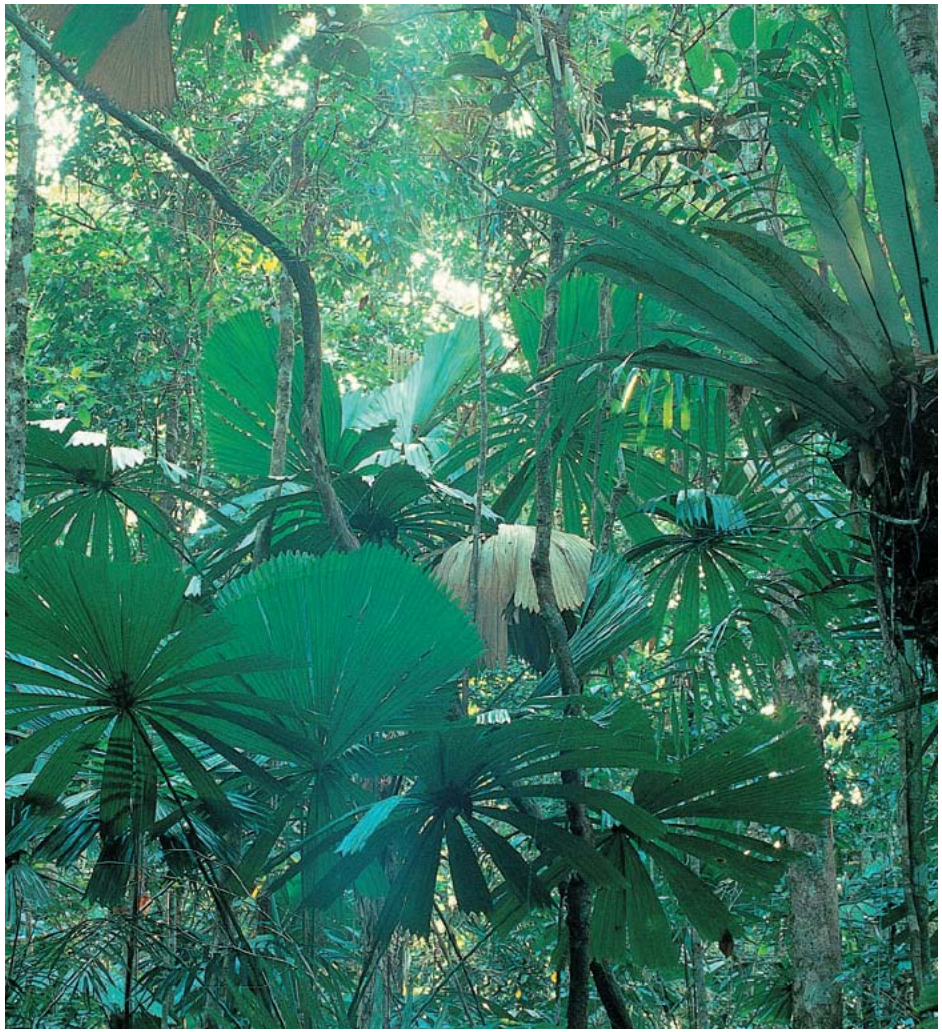
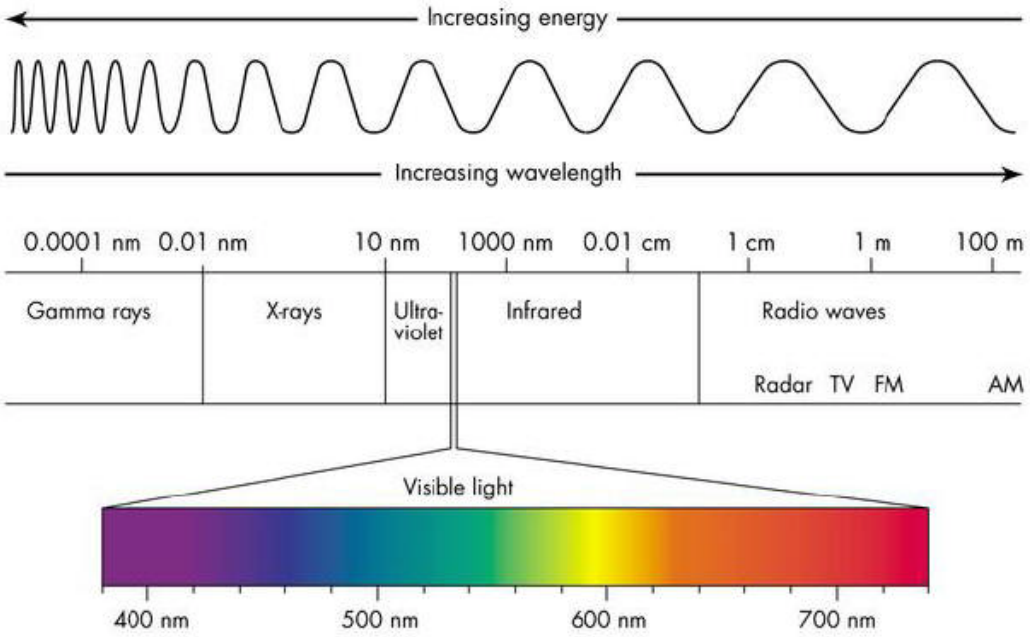
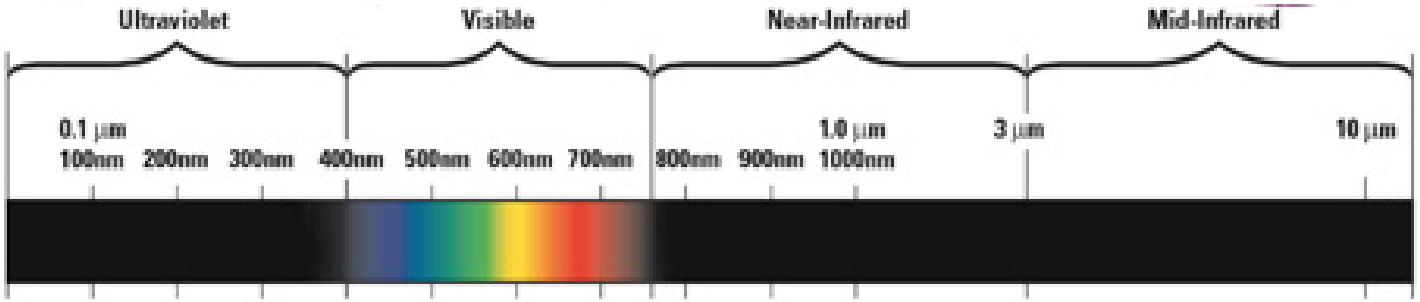
Spectral cleanup



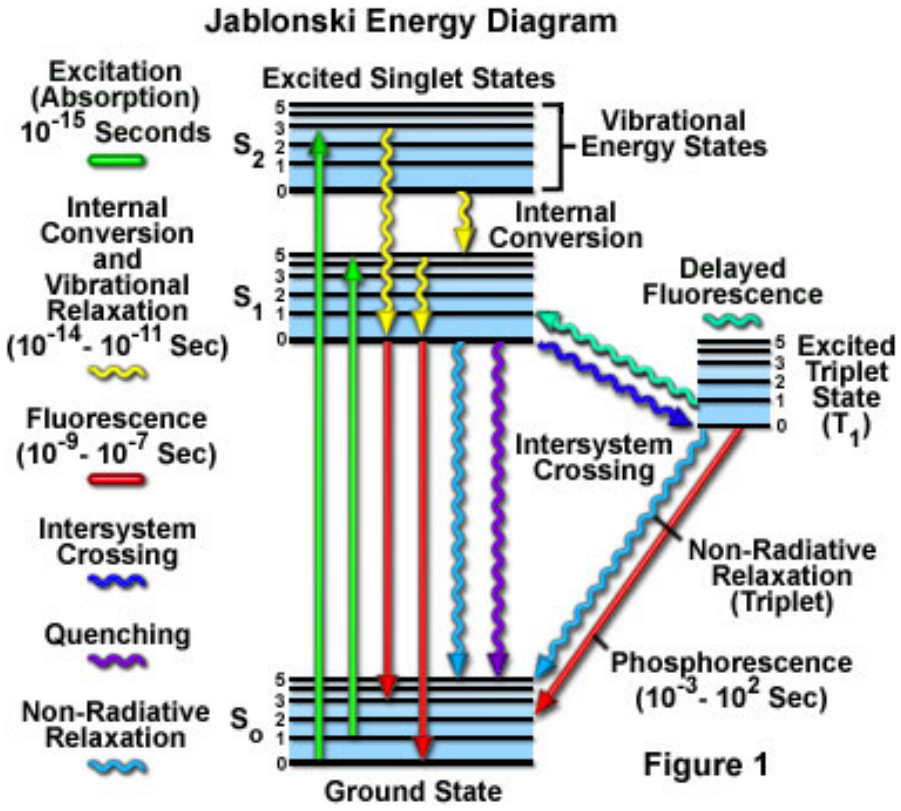
Overview

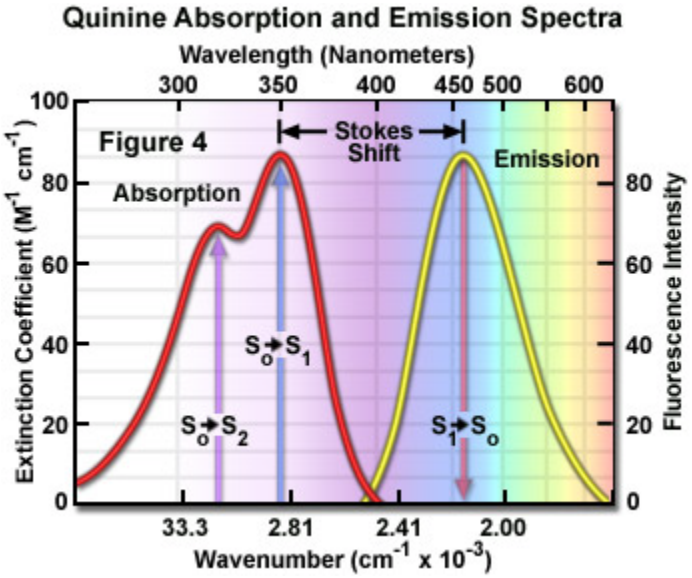
1. Electromagnetic radiation
2. Fluorescence, excitation and emission spectra
3. Multicolor imaging with cross-talk
4. Multicolor imaging of fluorophores with overlapping spectra
5. Hyperspectral and spectral imaging
6. Linear unmixing
7. Emission fingerprinting





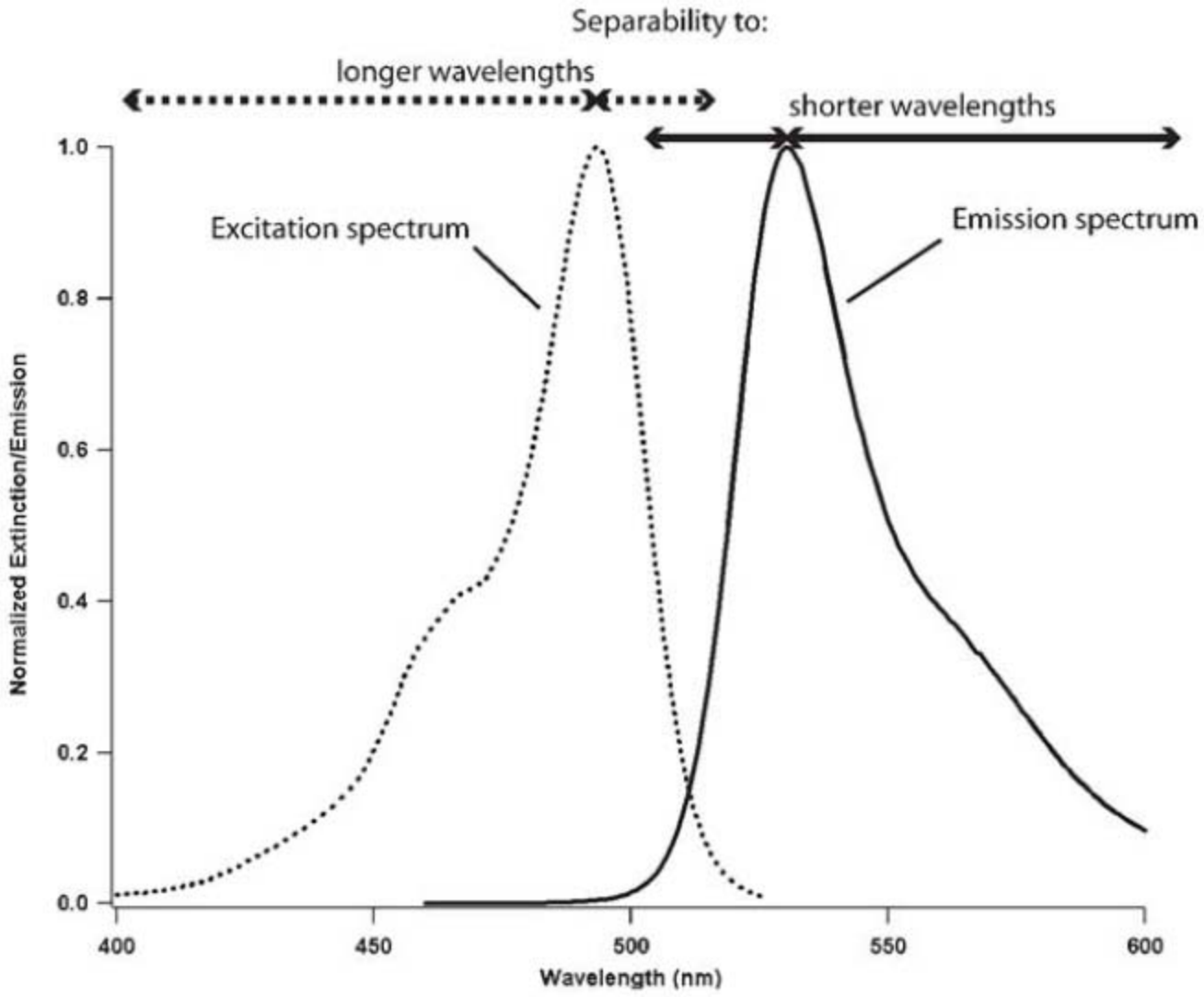
Fluorescence





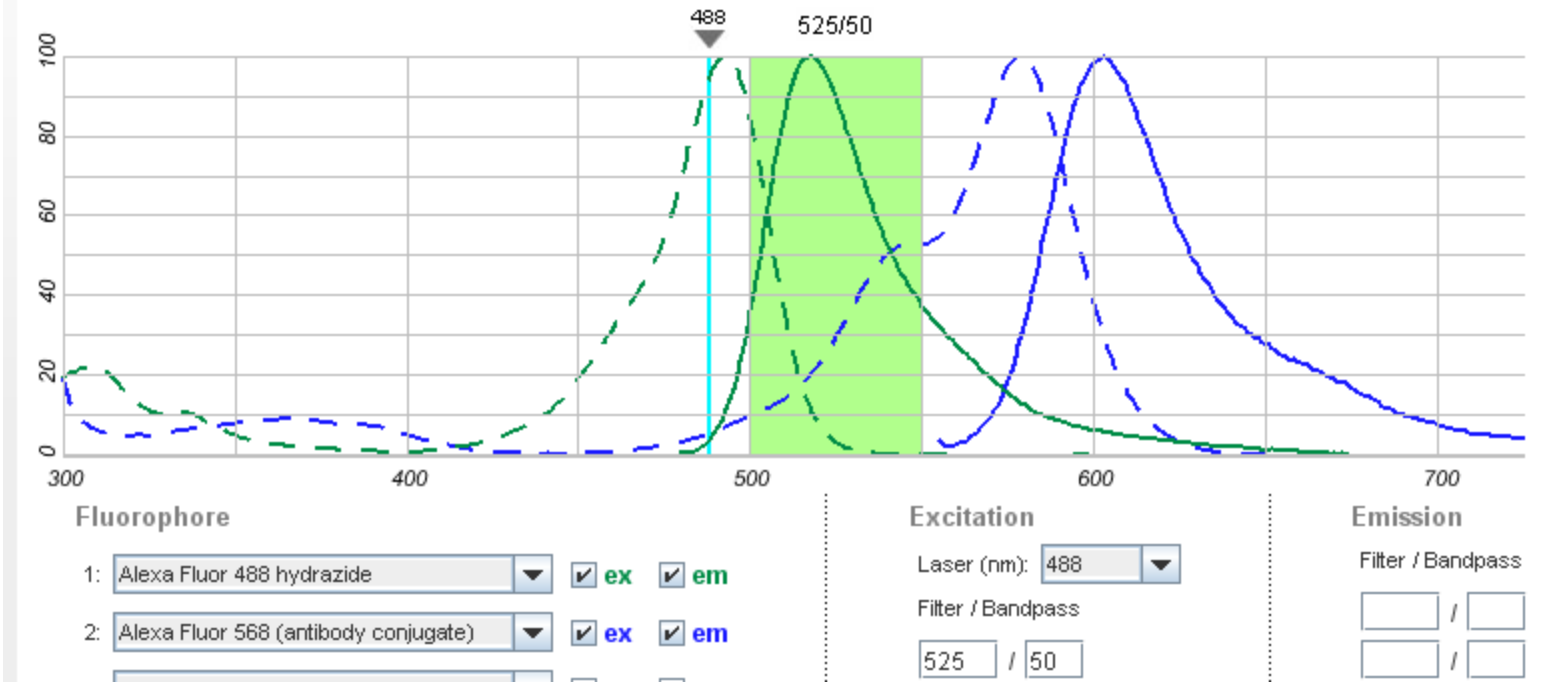
http://www.olympusmicro.com/primer/

Excitation and emission spectra

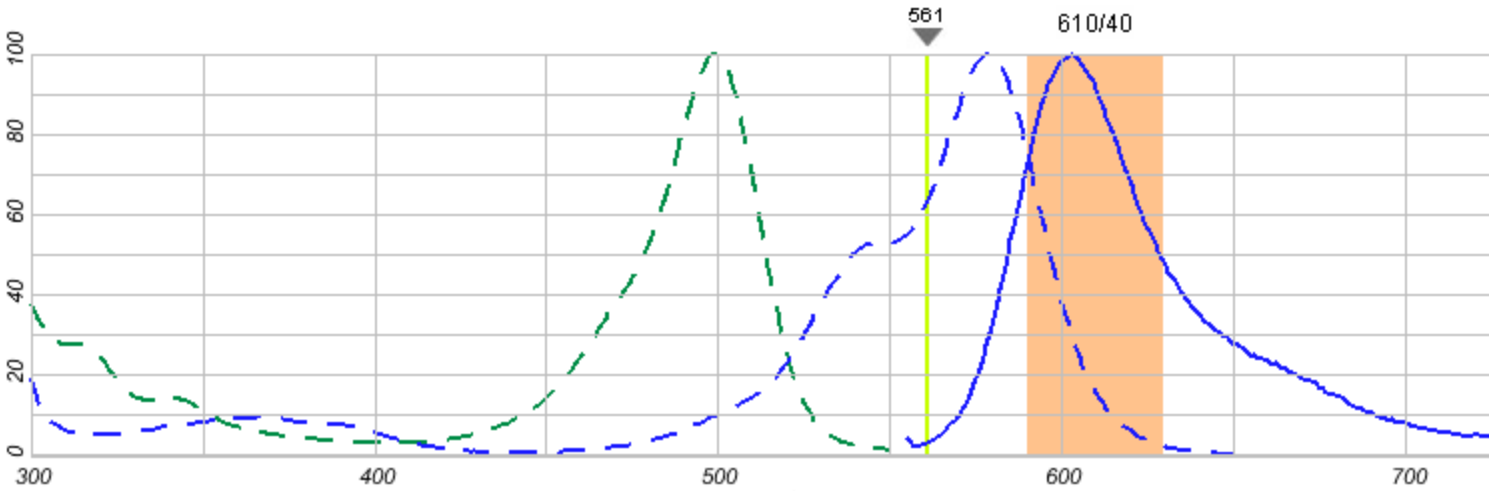


Zimmermann, 2005

Fluorescence SpectraViewer



Fluorescence SpectraViewer



Fluorophore

1:	Alexa Fluor 488 (antibody conjugate)	☑ ex	☐ em
2:	Alexa Fluor 568 (antibody conjugate)	☑ ex	☑ em
3:	None	☑ ex	☑ em
4:	None	☑ ex	☑ em

