LTI

- In practice a microscope is not LTI (cf spherical aberrations)

Convolution

- **Linear combination** of intensity values for neighbours

$$I[n] = a \cdot I[n-1] + b \cdot I[n] + c \cdot I[n+1]$$

- Coefficients $[a, b, c]$ define an array h called **filter**:

$$I[n] = h[0] \cdot I[n-1] + h[1] \cdot I[n] + h[2] \cdot I[n+1]$$

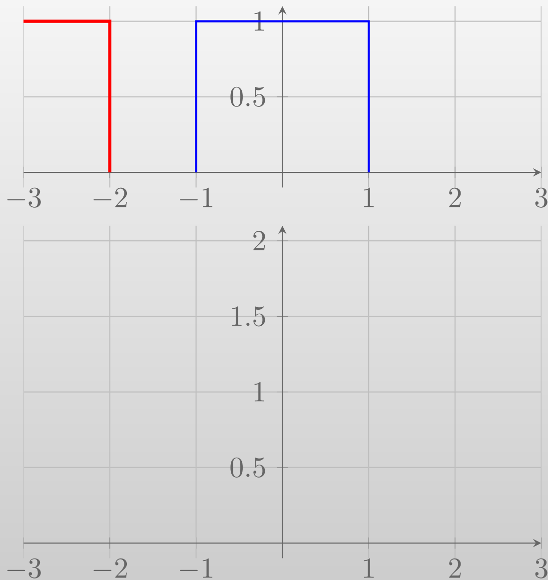
- This operation is called a **convolution** and we can denote $h * I$ the convolution of I by h.

123	121	100	120	122	115	138	100	120	109	117	113	128
139	117	123	130	95	103	103	102	112	113	121	104	108
134	118	157	141	110	125	102	106	100	134	134	139	113
112	134	174	130	136	128	152	109	105	123	118	103	110
143	132	157	159	170	189	159	136	127	125	103	105	105
105	130	170	208	184	220	170	144	151	146	144	156	123
112	130	124	230	191	182	167	174	160	181	162	170	114
118	95	120	117	166	151	148	143	136	156	116	135	137
121	123	112	116	102	134	127	123	122	117	111	132	108
98	101	121	135	130	110	144	101	102	99	110	121	116
96	111	140	117	99	106	121	93	98	93	98	118	107
102	115	115	80	98	101	93	117	114	86	84	83	96
103	94	79	120	83	112	83	103	95	76	91	111	81

Fig. 7: Convolution by a 3x3 filter

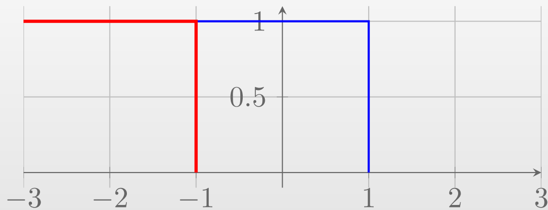
1D Example

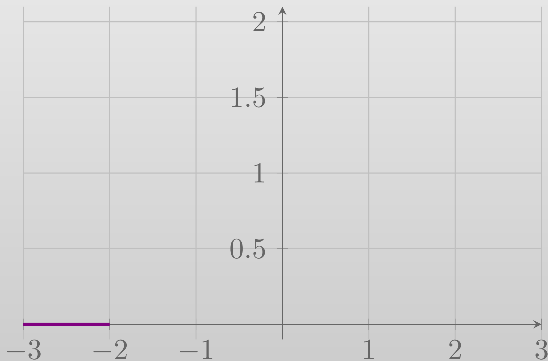
Convolutions of two rectangular functions



1D Example

Convolutions of two rectangular functions





1D Example

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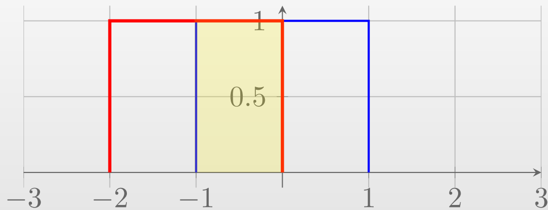

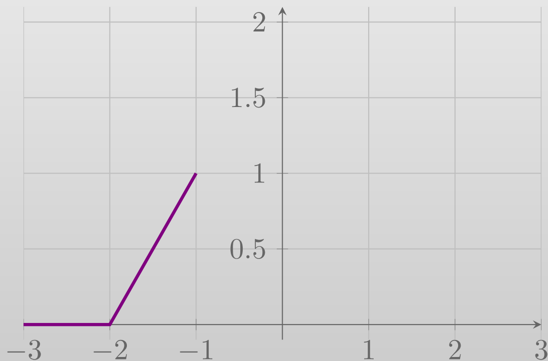

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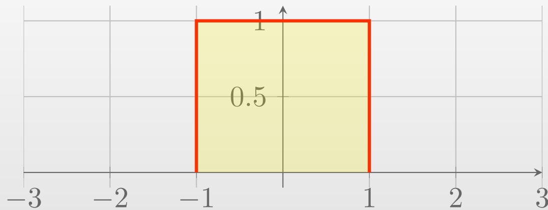

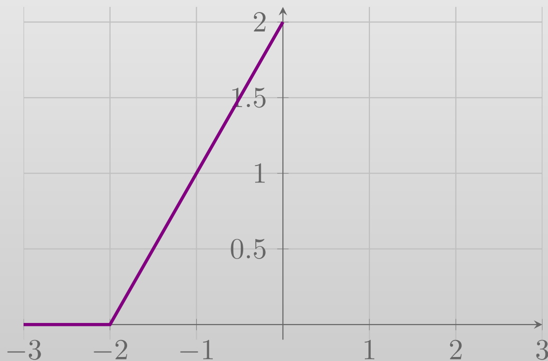

1D Example

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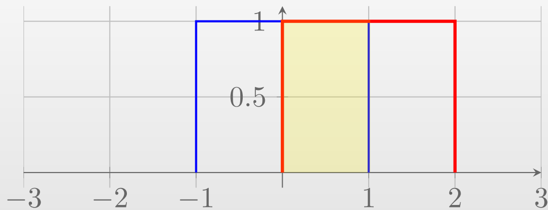