Excitation

Laser (nm): 561

Filter / Bandpass

/

610 / 40

/

Emission

Filter / Bandpass

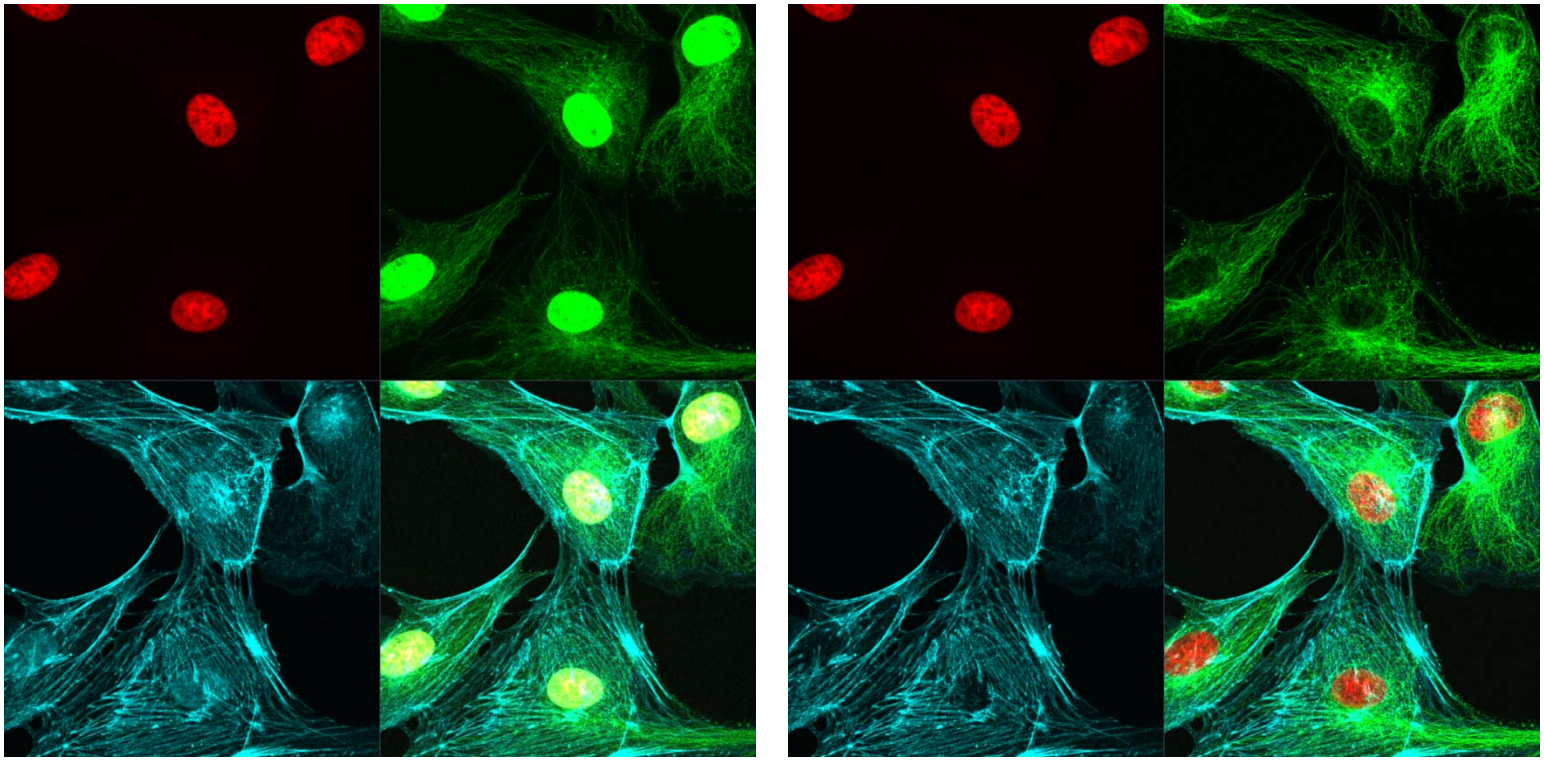
/

/

/

/

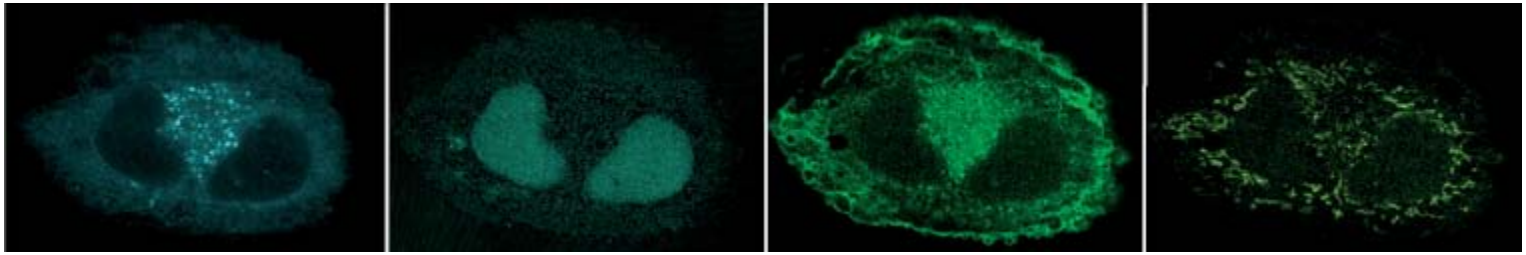
Confocal single - multitrack



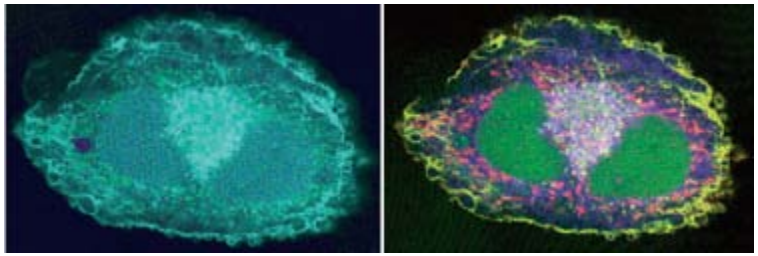
Take home message I

Sequential acquisition	speed
Bandpass emission filter allows only ca.50% of photons to pass	sensitivity
Doesn't work for fluorophores with closely overlapping spectra	labeling

Different fluorophores have distinct hues that are discernible by eye



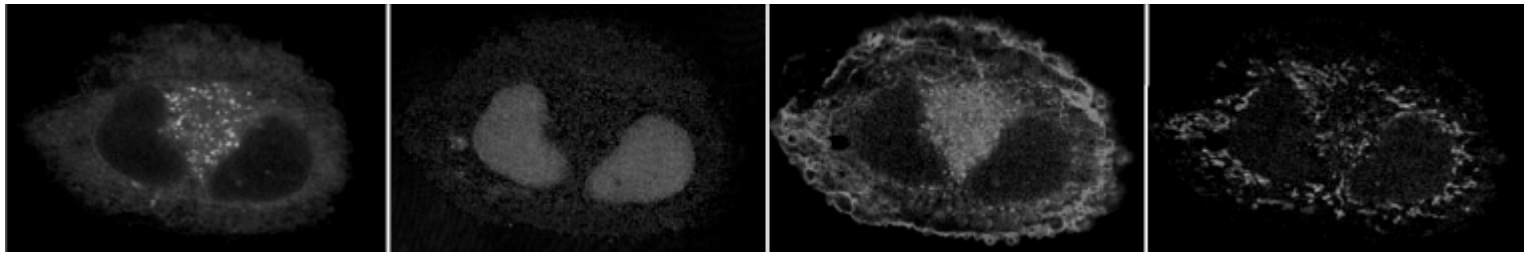
CFP *CGFP* *GFP* *YFP*



Quadruple-labeled cell - wavelength-coded projection of the spectral image stack.

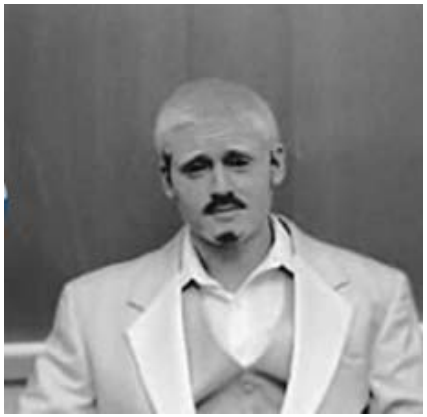
Pseudocolor-coded, spectrally unmixed quadruple labeling

At the time of acquisition, the spectral info – color – is lost



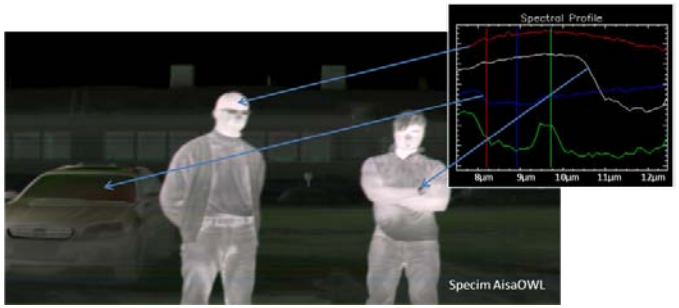
CFP *CGFP* *GFP* *YFP*

Hyperspectral imaging



Short Wave Infrared (SWIR)

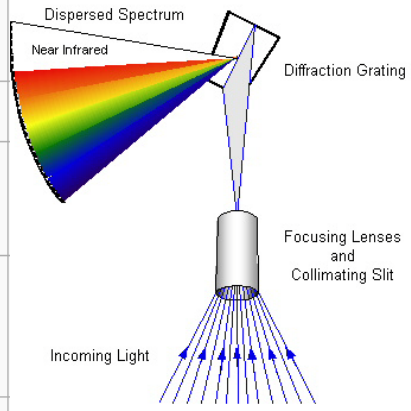
http://www.sensorsinc.com



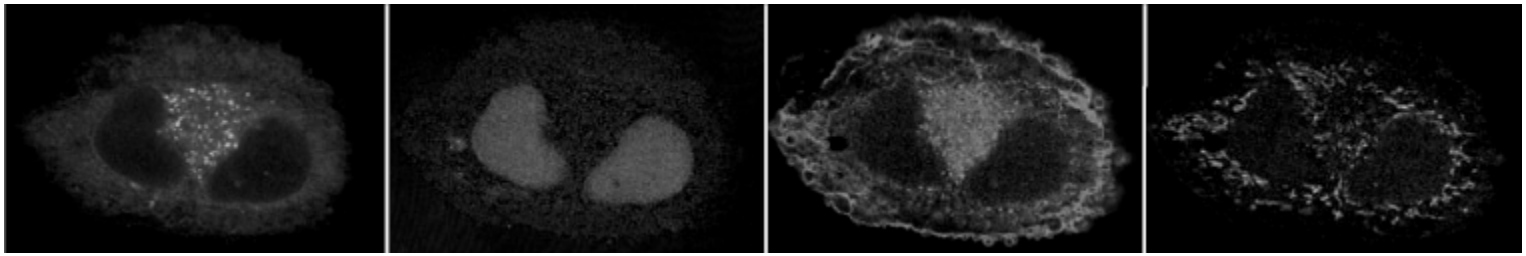
Holma, H., (2011)

Division Name	Abbreviation	Wavelength
Near-infrared	NIR, IR-A *DIN*	0.75-1.4 µm
Short-wavelength infrared	SWIR, IR-B *DIN*	1.4-3 µm
Mid-wavelength infrared	MWIR, IR-C *DIN*. Also called intermediate infrared (IIR)	3-8 µm
Long-wavelength infrared	LWIR, IR-C *DIN*	8–15 µm
Far infrared	FIR	15 - 1,000 µm

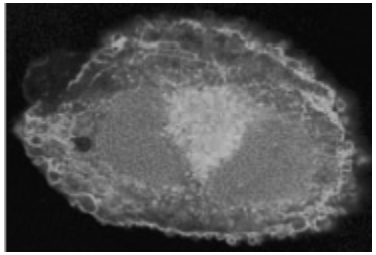
Byrnes, James (2009)



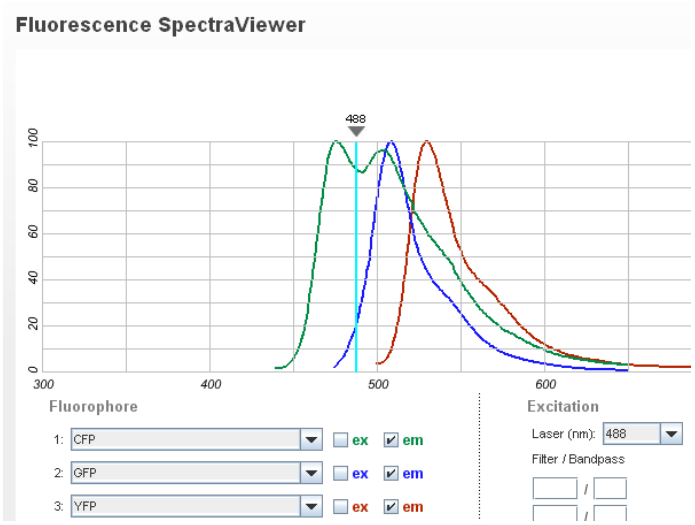
wikipedia



CFP *CGFP* *GFP* *YFP*



www.zeiss.de



http://www.invitrogen.com

Spectral imaging

Imaging
Provides the intensity at every pixel of the image I(x,y)

Imaging	Spatial resolution	250 nm (in plane) at $\lambda = 500$ nm
	Field of view	~50 μm (high magnification)
	Dynamic range	8–16 bits (256–65, 536 intensity levels)
	Lowest detectable signal	Shot-noise limited

Spectroscopy
Provides the spectral characteristics of matter I(λ).
The energy levels are intrinsic properties of the molecule and spectrum, therefore, provides a precise fingerprint of the molecule.

Spectroscopy	Spectral resolution	1–20 nm (may depend on λ)
	Spectral range	400–900 nm

Garini et al. 2006

Spectral Image provides spectrum at every pixel of the image I(x,y)

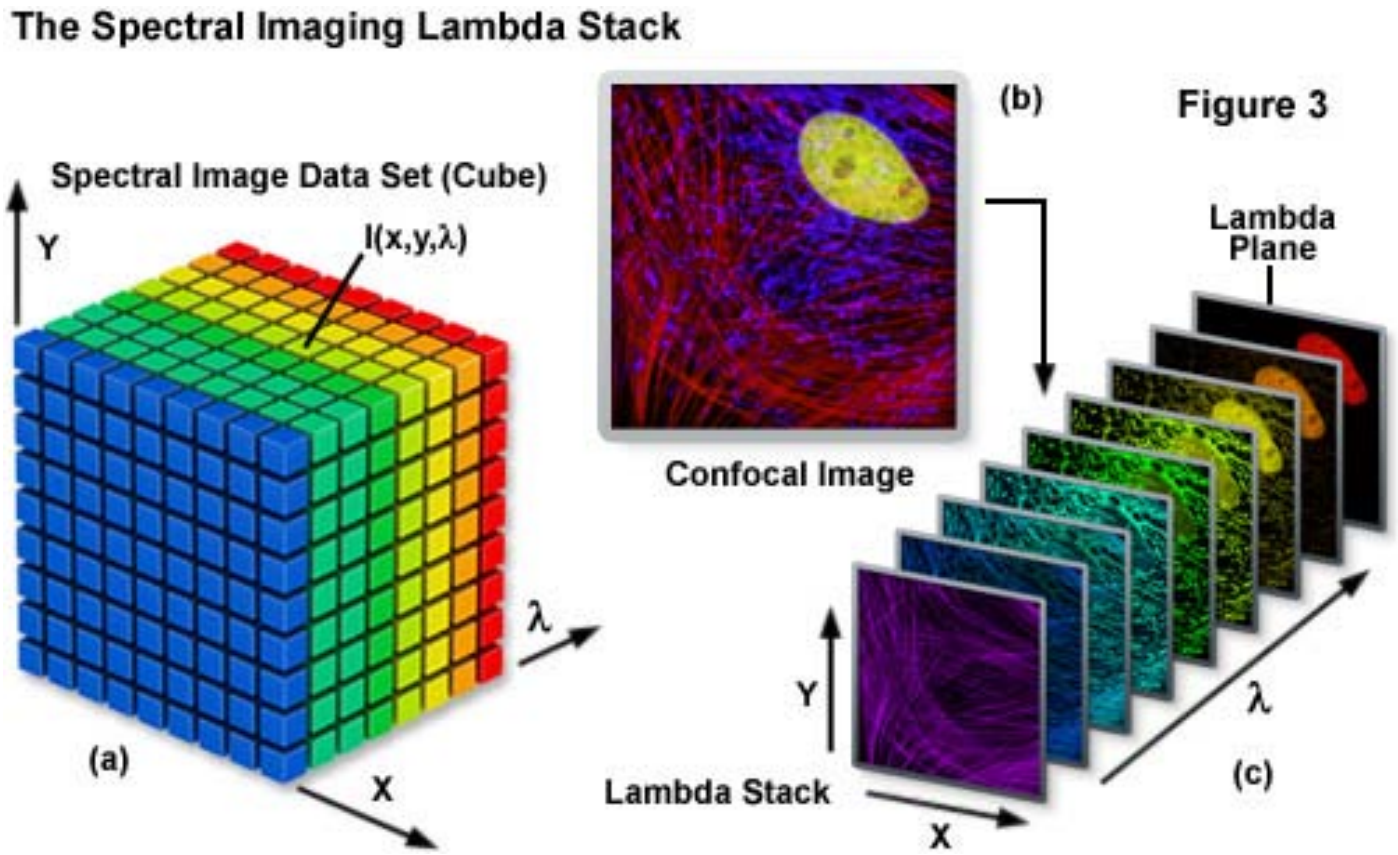


FIG. 2. Description of a spectral image data set. Each point in the cube represents a single number and the spectral image is described as $I(x,y,\lambda)$. It can be viewed either as an image $I(x,y)$ at each wavelength λ, or as a spectrum $I(\lambda)$ at every pixel (x,y).

http://zeiss-campus.magnet.fsu.edu Garini et al., 2006

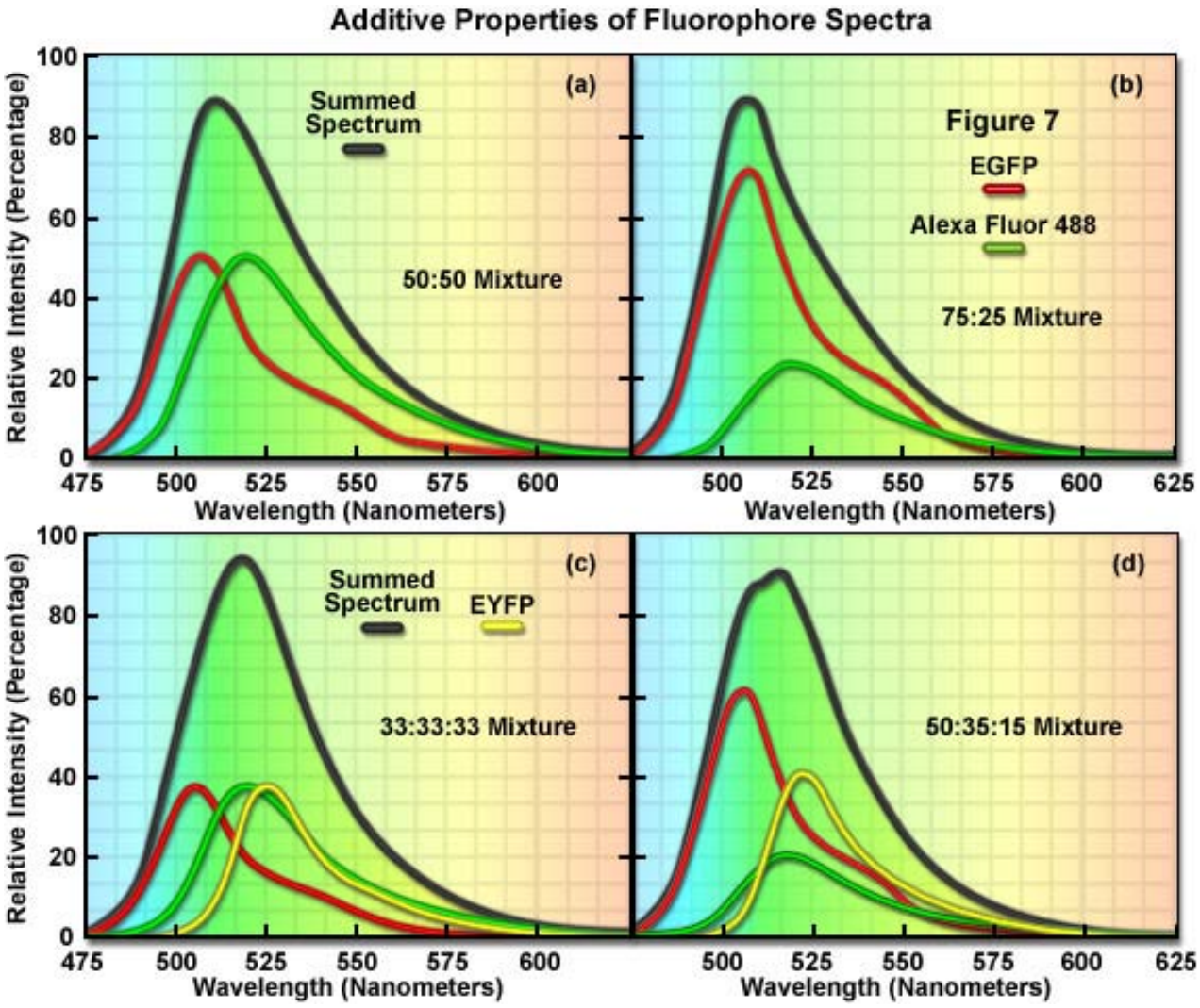
Linear Unmixing

This technology is based on the assumption that the total detected signal S for every channel λ can be expressed as a linear combination of the contributing fluorophores $S_{dye}(\lambda_{dye})$.

$$S(\lambda)_{sum} = \left[\text{Intensity}_{\text{dye A}} \cdot S(\lambda)_{\text{dye A}}\right] + \left[\text{Intensity}_{\text{dye B}} \cdot S(\lambda)_{\text{dye B}}\right] + \left[\text{Intensity}_{\text{dye C}} \cdot S(\lambda)_{\text{dye C}}\right]$$

With the signal S detected and the reference emission spectra S known, the contributions $\text{Intensity}_{dyeA,B,C}$ of the fluorophores in the sample are determined by calculating contribution values that most closely match the detected signals in the channels.

Zimmermann, 2005; www.zeiss.de


Additive Properties of Fluorophore Spectra
(a)
Summed Spectrum
50:50 Mixture
(b)
Figure 7
EGFP
Alexa Fluor 488
75:25 Mixture
(c)
Summed Spectrum
EYFP
33:33:33 Mixture
(d)
50:35:15 Mixture
Relative Intensity (Percentage)
Wavelength (Nanometers)
0
20
40
60
80
100
475
500
525
550
575
600
625

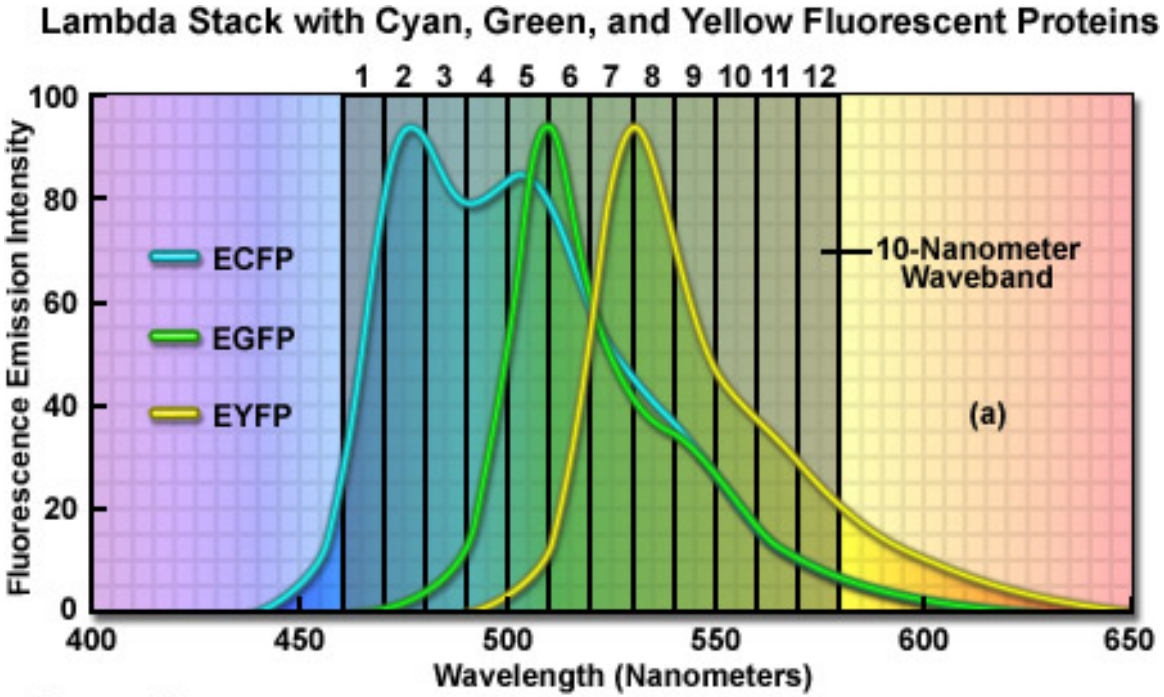
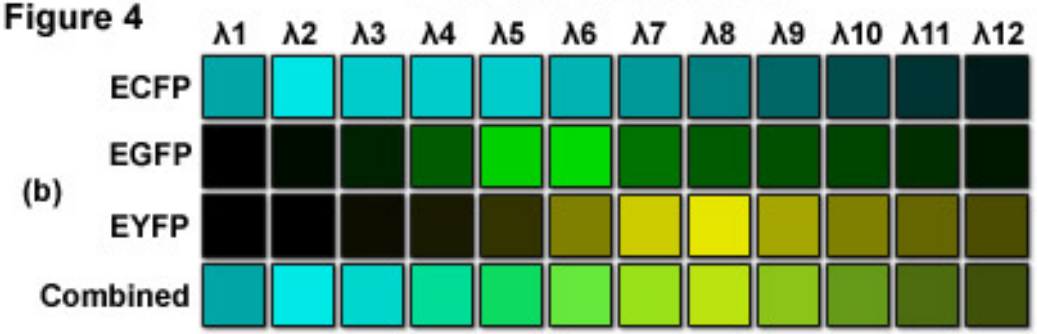
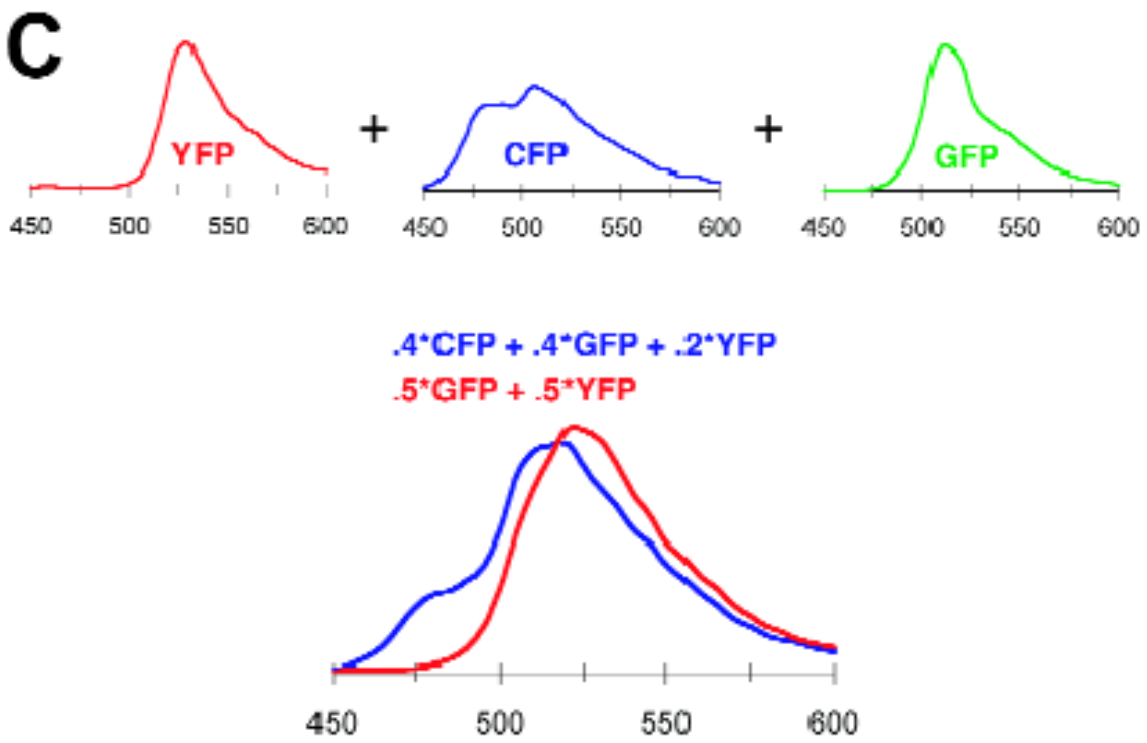

Lambda Stack with Cyan, Green, and Yellow Fluorescent Proteins
1 2 3 4 5 6 7 8 9 10 11 12
100
80
60
40
20
0
Fluorescence Emission Intensity
ECFP
EGFP
EYFP
10-Nanometer Waveband
(a)
400
450
500
550
600
650
Wavelength (Nanometers)

Figure 4

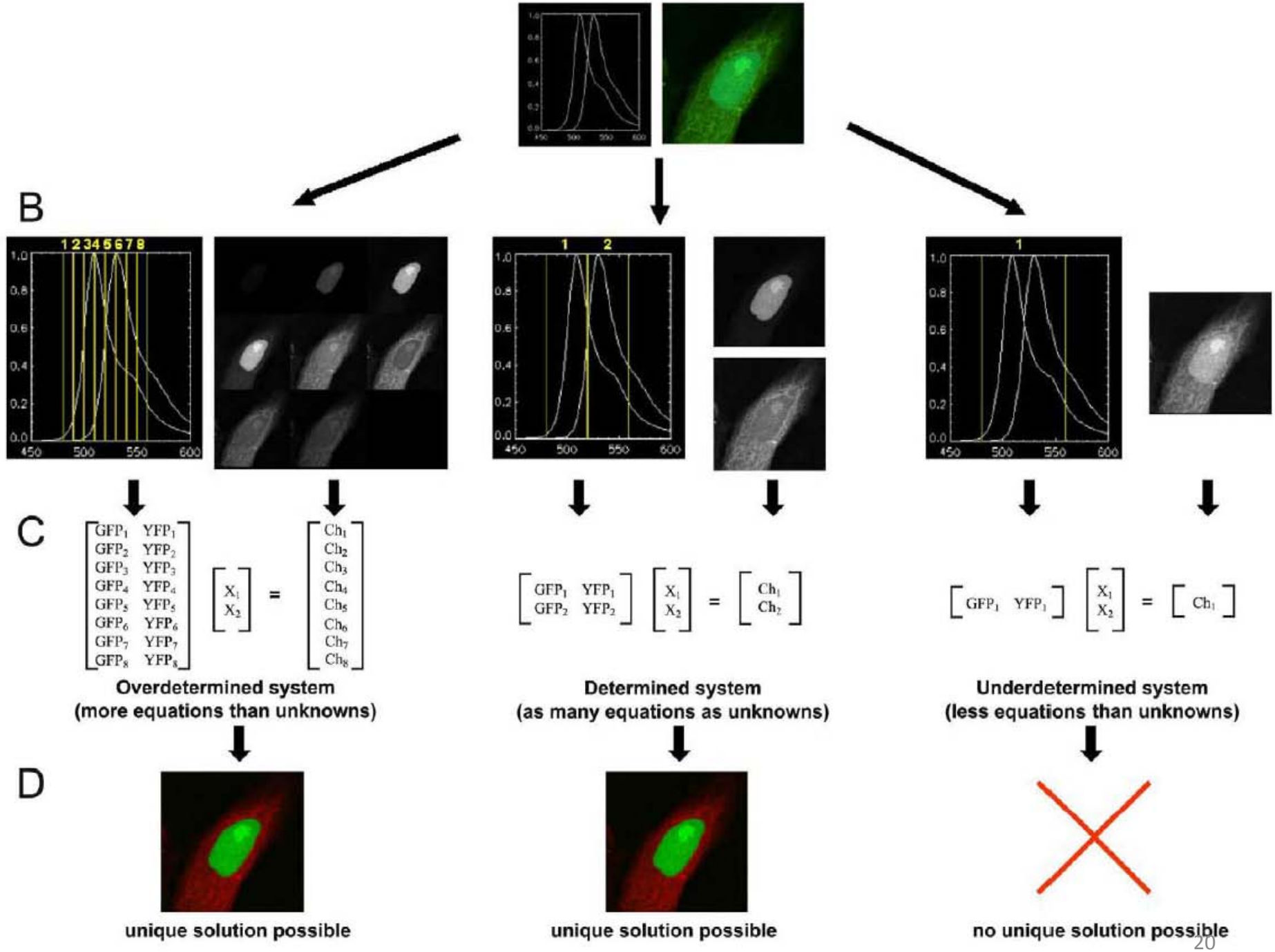

λ1 λ2 λ3 λ4 λ5 λ6 λ7 λ8 λ9 λ10 λ11 λ12
ECFP
EGFP
EYFP
Combined
(b)



Spectrally mixed pixels combine emission spectra to yield an observed spectrum that is a linear sum of the components, weighted by their local concentration.