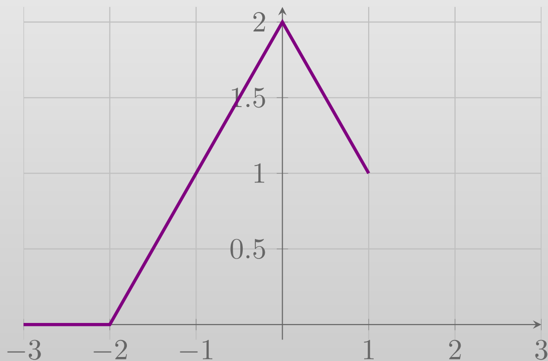

1D Example

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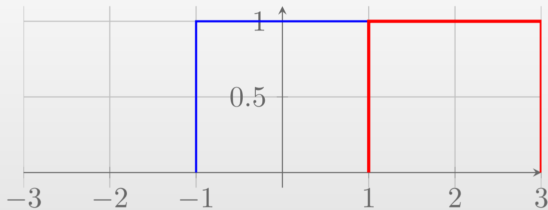
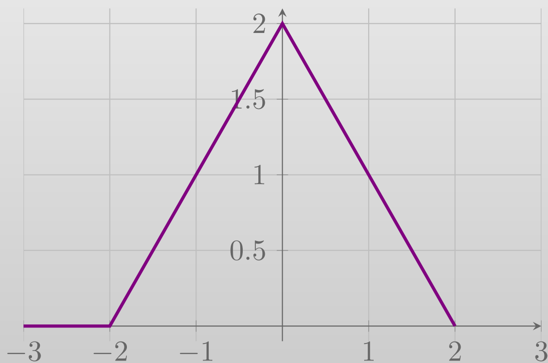
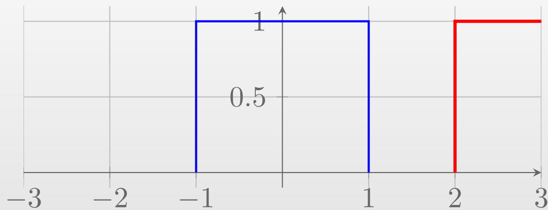
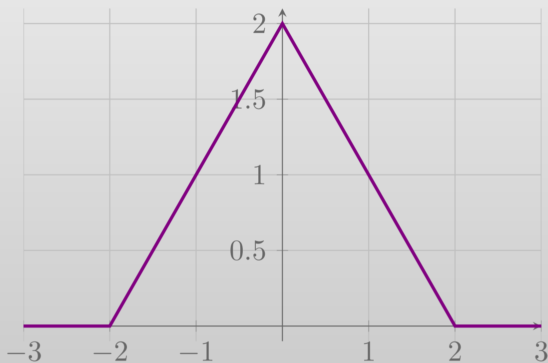




1D Example

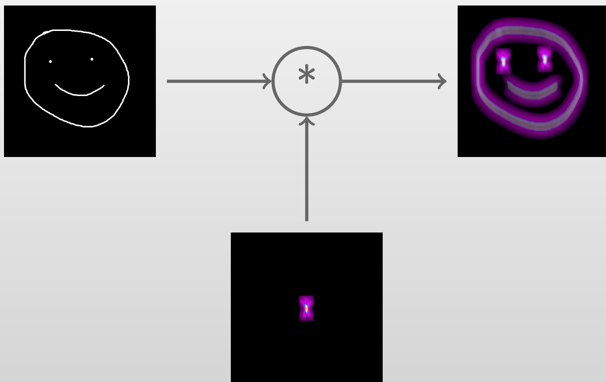
Convolutions of two rectangular functions





Thick brush

- The convolution can be seen a brush in used for painting



Decomposition in sine waves

- Signals can be decomposed into sine waves
- Example of a square wave:

$$f(x) = \sum_{n=1}^{\infty} \frac{1}{n} \sin nx = 1 + \sin(x) + \frac{1}{3}\sin(3x) + \frac{1}{5}\sin(5x) + \cdots$$

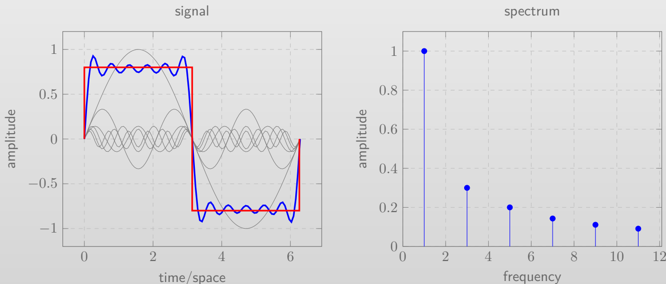


Fig. 8: Rectangle function decomposed into a series of sine function.

Frequency domain

- Representation as a sum of sine and cosine at different frequencies using the **Fourier transform**
- For discret signal we can define a **Discret Fourier Transform**.
- The **Fast Fourier Transform** (FFT) algorithm allows to speed-up the computation (from $\mathcal{O}(n^2)$ to $\mathcal{O}(n \log n)$).
- The cosine and sine functions are not localized
- A lens perform a Fourier transform (Fraunhofer approx.)
- Convolution is equivalent to a multiplication in Fourier space.