This figure illustrates a few examples that produce relatively similar summed spectra that cannot be unraveled using filters. It is a linear algebra problem to deconvolve the weighting coefficients of the component spectra from the measured sum spectra.

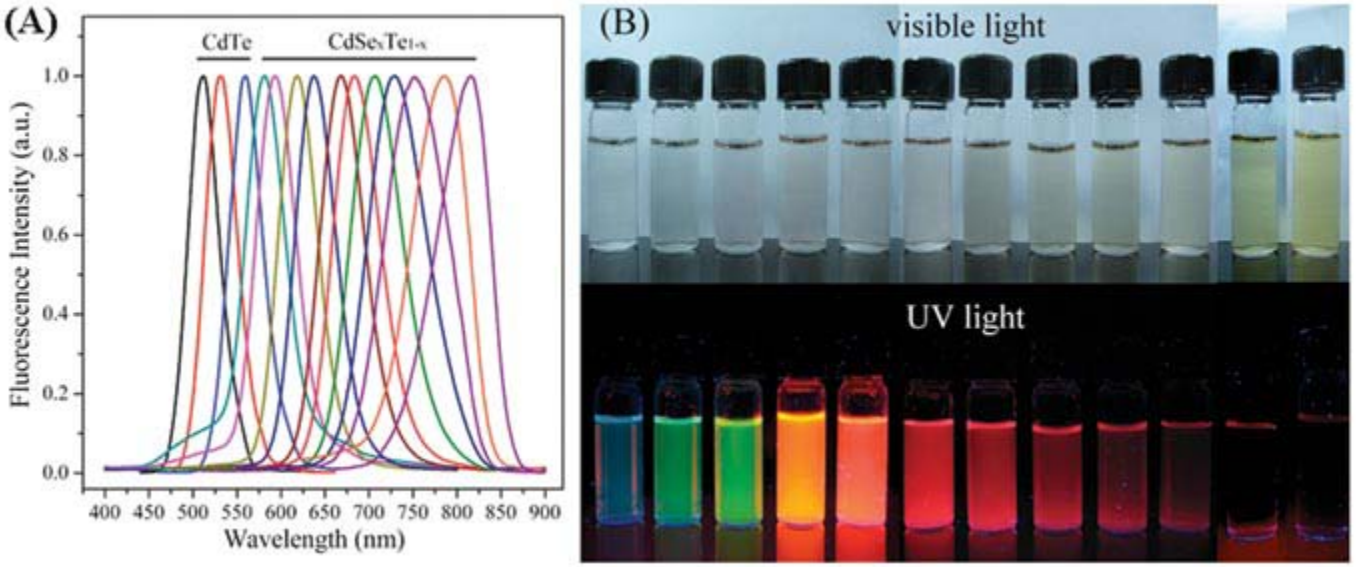
Dickinson at al. 2001



Zimmermann, 2005;

Take home message II
Why do I want to use it?

Simultaneous acquisition	speed
Longpass emission filter allows most of photons to pass	sensitivity
Does work for fluorophores with closely overlapping spectra	labeling



http://nanotechweb.org/cws/article/lab/40874

Take home message III

For the linear unmixing of spectral data, several criteria have to be met:

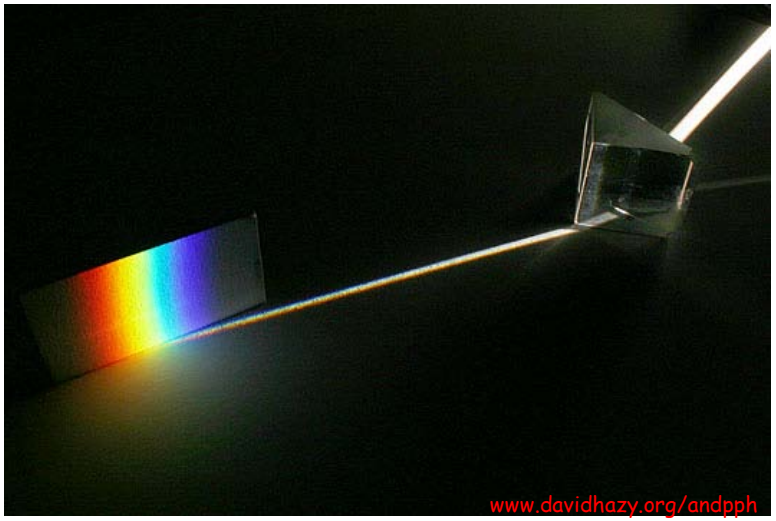
The number of spectral detection channels must be at least equal to the number of fluorophores in the sample. If this is not the case, multiple solutions are possible and no unique result can be attained for spectral separation.

All fluorophores present in the sample have to be considered for the unmixing calculation. If this is not done, the results will inevitably be false! The unmixing calculation is however not affected by taking into account fluorophores spectra in addition to the ones present in the sample.

Removing any signal not originating from the fluorophores to be analyzed by background subtraction is an essential prerequisite for the linear unmixing analysis. Spectrally homogeneous background can be considered as a further fluorophore

Zimmermann, 2005;

Equipment



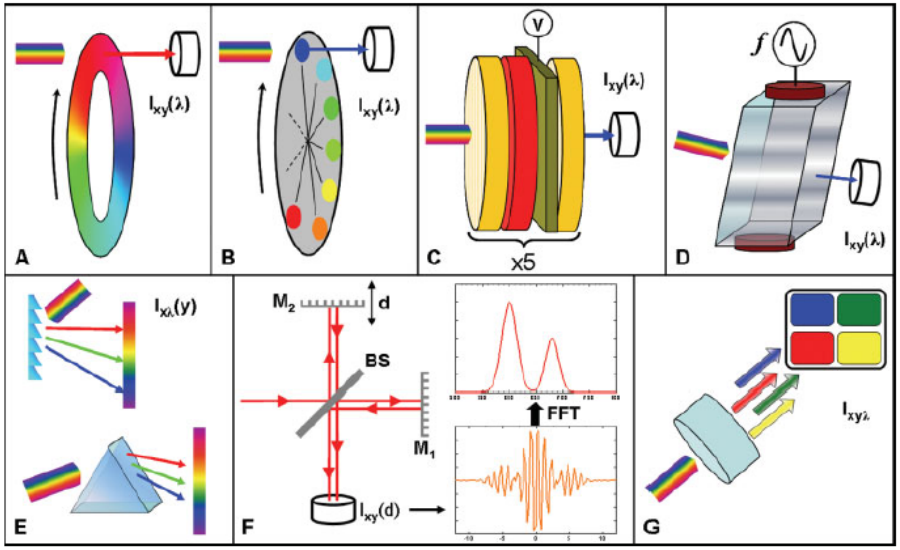


FIG. 3. Various methods of spectral imaging systems. They can be divided into four main methods: wavelength-scan (A–D), spatial scan (E), time scan (F) and "compromise" methods (G). In wavelength-scan methods, the whole image is measured one wavelength at a time. This can be realized using either a circular variable filter (A), a set of filters (B), a liquid crystal variable filter (C) or an acousto-optic variable filter (D). Spatial-scan methods use a dispersion element, either a grating or prism (E) and the image has to be scanned along at least one axis. There are also confocal microscopes that use a dispersive element and scan the image point by point. In time-scanning method (F), the whole image is measured after passing through an interferometer (or other optical elements). In order to calculate the spectrum at each pixel a mathematical transformation has to be carried out, for example, a Fourier transform. In "compromise" methods (G) only a few spectral ranges are measured and the FOV is limited, but the measurement is fast.

Garini et al., 2006

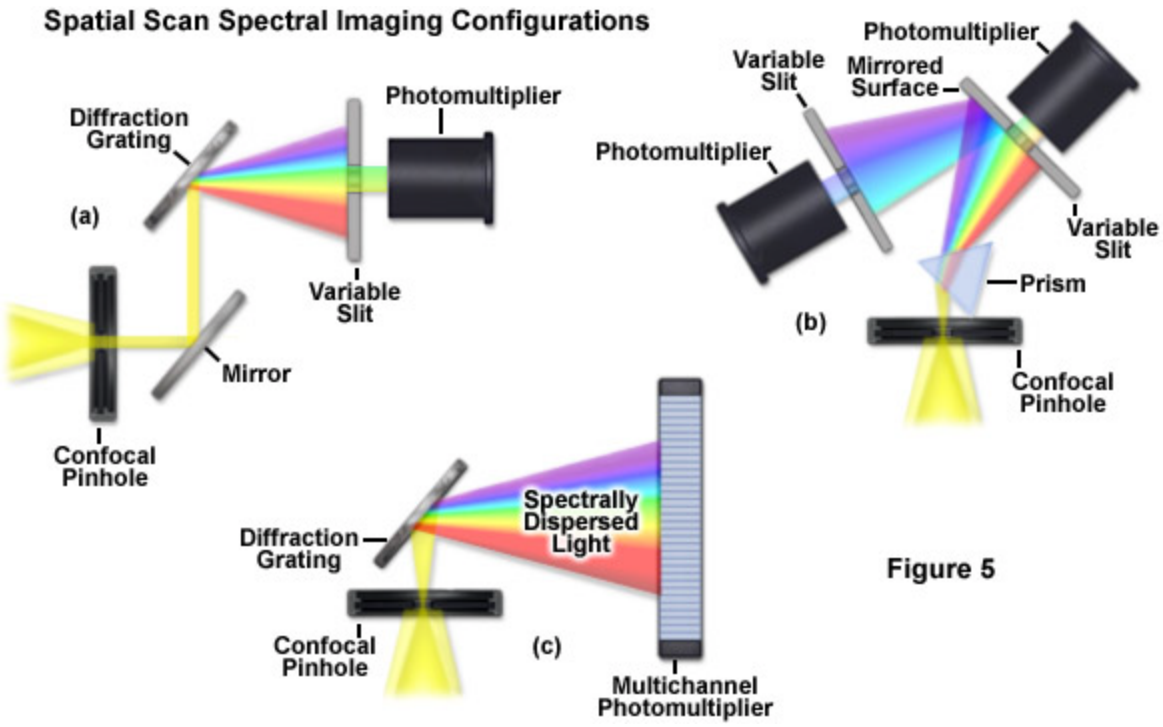
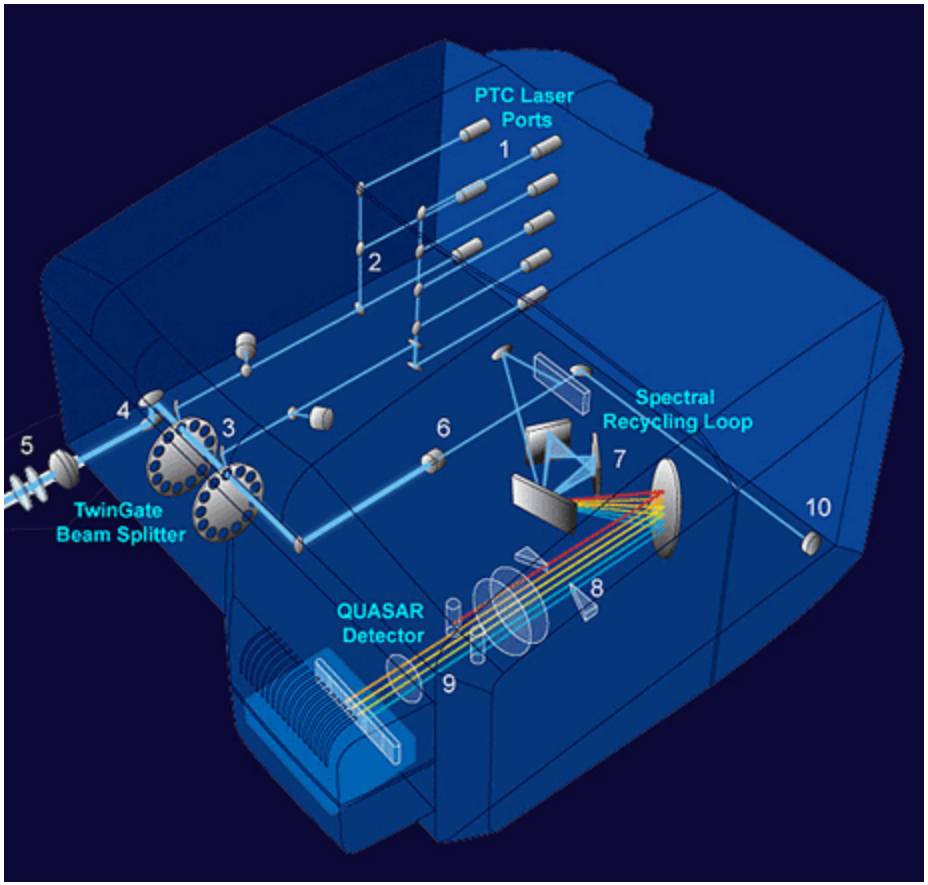
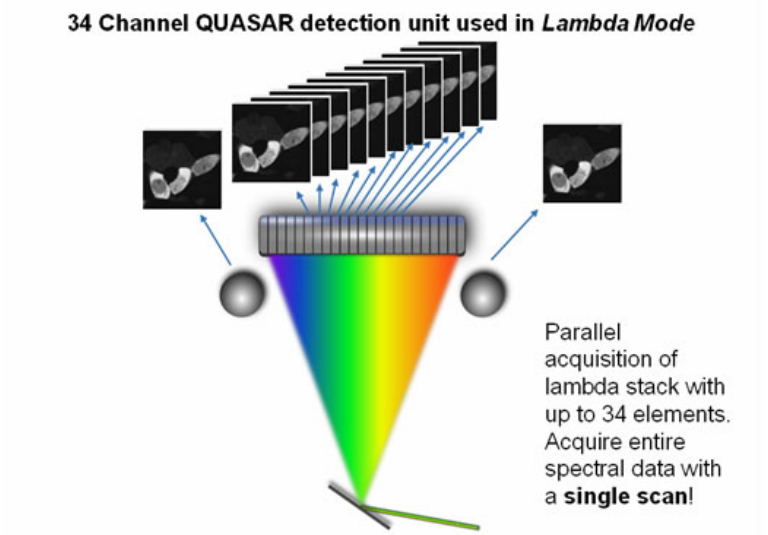


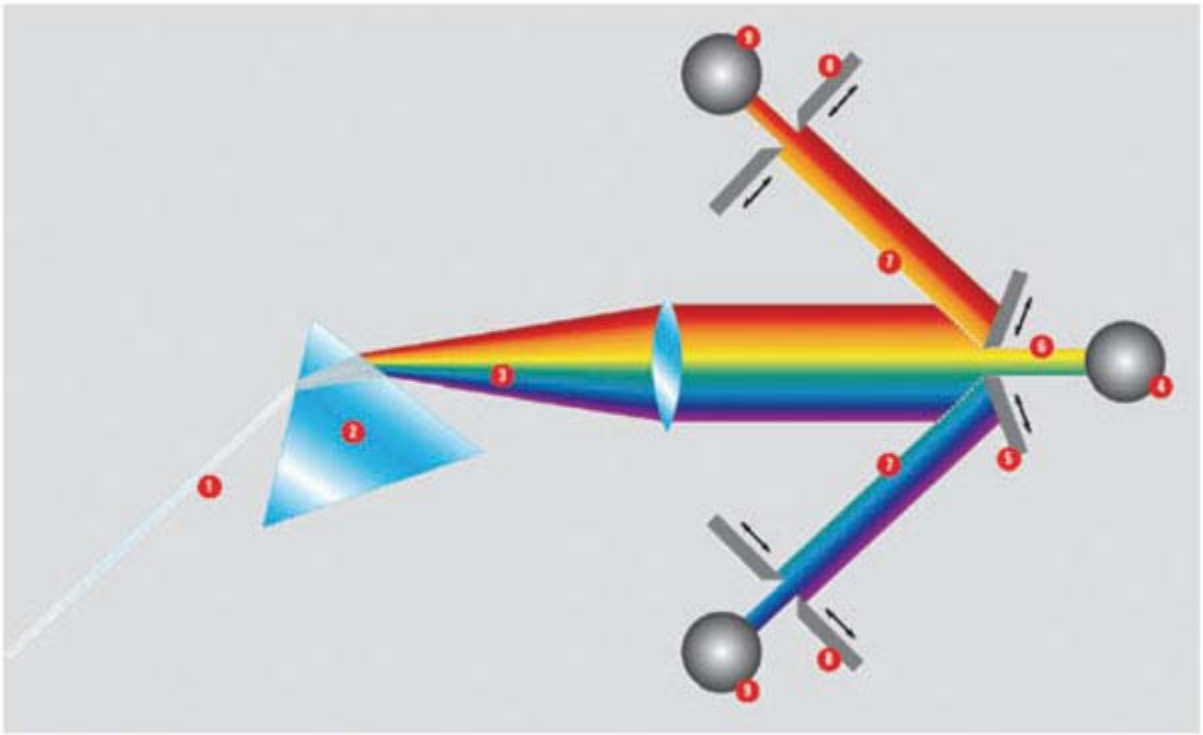
Figure 5

LSM 710 & 780


PTC Laser
Ports
1
2
3
4
5
6
7
8
9
10
Spectral
Recycling Loop
TwinGate
Beam Splitter
QUASAR
Detector


34 Channel QUASAR detection unit used in *Lambda Mode*
Parallel acquisition of lambda stack with up to 34 elements. Acquire entire spectral data with a **single scan**!