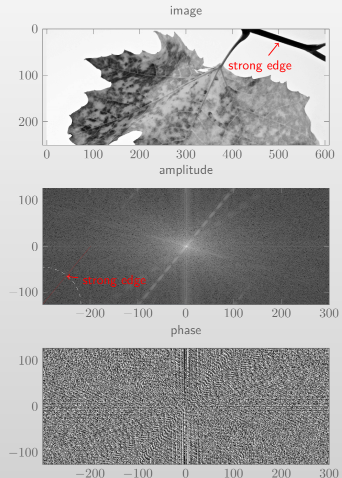


Fig. 9: Amplitude and phase of the Fourier transform of the top images.

Optical transfer function

Definition

The **optical transfer function** is the Fourier transform of the point spread function.

Properties

- Complexe values
- Contains the same information that the point spread function
- The modulus is called the **modulation transfer function** (MTF)
- Its value vanishes beyond the cut-off frequency (resolution limit)

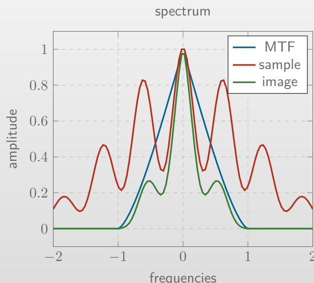


Fig. 10: MTF in 2D computed as the autocorrelation of a disk.

Widefield & Confocal PSF

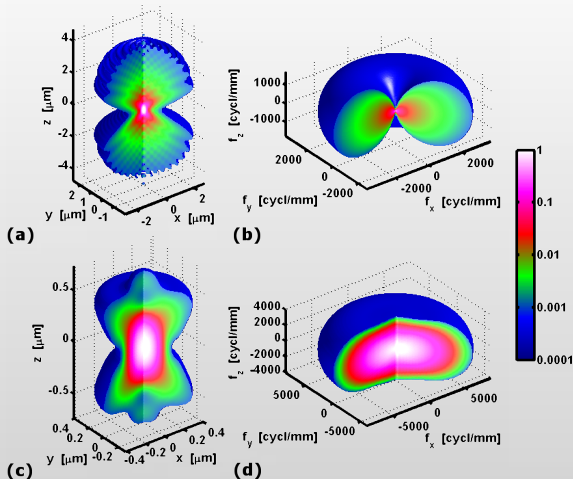


Fig. 11: The three-dimensional point spread functions (a,c) and corresponding modulation transfer functions (b,d) of a wide-field microscope (a,b) and confocal microscope (c,d). In both cases the numerical aperture of the objective is 1.49 and the refractive index of the medium 1.52. The wavelength of the emitted light is assumed to be 600 nm and, in case of the confocal microscope, that of the excitation light 500 nm with circular polarization. The colors as shown on the logarithmic color bar indicate the irradiance (a,c) and spectral density (b,d) normalized to the maximum value. (cc) Tom.vettenburg at English Wikipedia

Spherical aberrations

- The rays on the edge of the lens do not intersect at the same point than the ones coming from the center.
- Microscope objectives are corrected for spherical aberrations
- In practice spherical aberration comes from
  - index mismatch (use the right lens: oil/water/air)
  - coverslip thickness (objectives are optimized for 0.17mm coverslip)
  - depth (across the z-stack, the PSF is varying due to the intermediate layers of different refractive index)

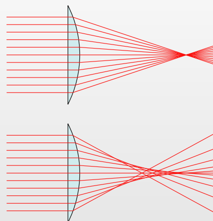


Fig. 12: Origin of spherical aberration.

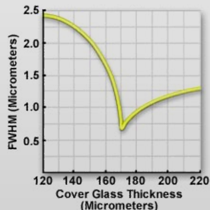


Fig. 13: Cover glass thickness vs half-width intensity distribution.
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Spherical aberrations

- Characterized by an asymmetry in axial (Z) direction

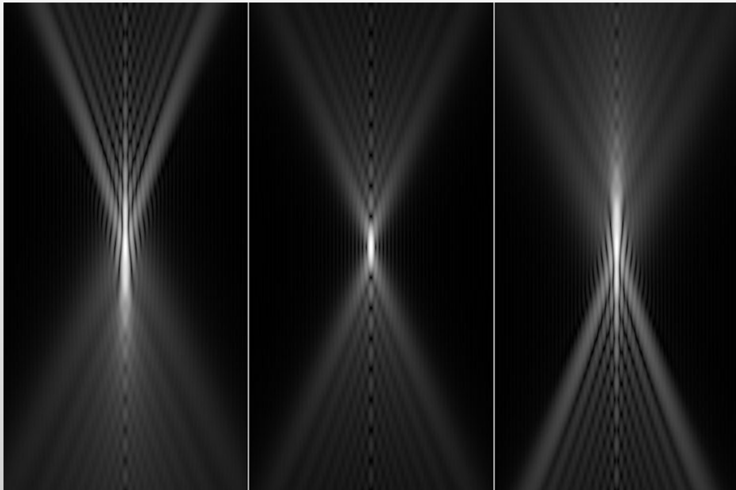


Fig. 14: Longitudinal sections through a focused beam with negative (top row), zero (middle row), and positive spherical aberration (bottom row). The lens is to the bottom.

Chromatic aberrations

- The refractive index of materials depends on the wavelength λ
- The focus point of the lens depends on λ
- High quality objective are corrected for achromatism by combining doublet of lens (flint/crown)
- A change of dichroic filter might affect the lateral position of the focal point

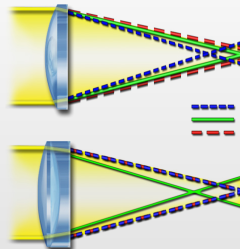


Fig. 15: Axial chromatic aberration © www.microscopyu.com

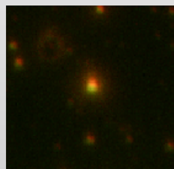


Fig. 16: Image of a Tetraspeck beads preparation green and far red channels © Nick Barry.