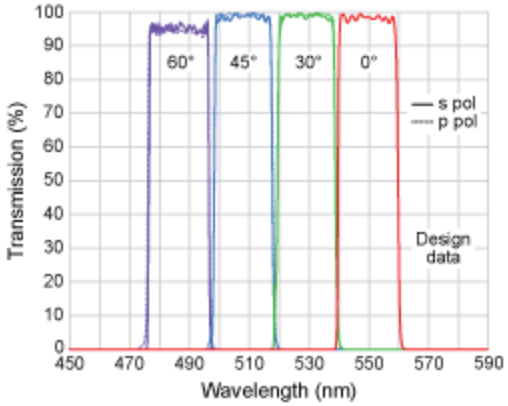
Leica SP5



www.leica.de

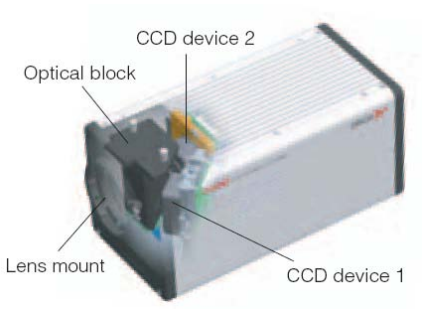
Additional recent developments

Tuneable bandpass filters



www.ahf.de

Dual-wavelength cameras



www.hamamatsu.com

Emission fingerprinting

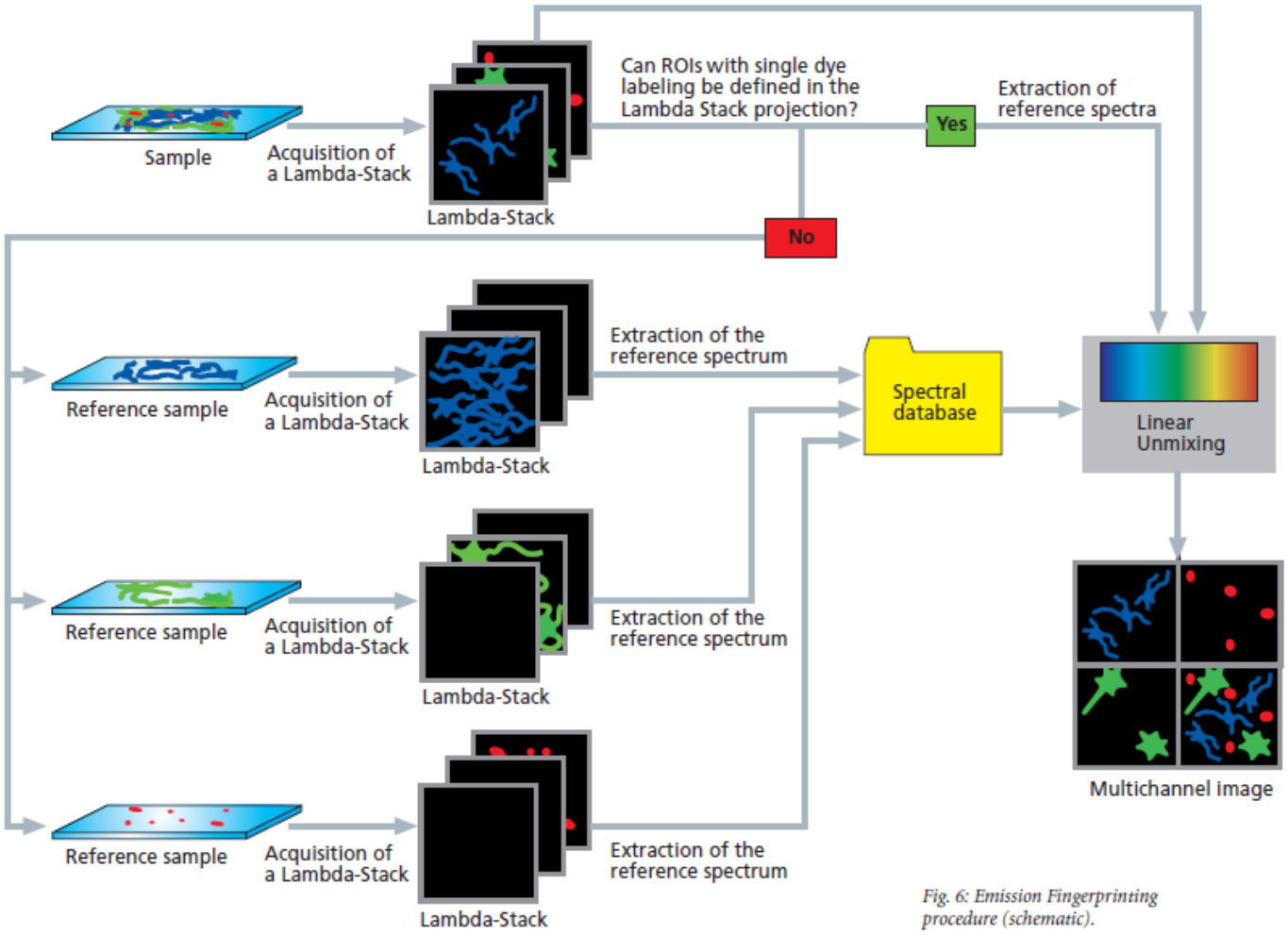
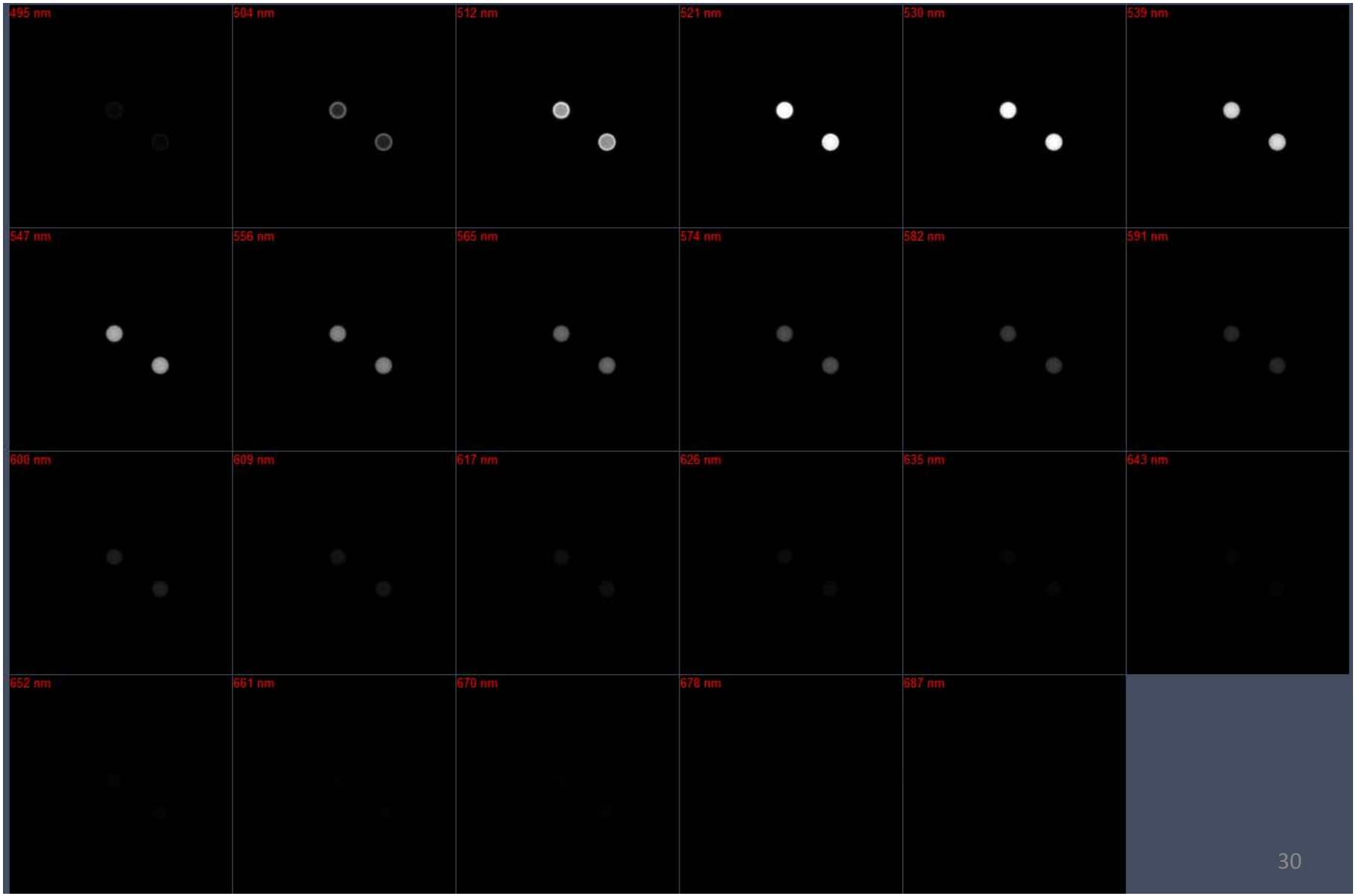
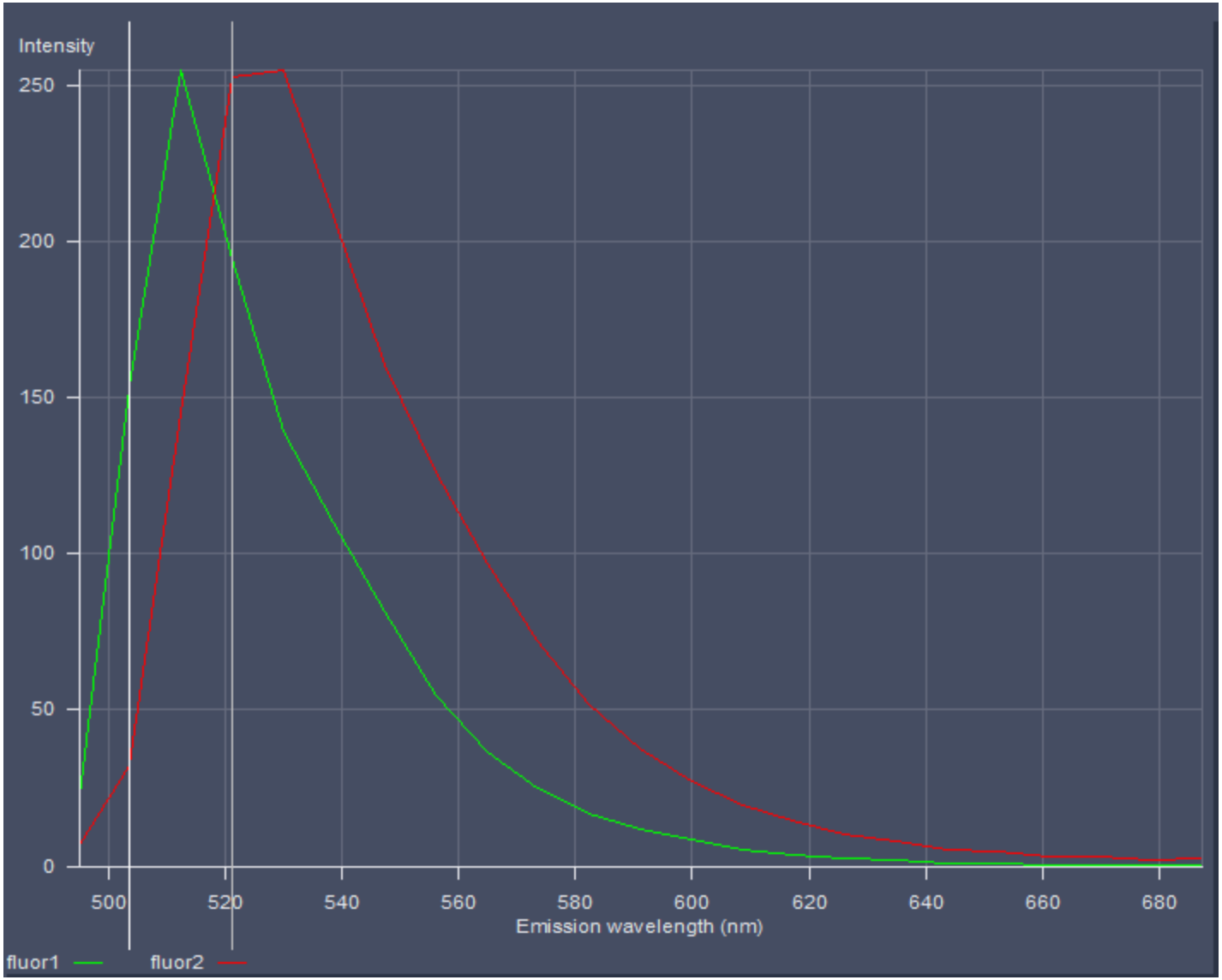
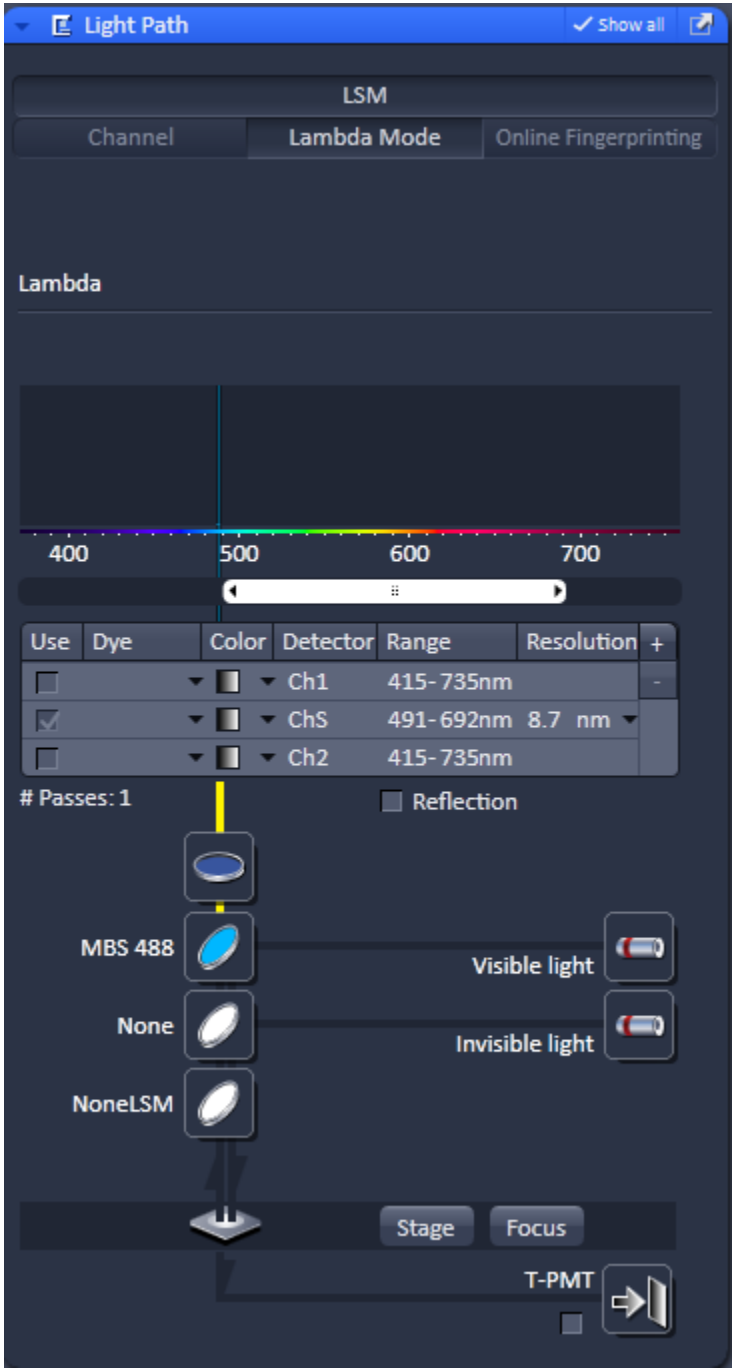


Fig. 6: Emission Fingerprinting procedure (schematic).