Noise sources

Definition [signal processing]

In signal processing, noise is defined as un-wanted signal...

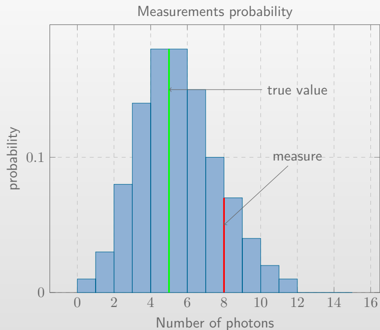
- Out of focus light, autofluorescence, dark current, shot noise, hot pixels?

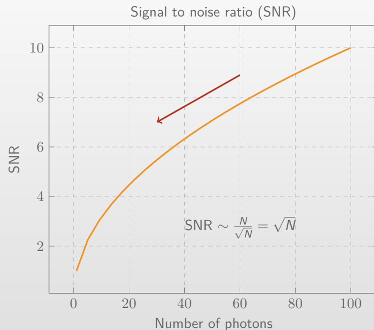
Definition [statistic]

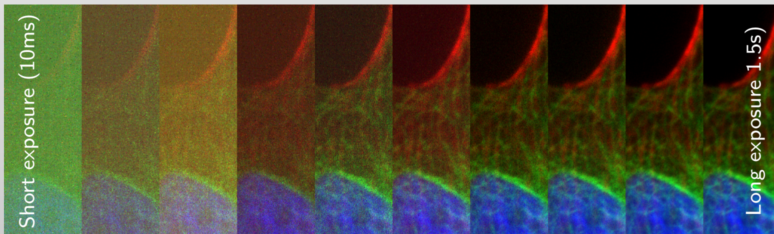
Noise is the variation of signal obtained by repeating the measurement.

- **shot noise**: the counting process lead to a Poisson distribution for the number of generated photo-electrons.
- **dark current**: even in the total obscurity, photo-electron are created and generate a tiny current
- **read-out**: errors made when measuring the voltage at each pixel

Random measurements







Noise model & characterization

Assuming for each pixel i of the image:

$$Y_i = g_0 N_i + \varepsilon_i$$

where

- Y_i intensity
- $N_i \sim \mathcal{P}(\mu_i)$ # photo-electron
- $\varepsilon_i \sim \mathcal{N}(m, \sigma_\varepsilon^2)$ thermal fluctuations

this leads to

$$\mathrm{Var}[Y_i] = g_0\, \mathbb{E}[Y_i] + \underbrace{\sigma_\varepsilon^2 - g_0 m}_{e_{DC}}$$

Variance stabilization transform

$$\mathcal{T}(Y_i) = \frac{2}{g_0}\sqrt{g_0 Y_i + \frac{3}{8} g_0{}^2 + e_{DC}} \quad \sim \quad \mathcal{N}(0,1)$$

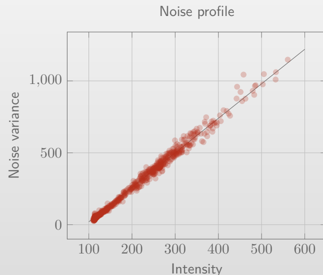
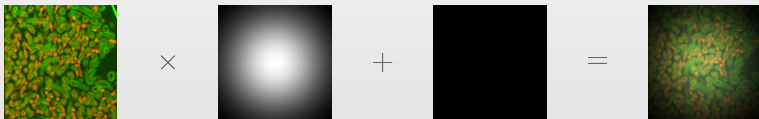


Fig. 17: Noise variance vs intensity empirically measured on a sCMOS camera.

Background and uneven illumination

- Intensities are corrupted by a background and an non-uniform illumination profile.



- Non uniformity introduces
  - **bias** in intensity quantification
  - problem when tiling images.
- Background subtraction is not valid and make things worse in some case.

Summary

- The microscope produces blurred and noisy images...

Outline

1. Image formation
2. Signal-to-noise enhancement
3. Deconvolution with Huygens
4. Visualization & analysis with Imaris
5. Access & training

Non-local image denoising

Non–Local Means principle

- Compare **patches** instead of pixels
- Average repeats in a large neighborhood
- Minimize the sum of the difference between patches

Iterative refinement

- Growing **4D** neighborhood
- Statistical patch distance
- Bias-variance trade-off

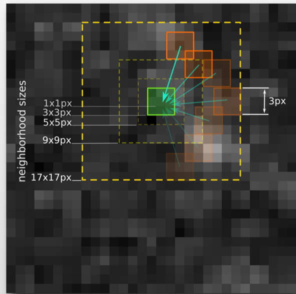
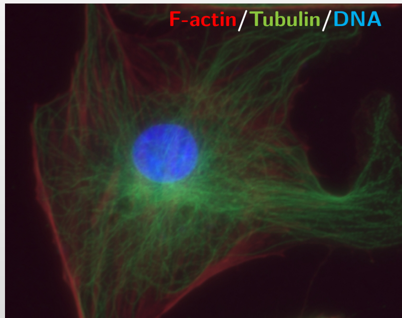


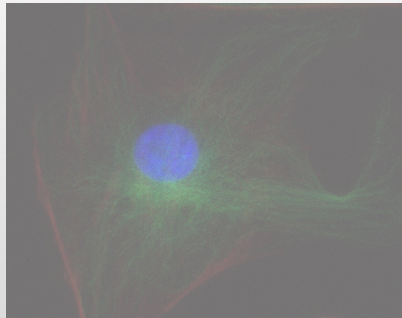
Fig. 18: Patch exploration

Denoising benchmark

Using acquired images

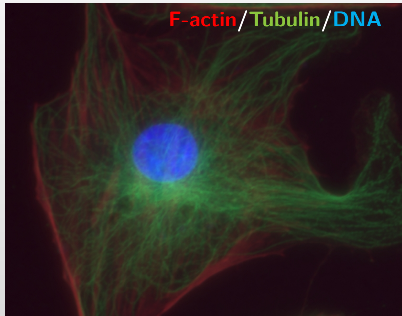


3.5s exposure time

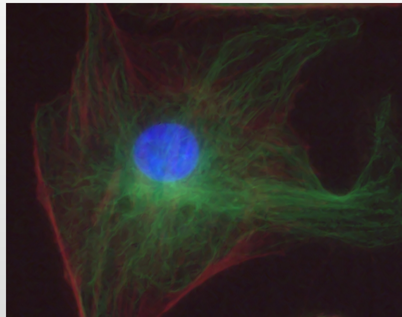

50ms exposure time

Denoising benchmark

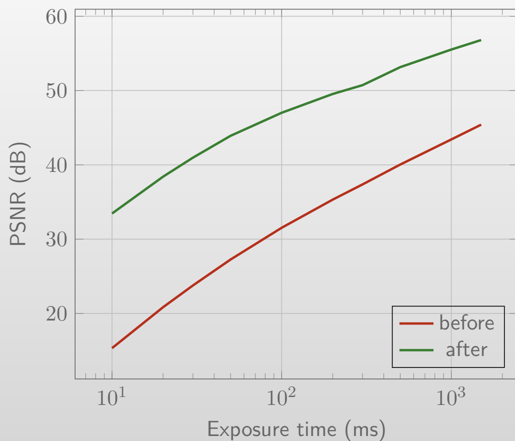
Using acquired images



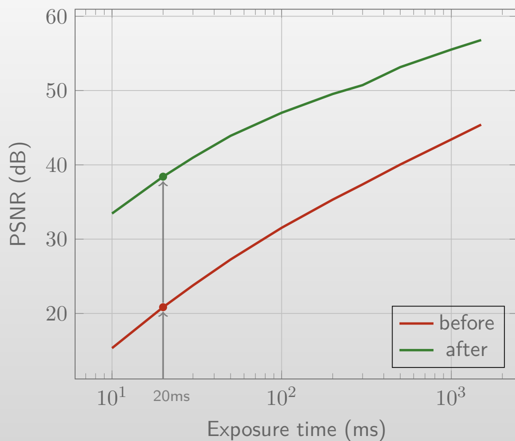
3.5s exposure time


50ms exposure time **denoising**

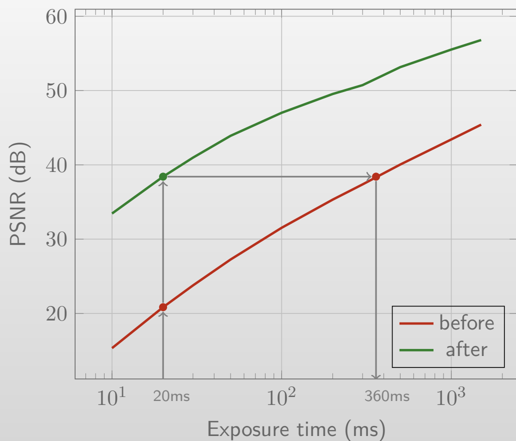
Signal-to-noise improvement



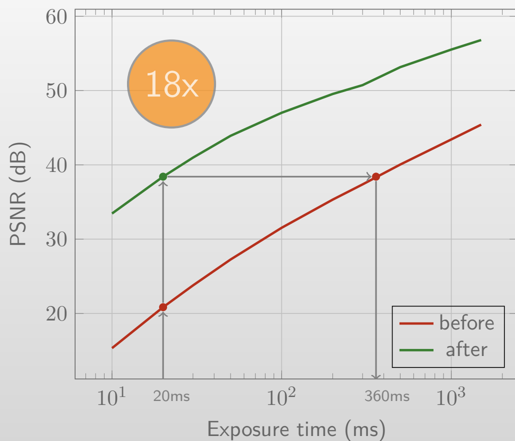
Signal-to-noise improvement



Signal-to-noise improvement



Signal-to-noise improvement



Examples

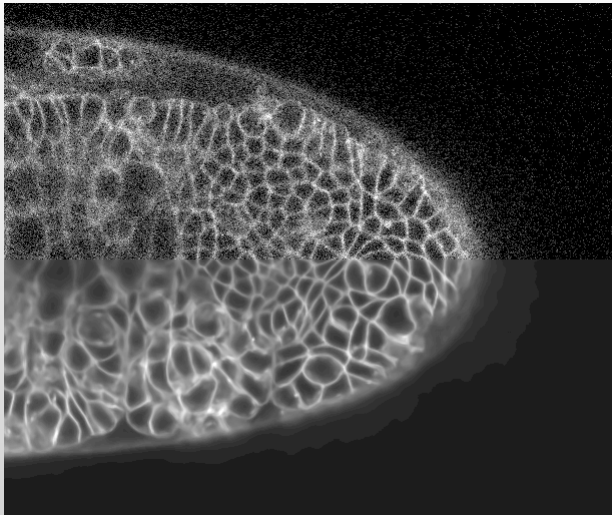
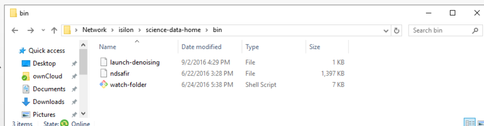


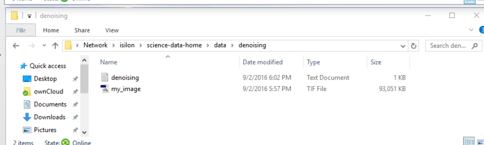
Fig. 19: Confocal image of developing Drosophila – Yara Sanchez-Corrales