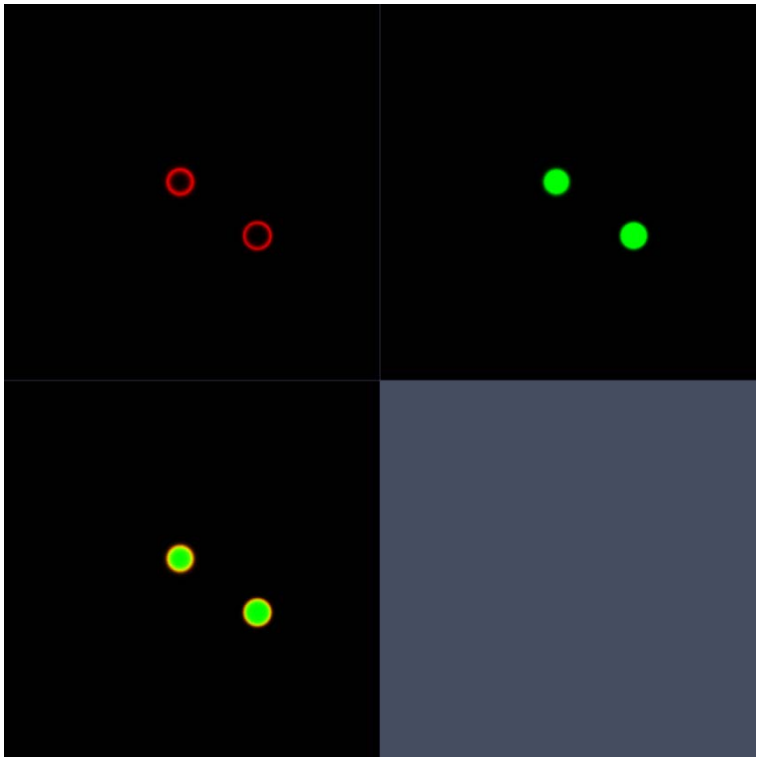
Example from M21

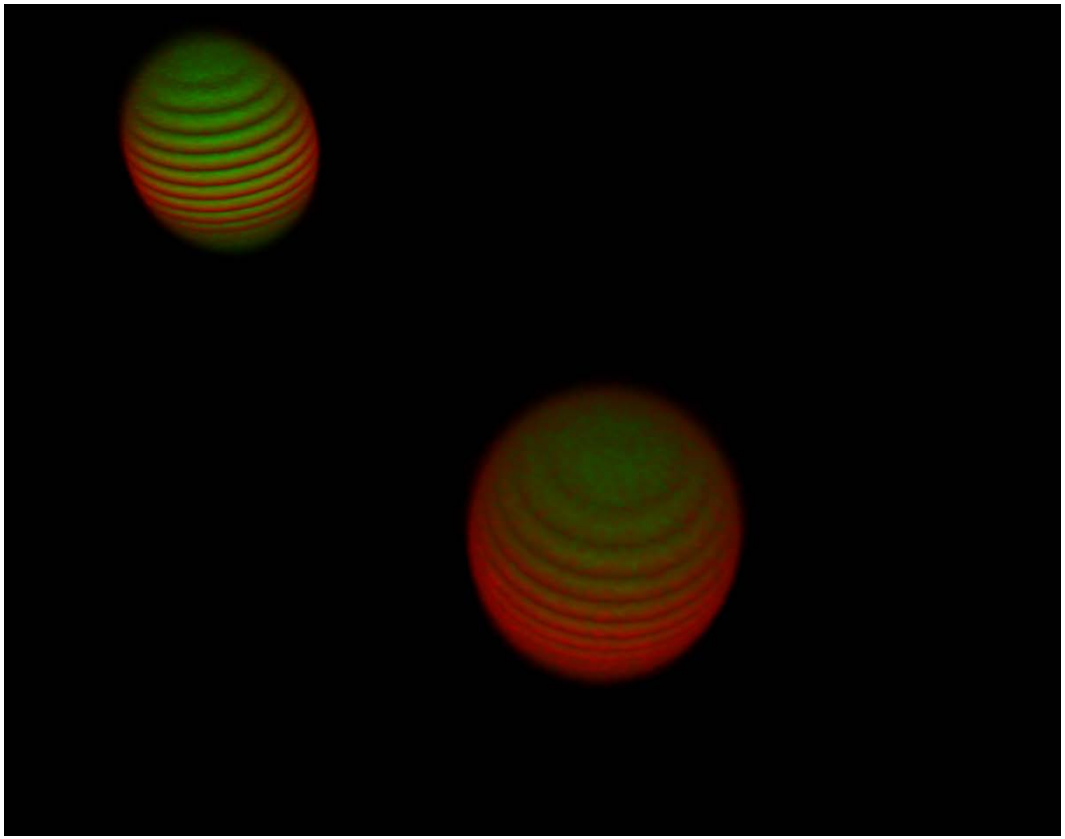


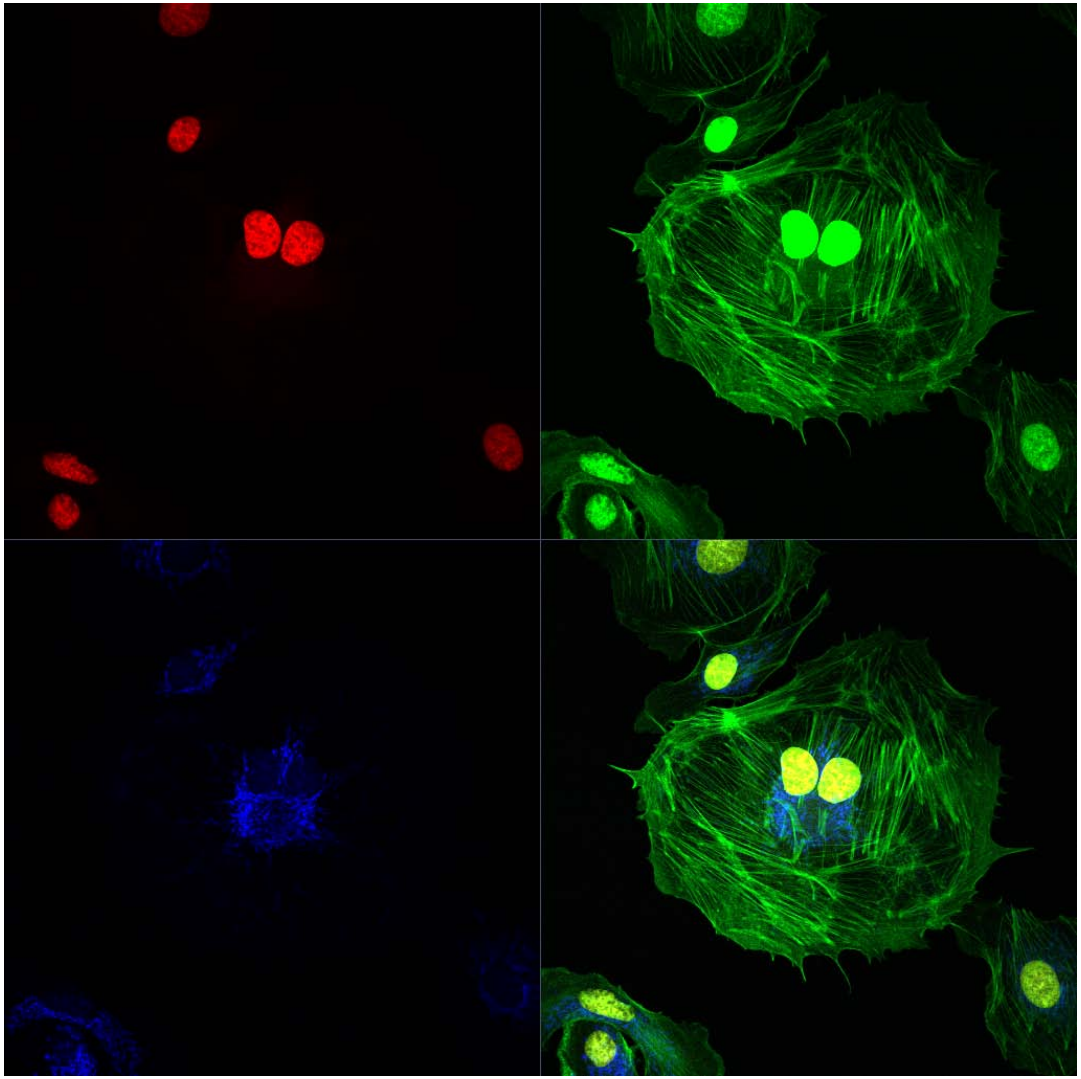

Intensity
250
200
150
100
50
0
500
520
540
560
580
600
620
640
660
680
Emission wavelength (nm)
fluor1
fluor2

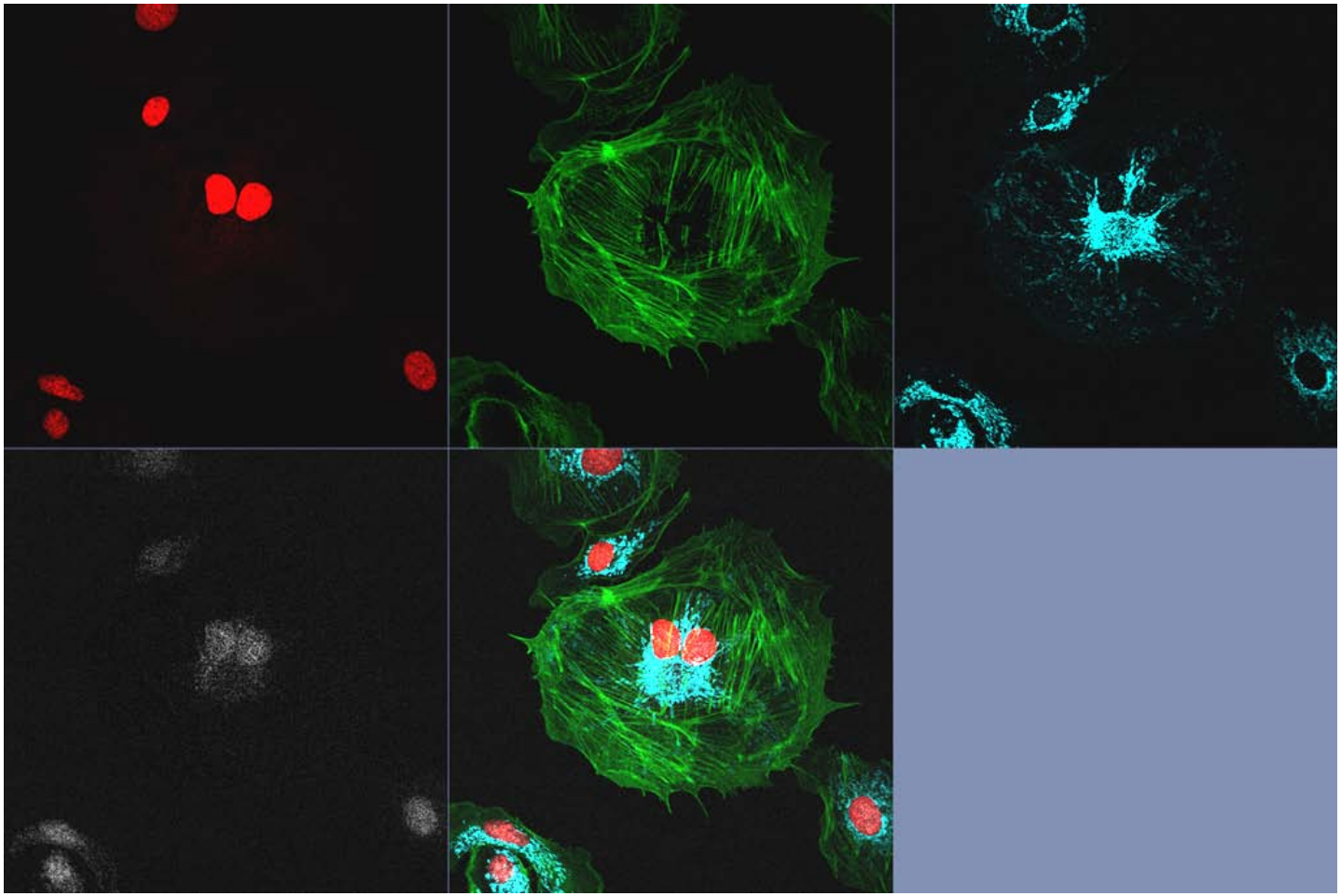

Light Path
Show all
LSM
Channel
Lambda Mode
Online Fingerprinting
Lambda
400
500
600
700
Use
Dye
Color
Detector
Range
Resolution
+
Ch1
415-735nm
ChS
491-692nm
8.7
nm
Ch2
415-735nm
Passes: 1
Reflection
MBS 488
None
NoneLSM
Visible light
Invisible light
Stage
Focus
T-PMT

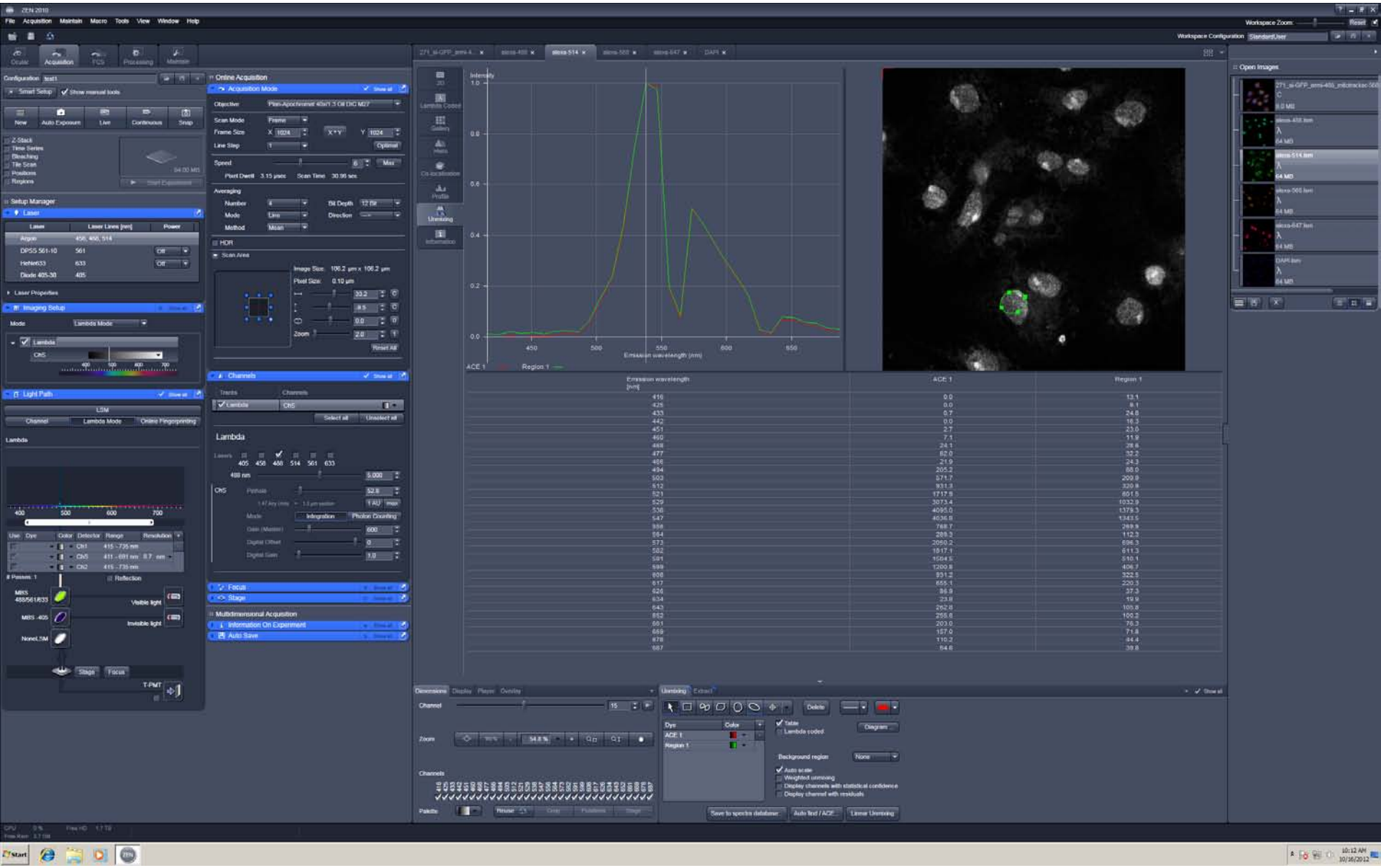

Lambda
400
500
600
700
Use
Dye
Color
Detector
Range
Resolution
+
fluor1
RS1
491-692nm
8.7
nm
fluor2
RS2
491-692nm
Passes: 1
Reflection