Batch processing on the cluster

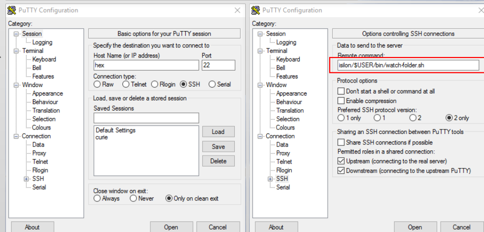
1) copy the software on istore

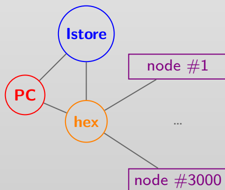


2) copy the images on istore



3) launch the command





Denoising from ImageJ/Fiji

- Integration as a macro
- Calling the native code from Fiji

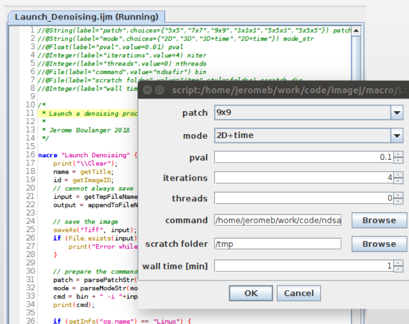


Fig. 20: Denoising interface and macro from Fiji

nd-safir software

n-dimensional spatially-adaptive filtering

- Process multi-dimensional data (5D)
- Parallel software rewritten (c++) at MRC-LMB
- Running on the cluster (single node) with helper scripts
- Bridge with ImageJ/Fiji

Integration in commercial solutions:

- OMERO image management tool
- OMX
- Metamorph plugin (Roper Scientific SAS)
- Photometrics Prime Enhance sCMOS camera (FPGA)

Outline

1. Image formation
2. Signal-to-noise enhancement
3. **Deconvolution with Huygens**
4. Visualization & analysis with Imaris
5. Access & training

Deconvolution with Huygens

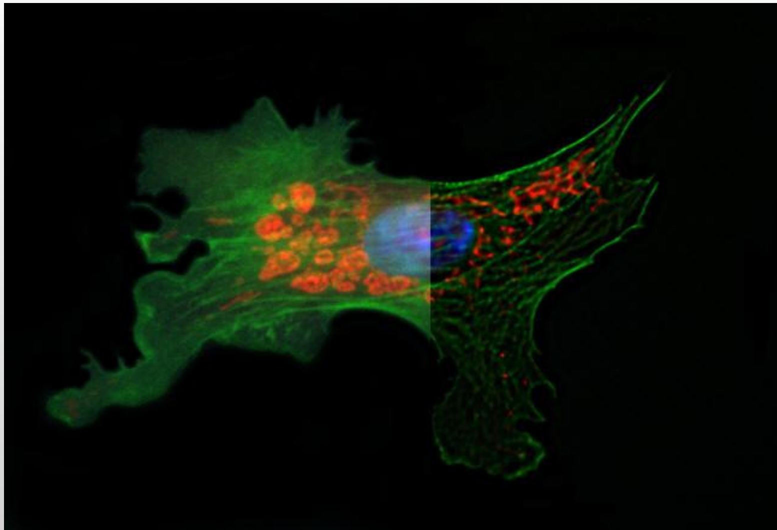


Fig. 21: Before (left) and after (right) deconvolution images. Experimental point spread functions were generated for the red, green, and blue channels on an epifluorescence microscope and then used to deconvolve a standard Invitrogen Fluocells #1 prepared slide, containing bovine pulmonary artery endothelial cells stained for mitochondria (red), F-actin (green), and nuclei (blue).

Why is it difficult?

- Convolution is equivalent to a multiplication by the OTF in Fourier space:

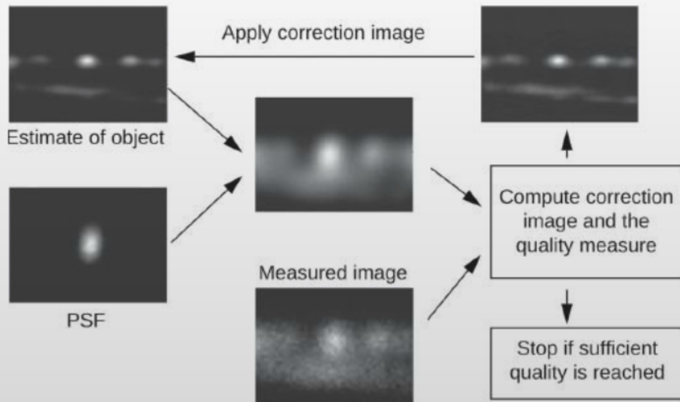
$$F = H \times U$$

- Can we deconvolve an image by dividing the image by the OTF?

$$U = \frac{F}{H}$$

- We saw that
  - The OTF vanishes beyond the resolution limit.
  - The noise kicks in even before this limit and would be amplified.
- Deconvolution is an “ill-posed” problem, we need to add constraints:
  - Positivity,
  - Smoothness (limit the number of iterations/add constraints).