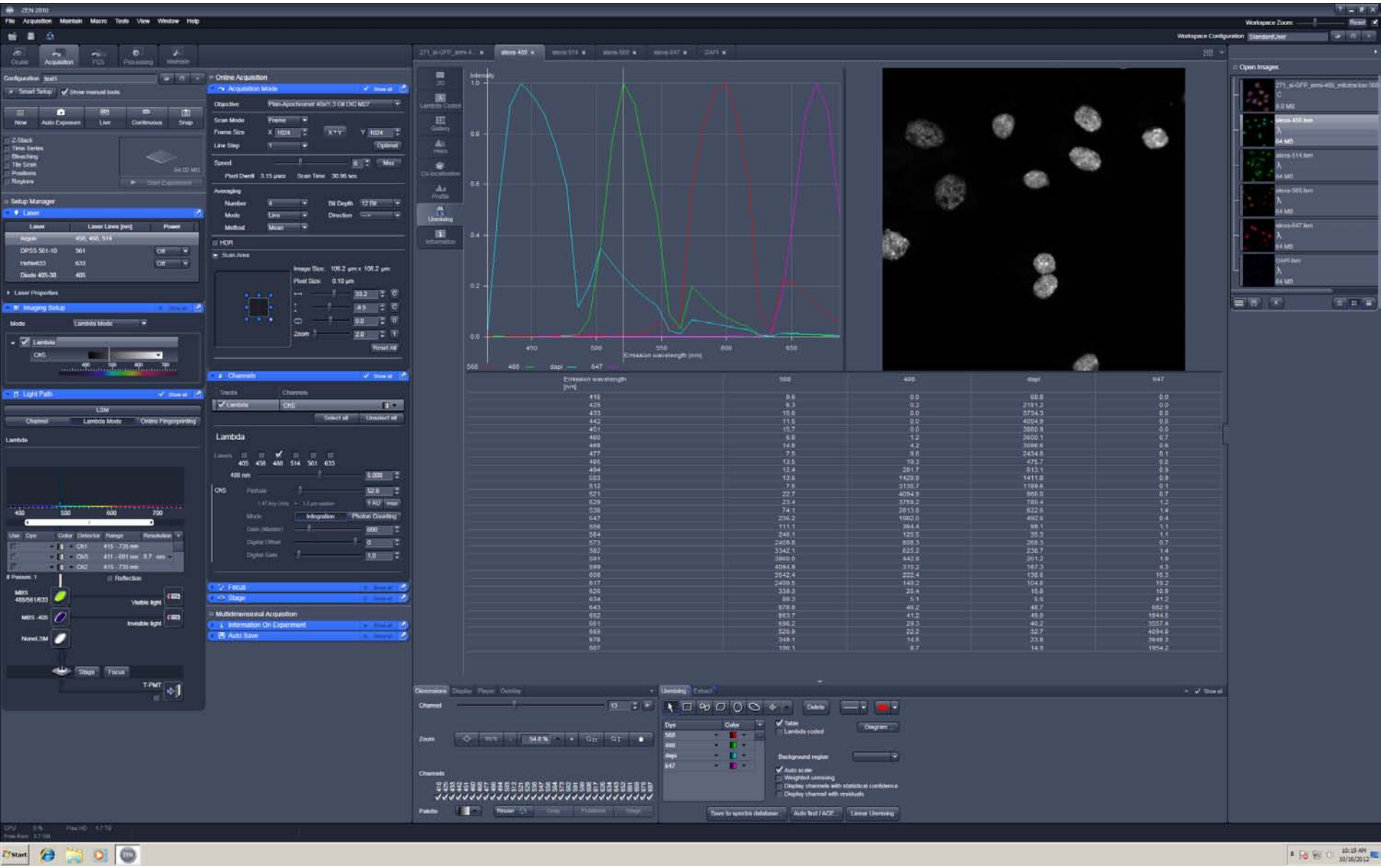


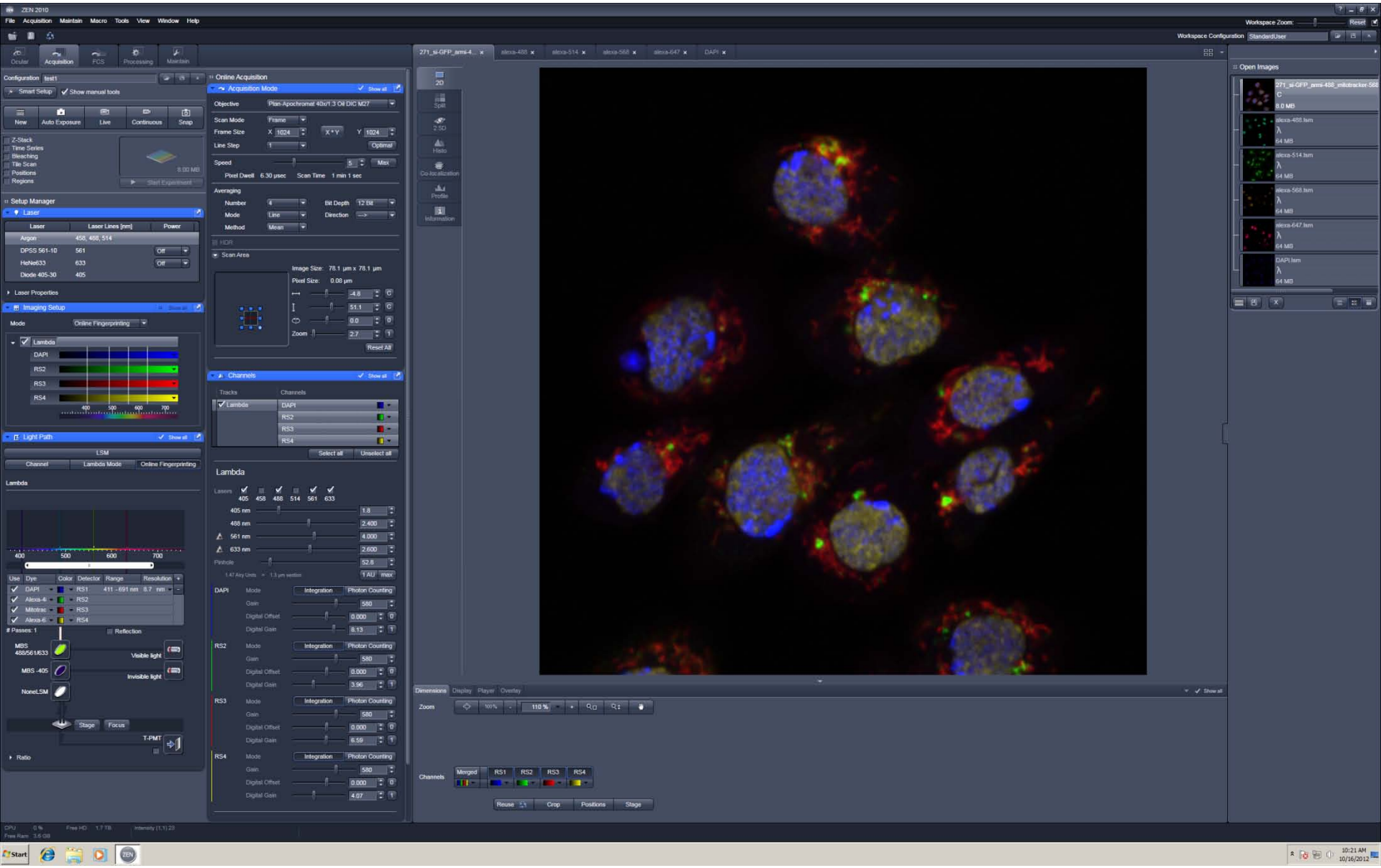









ZEN 2010
File Acquisition Maintain Macro Tools View Window Help
Online Acquisition
Acquisition Mode
Setup Manager
Laser
Imaging Setup
Light Path
Channels
Lambda
Focus
Stage
Multidimensional Acquisition
Information On Experiment
Auto Save
Open Images
Intensity
Emission wavelength (nm)
568
488
dapi
647


ZEN 2010
File Acquisition Maintain Macro Tools View Window Help
Ocular Acquisition FCS Processing Maintain
Online Acquisition
Acquisition Mode
Objective Plan-Apochromat 40x/1.3 Oil DIC M27
Scan Mode Frame
Frame Size X 1024 Y 1024
Line Step 1
Speed 5
Pixel Dwell 6.30 µsec Scan Time 1 min 1 sec
Averaging
Number 4 Bit Depth 12 Bit
Mode Line Direction
Method Mean
Scan Area
Image Size: 78.1 µm x 78.1 µm
Pixel Size: 0.08 µm
Zoom 2.7
Reset All
Setup Manager
Laser
Laser Lines [nm] Power
Argon 458, 488, 514
DPSS 561-10 561
HeNe633 633
Diode 405-30 405
Laser Properties
Imaging Setup
Mode Online Fingerprinting
Lambda
DAPI
RS2
RS3
RS4
Light Path
LSM
Channel Lambda Mode Online Fingerprinting
Lambda
Use Dye Color Detector Range Resolution
DAPI RS1 411 - 691 nm 8.7 nm
Alexa-4 RS2
Mitotrac RS3
Alexa-6 RS4
Passes: 1
Reflection
MBS 488/561/633
MBS -405
NoneLSM
Visible light
Invisible light
Stage Focus
T-PMT
Ratio
Channels
Tracks Channels
Lambda DAPI
RS2
RS3
RS4
Select all Unselect all
Lambda
Lasers 405 458 488 514 561 633
405 nm 1.8
488 nm 2.400
561 nm 4.000
633 nm 2.600
Pinhole 52.8
1 AU max
DAPI Mode Integration Photon Counting
Gain 580
Digital Offset 0.000
Digital Gain 8.13
RS2 Mode Integration Photon Counting
Gain 580
Digital Offset 0.000
Digital Gain 3.96
RS3 Mode Integration Photon Counting
Gain 580
Digital Offset 0.000
Digital Gain 6.59
RS4 Mode Integration Photon Counting
Gain 580
Digital Offset 0.000
Digital Gain 4.07
271_si-GFP_arms-4...
alexa-488
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DAPI
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2.5D
Histo
Co-localization
Profile
Information
Open Images
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alexa-488.lsm
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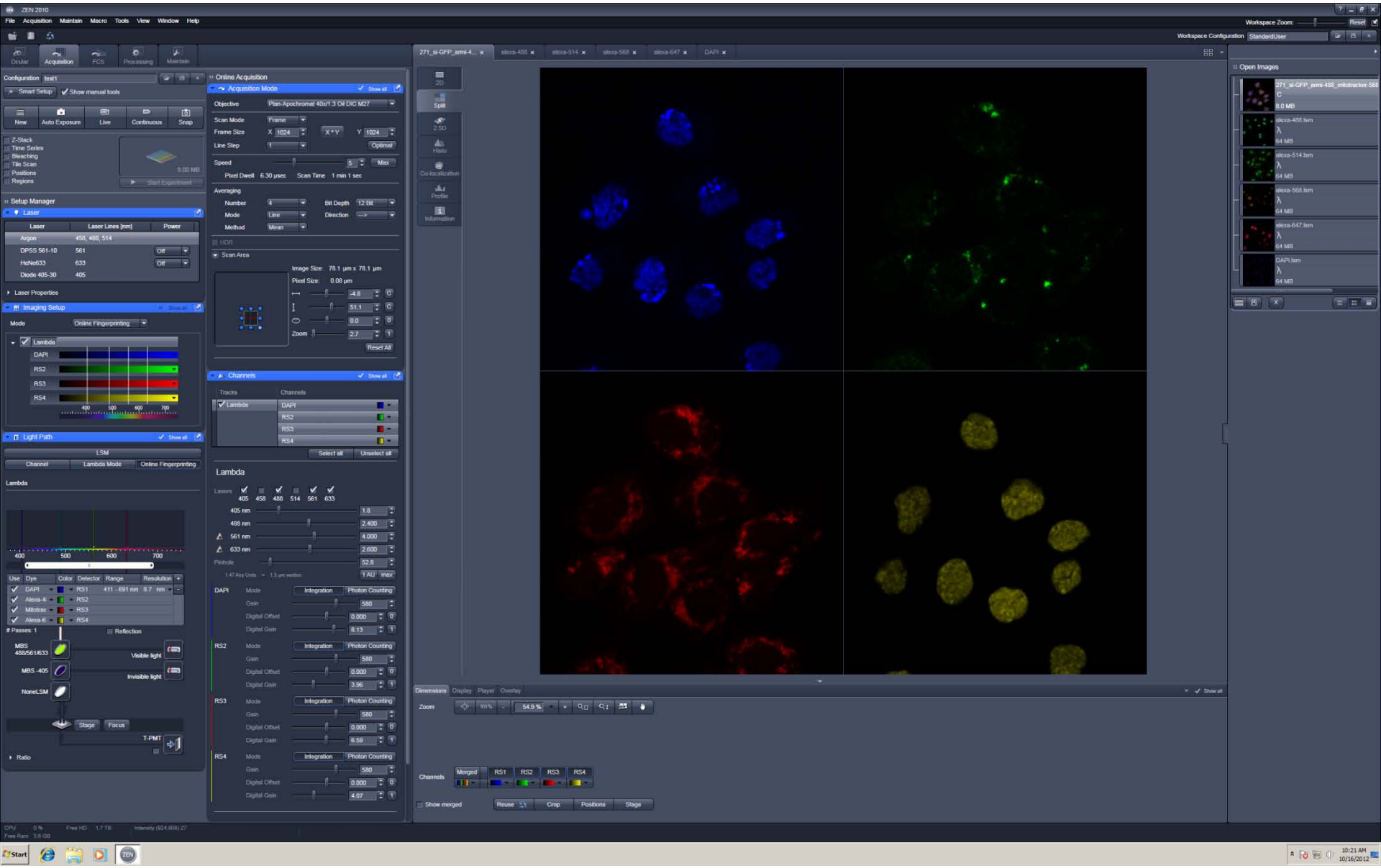
alexa-568.lsm
alexa-647.lsm
DAPI.lsm
Dimensions Display Player Overlay
Zoom 110 %
Channels Merged RS1 RS2 RS3 RS4
Reuse Crop Positions Stage
Workspace Zoom:
Workspace Configuration StandardUser


ZEN 2010
File Acquisition Maintain Macro Tools View Window Help
Workspace Zoom:
Workspace Configuration StandardUser
Ocular Acquisition FCS Processing Maintain
Configuration test1
Smart Setup
Show manual tools
New Auto Exposure Live Continuous Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
Start Experiment
Setup Manager
Laser
Laser Lines [nm]
Power
Argon 458, 488, 514
DPSS 561-10 561
HeNe633 633
Diode 405-30 405
Laser Properties
Imaging Setup
Mode Online Fingerprinting
Lambda
DAPI
RS2
RS3
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Light Path
LSM
Channel
Lambda Mode
Online Fingerprinting
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Use Dye Color Detector Range Resolution
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Reflection
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Visible light
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Stage
Focus
T-PMT
Ratio
Online Acquisition
Acquisition Mode
Objective Plan-Apochromat 40x/1.3 Oil DIC M27
Scan Mode Frame
Frame Size X 1024 X*Y Y 1024
Line Step 1
Optimal
Speed 5 Max
Pixel Dwell 6.30 µsec Scan Time 1 min 1 sec
Averaging
Number 4
Bit Depth 12 Bit
Mode Line
Direction
Method Mean
HDR
Scan Area
Image Size: 78.1 µm x 78.1 µm
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Zoom 2.7
Reset All
Channels
Tracks
Channels
Lambda
DAPI
RS2
RS3
RS4
Select all
Unselect all
Lambda
Lasers 405 458 488 514 561 633
405 nm 1.8
488 nm 2.400
561 nm 4.000
633 nm 2.600
Pinhole 52.8
1 AU max
DAPI Mode Integration Photon Counting
Gain 580
Digital Offset 0.000
Digital Gain 8.13
RS2 Mode Integration Photon Counting
Gain 580
Digital Offset 0.000
Digital Gain 3.96
RS3 Mode Integration Photon Counting
Gain 580
Digital Offset 0.000
Digital Gain 6.59
RS4 Mode Integration Photon Counting
Gain 580
Digital Offset 0.000
Digital Gain 4.07
271_si-GFP_arms-4...
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alexa-568
alexa-647
DAPI
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2.5D
Histo
Co-localization
Profile
Information
Open Images
271_si-GFP_arms-488_mitotracker-568
8.0 MB
alexa-488.lsm
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alexa-514.lsm
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DAPI.lsm
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Dimensions Display Player Overlay
Zoom 54.9 %
Channels Merged RS1 RS2 RS3 RS4
Show merged
Reuse Crop Positions Stage
CPU 0 %
Free HD 1.7 TB
Free Ram 3.6 GB
Intensity (924,608) 27
Start
10:21 AM
10/16/2012

How to do the acquisition ?

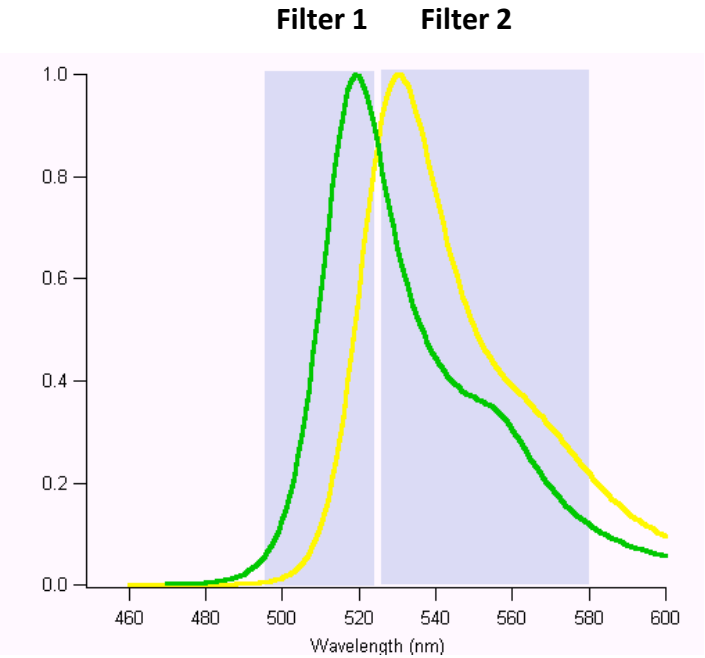
1. Never oversaturate the images – information lost!!!

2. Keep the hardware settings same for reference spectra and actual spectral image:

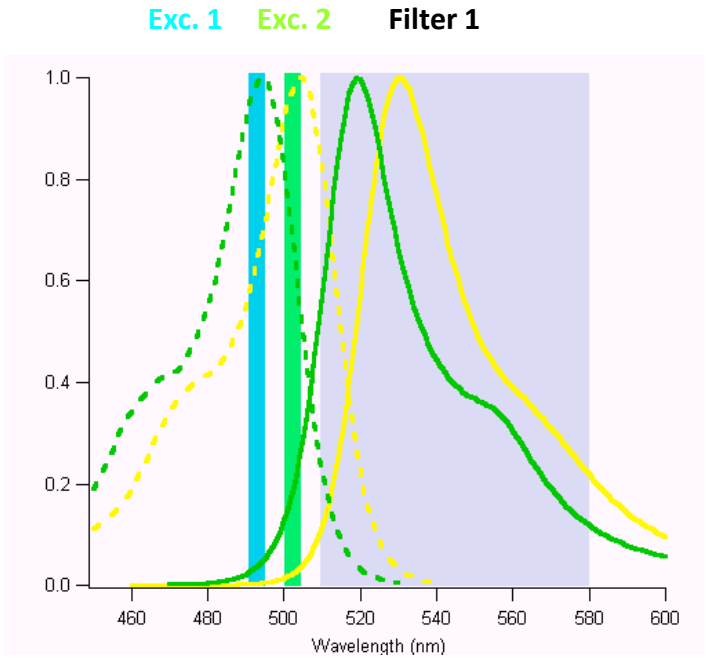
   Same beamsplitters
   Same detection window
   Same number of channels