Deconvolution algorithm



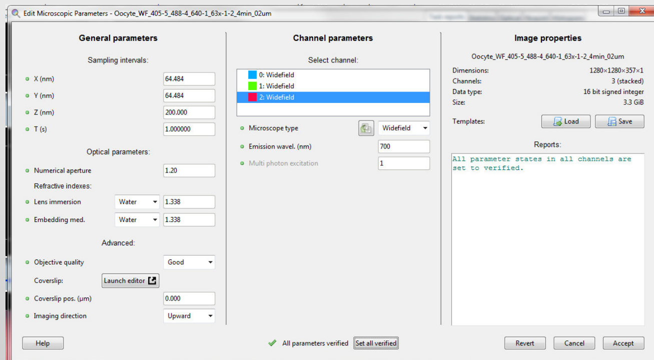
Step by step

1. Load the data (keep original format & metadata)
2. Edit the parameters
3. Inspect the data (hot pixels, saturation)
4. Define a PSF (theoretical vs measured)
5. Launch a deconvolution (several algorithm available)
6. Inspect the result (3D rendering, twin slicer)
7. Perform a chromatic correction

Main interface

Microscopy parameters

- Display the required informations related to the acquisition
- Indicate if these parameters are consistent
- Can be read in from metadata which accompany the RAW images.
- Keep the data in the original format



Algorithm selection and tuning

Deconvolution wizard

File Options Help

Images

Oocyte_WF_405-5_4

Oocyte_WF_405-5_4

decon:Ch0

Oocyte_WF_405-5_4

Comparison view

Image: decon:Ch0:brick
Position: (-86.32 105.26 0.10) µm
Value: ch 0: -

Help Reports

Iteration 17: quality 8.093
Iteration 18: quality 8.116
Iteration 19: quality 8.135
Iteration 20: quality 8.152
Iteration 21: quality 8.165
Iteration 22: quality 8.177
Iteration 23: quality 8.188
Iteration 24: quality 8.197
Iteration 25: quality 8.204
Iteration 26: quality 8.211
Iteration 27: quality 8.217
Iteration 28: quality 8.223
Iteration 29: quality 8.228
Iteration 30: quality 8.232

Wizard status

Obtained initial parameters from image Oocyte_WF_405-5_488-4_640-1_63x-1-2_4min_02um.

Deconvolution started

Deconvolution setup

Deconvolving the image

In this stage channel 0 of the original image will be deconvolved on the basis of the PSF and background as computed in the previous stages. Selected algorithm: CMLE.
The result will be stored in image decon:Ch0.

Below are the default values for deconvolving images. If necessary you can change these values.

Maximum iterations	50
Signal to noise ratio	20
Quality threshold	0.01
Iteration mode	Optimized
Bleaching correction	Off
Brick layout	Auto

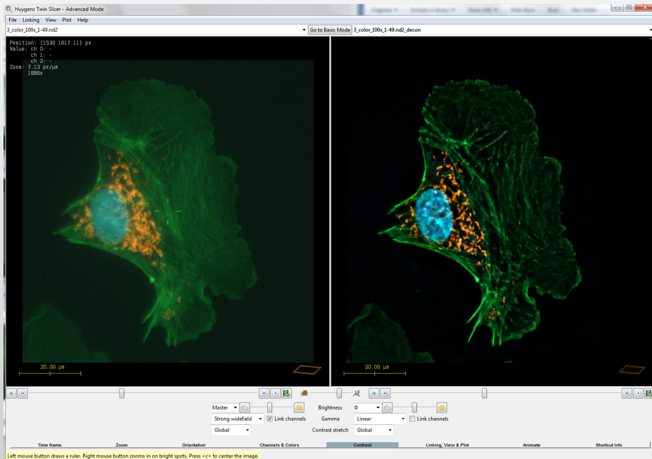
Stop deconvolution

Deconvolve

Progress: 55%

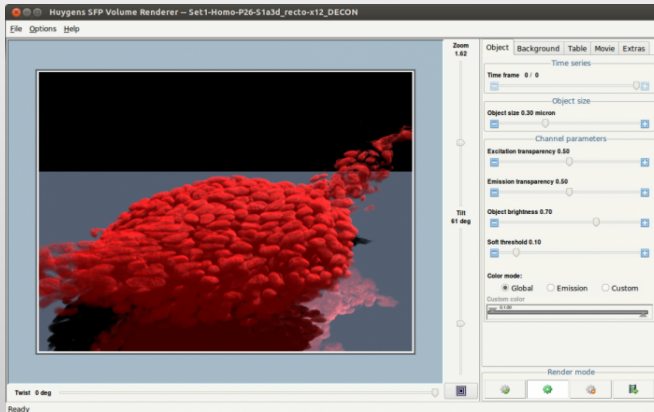
Twin slicer

- Synchronize views of 2 images
- Allows to inspect the image after deconvolution



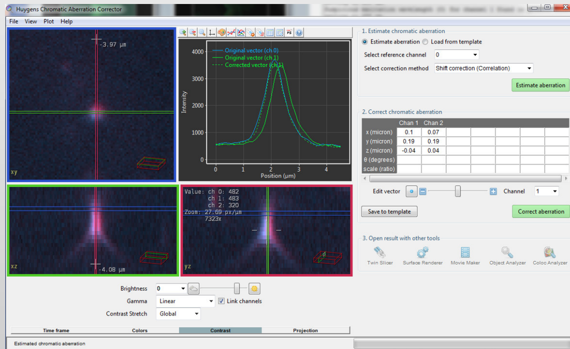
Simulated Fluorescence Process renderer

- Simulates how the object's excited matter emits light forming a scene
- Based on ray tracing



Chromatic Aberration Corrector

- Register channels using
  - Cross correlation
  - Center of mass
- When performed after deconvolution, the higer SNR improves the registration
- Improves colocalization analysis



STED deconvolution

- Not only wide-field image can be deconvolved
- Huygens supports various imaging modality
  - Wide-field
  - Confocal
  - Light-sheet
  - STED
- Due to the non-linearity of the deconvolution process, extra resolution can be claimed.
- Here the lateral jitter correction, proved essential in improving the image quality.

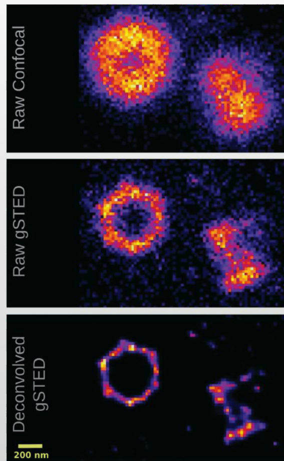


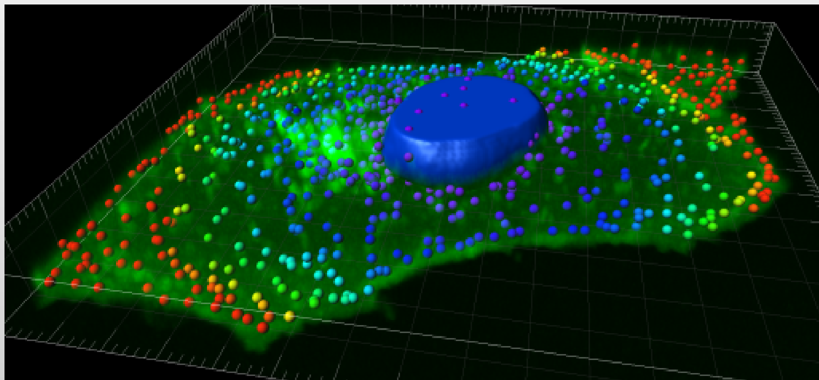
Fig. 22: FWHM improvement toward 22nm ©Microscopy Today Nov 2013

Outline

1. Image formation
2. Signal-to-noise enhancement
3. Deconvolution with Huygens
4. **Visualization & analysis with Imaris**
5. Access & training

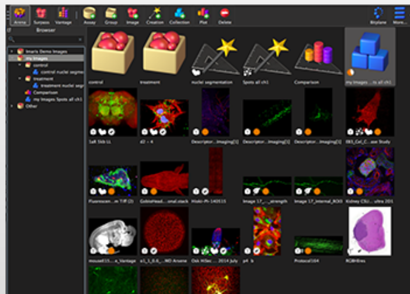
Bitplane Imaris

- Organize (group/conditions)
- Visualize (Volume rendering, isosurfaces)
- Analyze (spot detection, tracking, segmentation)



Organize

- All data are stored in an **Arena**
- Files can be grouped in an **assay** which can contain several **groups**.



Visualization

- A scene can be composed of several objects
  - Digital rendering
  - Surfaces
  - Spots (3D balls)
  - Tracks (3D filaments)
- A movie editors allows to create 3D animations easily

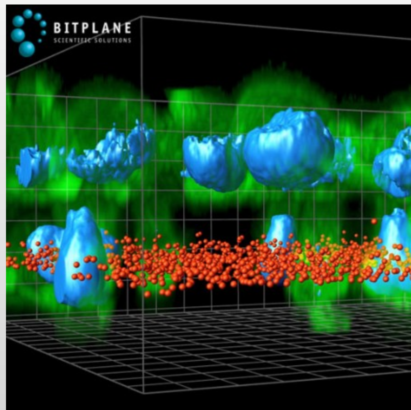


Image analysis

- Several analysis methods are available:
  - Spot detection and tracking
  - Segmentation
  - Neuron tracing
- You can then export extracted measurements
  - to Excel
  - via ImarisXT to make a bridge with Matlab
- Or use the statistical data plotting in Imaris.

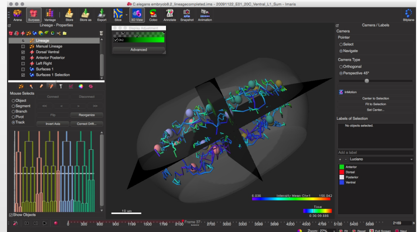


Fig. 23: Spot tracking and visualization in Imaris

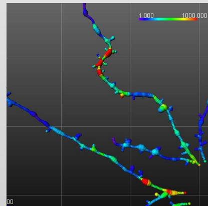


Fig. 24: Neuron tracing feature in Imaris

Outline

1. Image formation
2. Signal-to-noise enhancement
3. Deconvolution with Huygens
4. Visualization & analysis with Imaris
5. **Access & training**

Image analysis softwares

- Analysis room located in room 1N120
- Softwares:
  - SVI Huygens (2)
  - Bitplane Imaris (3)
  - Nikon NIS Element General Analysis (1)
  - Offline version of
    - Leica LAS
    - Nilon NIS Element
    - Zeiss Zen
  - ImageJ/Fiji (6)
  - Mathworks Matlab (1)
  - R (1)

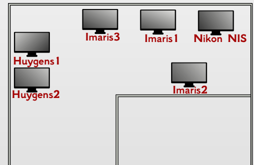


Fig. 25: Analysis room 1N120

Data management

- Acquisition stations
  - Temporary (regular clean up)
  - When disk are full the system slows down
- Temporary storage `micro-nas-1`
  - $\sim$ 14TB NAS
  - **Shared** with all users
  - Temporary (regular clean up)
  - No mirroring (less secure)
  - RAID (support disk failure)
  - Good for exchanging data with us
- Long term achiving with `istore`
  - 500GB max
  - **secure** (mirrored outside the building)
  - accessible only by you
  - Good for keeping published raw data

Online learning ressources

- ImageJ
  - http://imagej.nih.gov
  - http://fiji.sc
  - http://cmci.embl.de/documents/ijcourses
  - https://github.com/jboulanger/introduction-to-imagej-macros/releases/tag/1.0.0
- SVI Huygens https://svi.nl
  - register to the wiki
  - get in touch directly with SVI team (TCL scripting)
- Bitplane Imaris http://www.bitplane.com/learning
  - Step-by-step tutorials
  - Webinars
  - Case studies

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