3. Include all the fluorophores present in your sample during acquisition for reference spectra

Unmixing on the excitation side



Emission unmixing



Excitation unmixing

Only one wide emission filter required
=> high detection efficiency

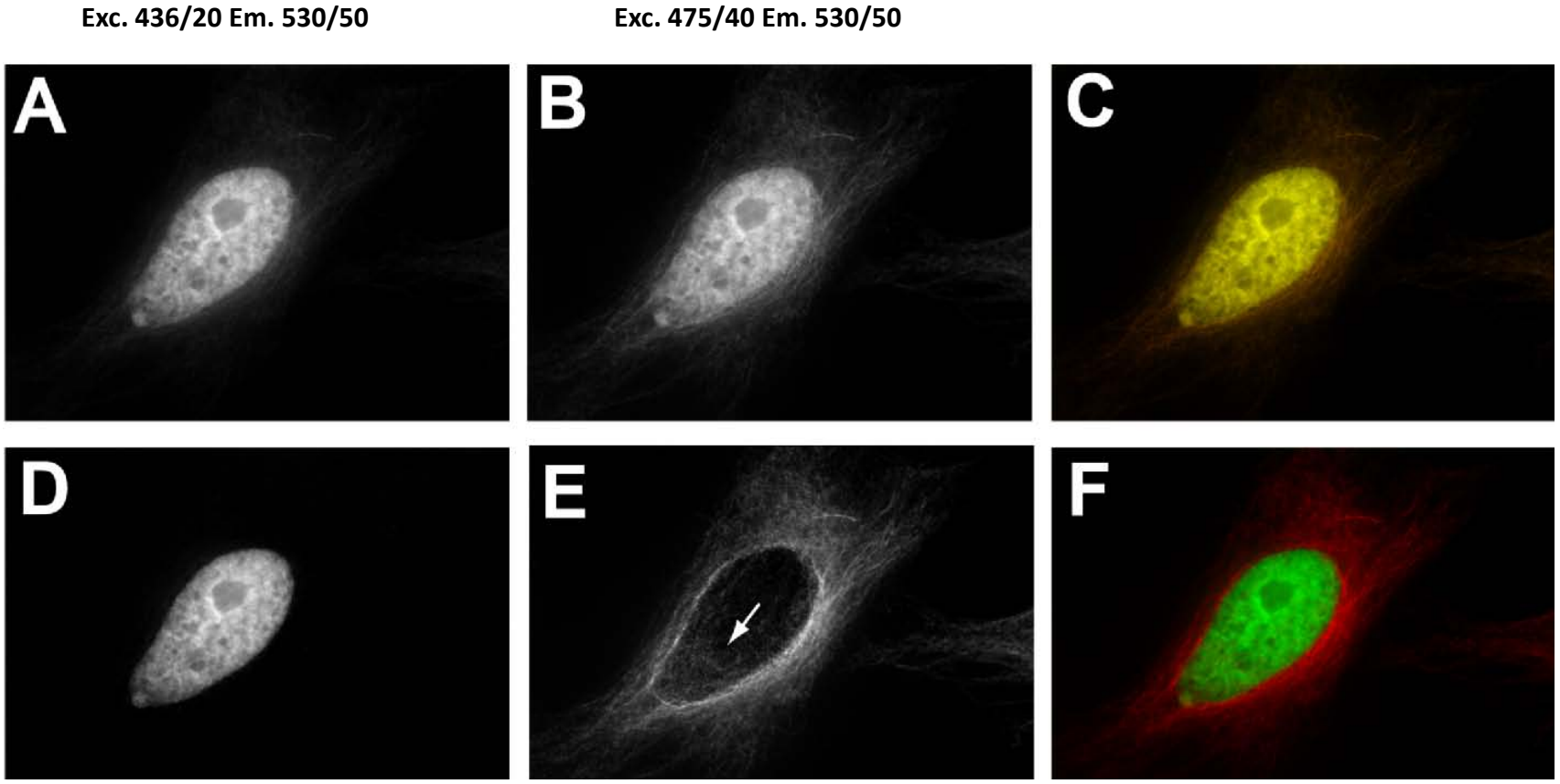
Linear unmixing formula identical to emission unmixing

-Exc. Filterwheel
- Monochromator
- Laserlines

However: Sequential

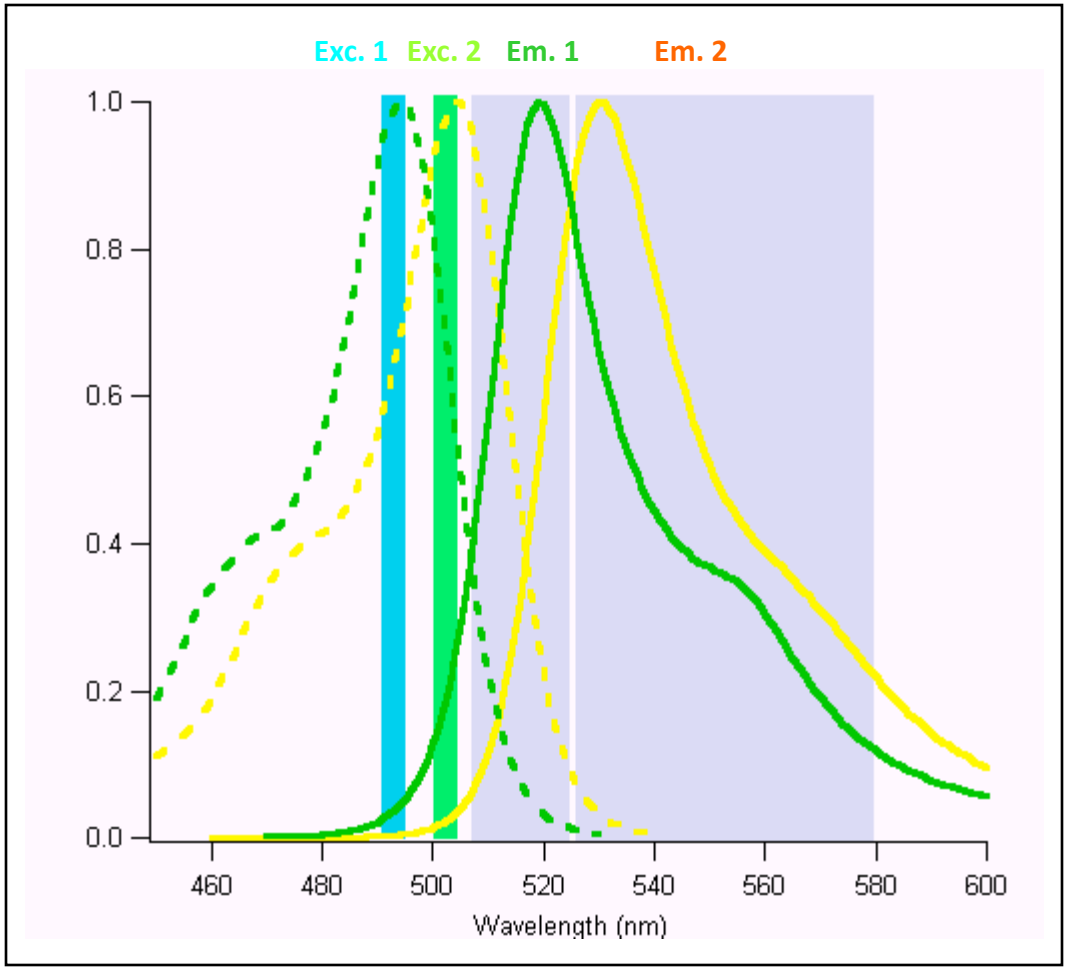
Zimmermann, 2005;

Unmixing on the excitation side



Zimmermann, 2005;

Combination of excitation and emission unmixing

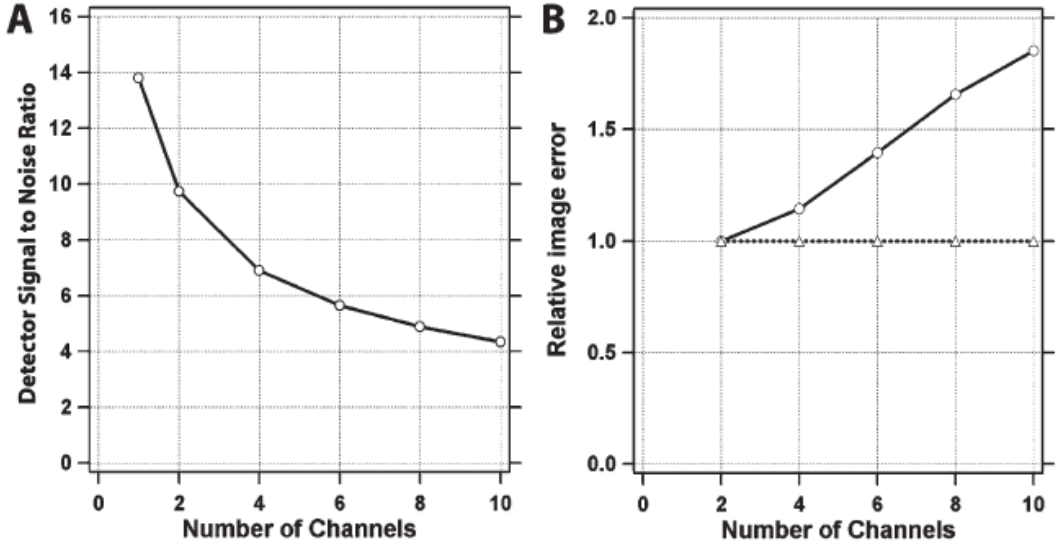


2 excitation + 2 emission filters => 4 distinct channels:
a) Exc.1/Em.1 b) Exc.1/Em.2 c) Exc.2/Em.1 d) Exc.2/Em.2
=> Separation of up to 4 fluorophores possible

Zimmermann, 2005;

Limitations

Examples of factors influencing the efficiency of spectral unmixing



A. Decrease of the detector signal to noise ratio in dependence of the number of channels used for sampling the spectral information
B. Dependence of unmixing efficiency on the number of detection channels in the absence and presence of noise.

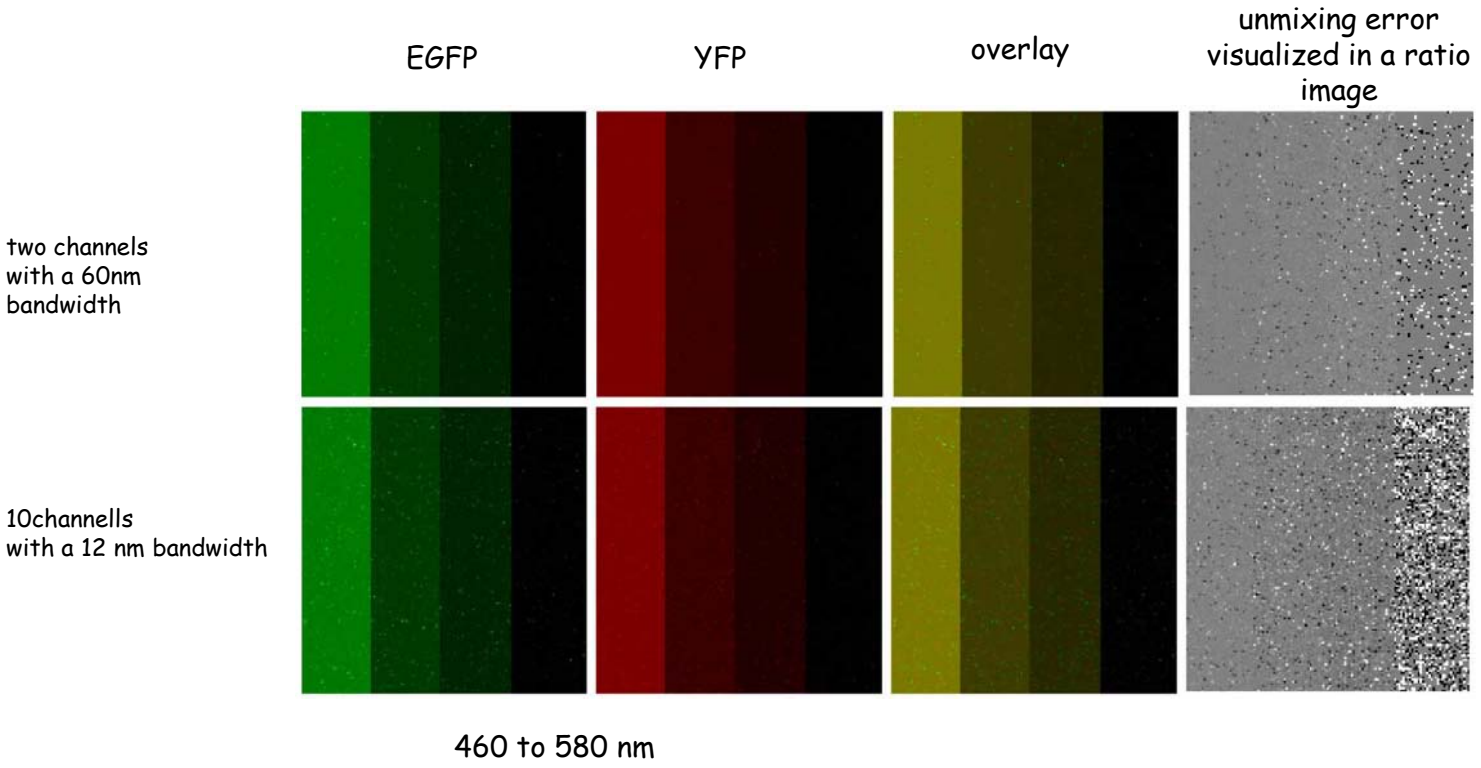
The relative increase of the unmixing error is independent of the actual noise level. Image error values in the graph are normalized to the image error obtained for two channel unmixing.

Solid line with circles: relative image error in the presence of detector noise. Broken line with triangles: relative image error without noise

The dSNR decreases according to n–1/2 and thus **sampling the spectral information into few detection channels with broad bandwidth should result in superior quality of the unmixed data compared to sampling into a large number of detection channels with narrow bandwidths**. This observation is relevant for measurements with high detector readout noise or with low signals

Zimmermann, 2005

Influence of detector noise and the number of channels on unmixing efficiency



For this purpose, test datasets of simulated EGFP (green) and YFP signals (red) of varying intensities were generated and combined with empty images acquired on a Zeiss LSM510confoca l microscope under speed and sensitivity settings suitable for in vivo imaging. These background images introduce realistic readout noise into the simulated images. The created gray values in the EGFP and YFP images are identical and thus co-localize with a 1:1 ratio in every image pixel and thus the signals appear in the overlay images (third column) as yellow. The number of detectors covering the spectral range between 460and 580 nm was either set to two (top row, each channel with a 60nm bandwidth) or 10channels (bottom row, each channel with a 12 nm bandwidth). The unmixing error for two and 10 channels can be visualized in a ratio image (gray scale) of the unmixed EGFP and YFP images. Correctly unmixed pixels should have values of 1.0(gray) whereas deviations are visible as darker or brighter pixels. The ratio image created with two channels (top row) contains less noise errors than the image created with 10 channels (bottom row). As can be seen for the stripes of deferent intensities, the errors become more significant for weaker intensities in the image.

Zimmermann, 2003

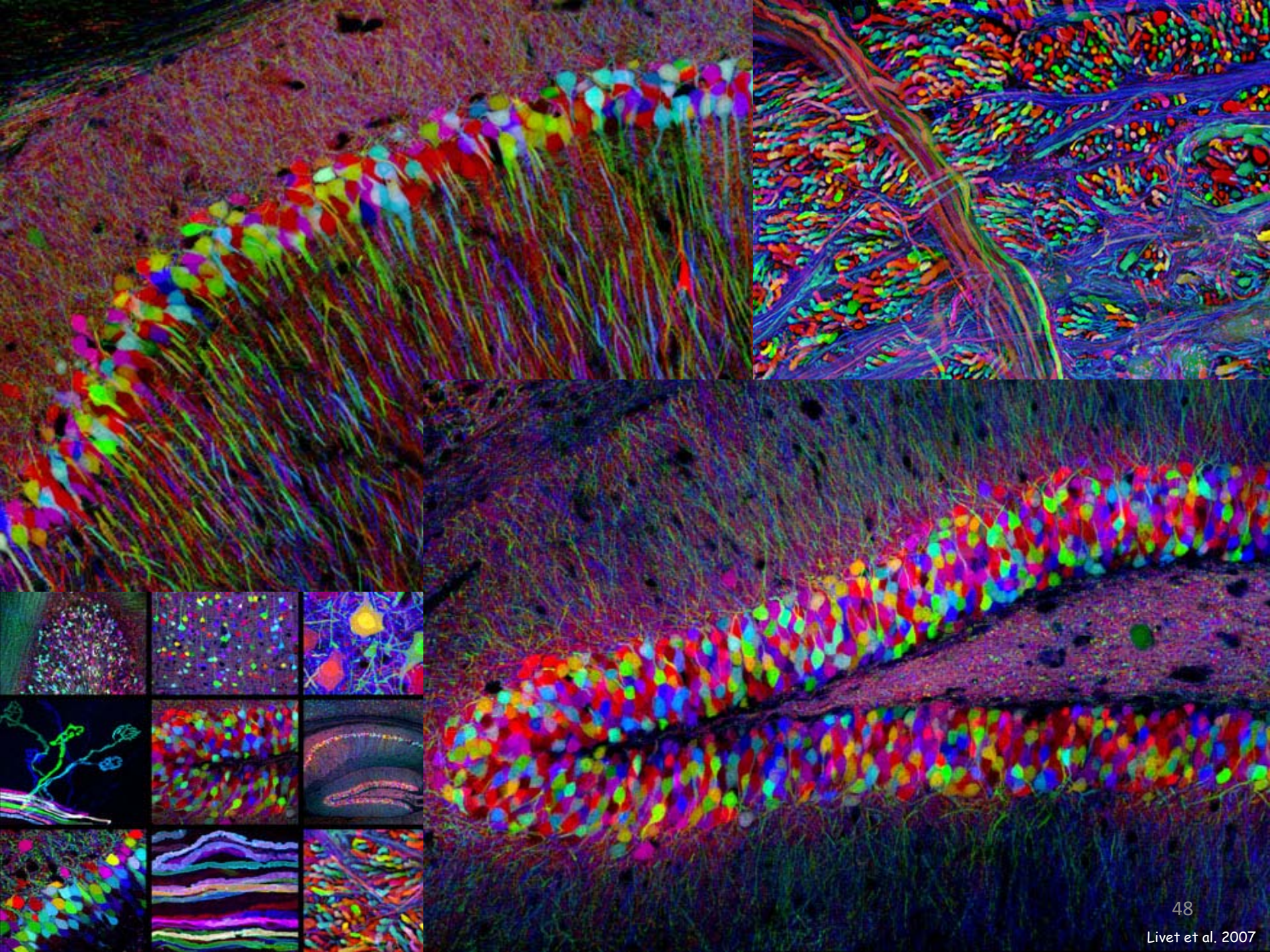
Take home message IV

The best unmixing results are obtained with few and wide channels as this gives higher signals per channel and minimizes possible readout noise problems.

If the gains of the channels can be set independently, the separability of the signals can be enhanced in this way. Such an approach does however require fine tuning of the settings, as only optimized settings will give an improved result. Not properly chosen settings will give inferior results.

Over-determined systems may not inherently give better results, but can be used without the fine tuning of filter settings.

Zimmermann, 2005



Livet et al. 2007