Confocal characterization and imaging calibration, SIPcharts.

Confocal fluorescence microscopy: characterization of imaging and calibration using thin uniform fluorescence layers, SIPcharts.

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Varenna 12 Juli 2010      International School of Physics Enrico Fermi
Advanced methods in optical fluorescence microscopy towards nanoscopy
Imaging by a lens
**Shading** or non-uniform intensity imaging:

![Diagram showing shading and focal points](image)

Object intensity → Image intensity

Focal point

Vignetting: partial block of light rays

Lower intensity at the edges!

Image of uniform layer:
Problems in microscopy affecting quantitative imaging

Shading effects

Imaging conditions difficult to characterize

Imaging conditions difficult to reproduce

Non-quantitative intensity imaging

Present both

in 2D - regular microscopy

3D - confocal microscopy

Propose correction/calibration procedures based on

Thin uniform fluorescent layers

Sectioned Imaging Property or SIPcharts analysis
Basis:
Spatially uniform fluorescing reference layer

0.01% fluorescing compound in polyvinylalcohol spin-coated on microscope slide.

Protected and sealed with a microscope cover glass

Thickness 100 to 200 nm.
Thickness uniform within a few %

Reference layers have to be to a high degree

spatially uniform and reproducible.
concentration of
N,N'-bis(2,5-di-tert-butylphenyl)-3,4,9,10-perylenebis(dicaboximide) in polystyrene/toluene

0.01 w% in polystyrene

congestion measured with absorption spectroscopy (ε(490nm) = 86000 l•mol•cm⁻¹) -> A = 0.09 -> 3.0•10⁶ mol/l -> in a layer of 100 nm thickness this leads to
1.8•10¹⁰ molecules/cm²
Average distance between molecules about 100 nm.

No quenching effects!
• Absorption spectrum

• Emission spectrum
Fluorescence specimen image:

\[ P_s(x,y) = I(x,y) \cdot D(x,y) \cdot F_s(x,y) \]

- illumination
- detection
- specimen fluorescence

Reference layer image: taken under same conditions!

\[ P_r(x,y) = I(x,y) \cdot D(x,y) \cdot F_r \]

Reference layer fluorescence: uniform

Calibrated image \( P_c(x,y) \)

\[ \frac{P_s(x,y)}{P_r(x,y)} = \frac{F(x,y)}{F_r} = \text{(FLU's)} \]

expressed in Fluorescence (of the reference) Layer Units

\"FLU's\"

Actual imaging conditions as described by \( I(x,y) \cdot D(x,y) \) have dropped out!

Specimen: Lipid DPPC two phase monolayer segregation

2D (regular microscopy)
Shading correction

Microscope
20x image

Microscope
40 x image

Images of object

Images of reference layer

Calibrated images

QuickTime™ and a TIFF (PackBits) decompressor are needed to see this picture.

Images with different magnifications can be well correlated!
Application:  
**Shading correction**

Specimen:  
Lipid DPPC monolayer,  
two phases  
with low (LC) and high (LE)  
NPB fluorescencer concentration

before shading correction  
after shading correction

A

B

c)  
d)
Are the reference layers really uniform?

Two images of the reference layer taken at two different locations are used one as image to be calibrated and the other as reference image.

Proves that our fluorescent reference layers for the calibration that are to a high degree

**spatially uniform**

2D (regular microscopy)
Image calibration in fluorescence microscopy

J. M. Zwier, G. J. Van Rooij, J. W. Hofstraat & G. J. Brakenhoff

J. of Micr., 216, 15-24, 2004
Confocal fluorescence microscopy sectioned images

(Anaphase, Allium Cepa (onion), DNA stain: mitrmycin)
Sectioned image characterization with thin uniform fluorescent layers

Method:
Making a z-stack (through focus) of the uniform polymer fluorescence reference layer (ca. 100 nm)

Through focus stack
Sectioned image characterization with thin uniform fluorescent layers

Axial Point Spread Function

Through-focus stack of “thin”
uniform fluorescence reference layer

a)

I intensity

1

2

3

4

5

b)

Super position plot axial PSF
responses over image field

c)

Confocal trough-focus response: local off axis responses
Sectioned image characterization with thin uniform fluorescent layers.

Layer thickness thin with respect to axial confocal dimensions. **Essential!**
How to characterize the spatial imaging conditions in confocal and (later) multi-photon microscopy?

Proposed parameters for characterization:

- $s = \frac{a}{b}$ skew
- $m$ max intensity
- FWHM resolution
- $Z_m$ axial position of maximum
- Laterally Integrated Intensity
Analysis of confocal imaging conditions

Resolution variation over image

exp 230603  100nm layer
pinhole 1 Airy
Sectioned image characterization with thin uniform fluorescent layers

x, y confocal intensity distribution

Confocal imaging determined by overlap of illumination and detection distributions

Beam scanning:
- no aberration
- off-axis chromatic aberration

Int. vs. x

Int. vs. x

Avg = 8.20187e+06
SD = 306548

s = a/b skew

m max intensity

fwhm resolution

z_m axial position of maximum
How to standardize the characterization of the spatial imaging conditions in confocal and (later) multi-photon microscopy?

Propose:

Sectioned Imaging Property charts

or

SIPcharts
Sectioned image characterization with thin uniform fluorescent layers

**I_{total}**

- **Avg** = 90.6395
- **SD** = 4.68024

**I_{max}**

- **Avg** = 79.9347
- **SD** = 10.0326

**Z_{max}**

- **Avg** = 6012.89
- **SD** = 75.4975

**fwhm**

- **Avg** = 1026.68
- **SD** = 92.209

**skew**

- **Avg** = -0.0488328
- **SD** = 0.0299356

**Axial PSF**

- **skew** = \( \frac{a - b}{a + b} \)

**SIP Chart**

- **Itot** integrated intensity under axial PSF
- **Z_{max}** axial position of maximum

**Sectioned Imaging Property chart**

- **I_{max}** max intensity
- **fwhm** resolution
- **I_{tot}** integrated intensity under axial PSF
Analysis 3D image formation with SIPcharts
Sectioned image characterization with thin uniform fluorescent layers

Comparing confocal systems, system1

SIP Chart

Confocal System 1
Obj. 63x, oil, 1.4 NA pinhole 1 Airy
NKI 12-08-04 Bin 8 from 512*512

<table>
<thead>
<tr>
<th>Point</th>
<th>params</th>
<th>bin_avg</th>
<th>bin_sd</th>
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<tr>
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<td>10.0326</td>
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<tr>
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<td>6012.89</td>
<td>75.4975</td>
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<tr>
<td>2</td>
<td>fwhm</td>
<td>1026.68</td>
<td>92.209</td>
</tr>
<tr>
<td>3</td>
<td>skew</td>
<td>-0.0488328</td>
<td>0.0299356</td>
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<tr>
<td>4</td>
<td>$I_{\text{total}}$</td>
<td>90.6395</td>
<td>4.68024</td>
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</tbody>
</table>

Point params

$\text{bin avg}$ $\text{bin sd}$

$0 I_{\text{max}} 79.9347 10.0326 1 z_{\text{max}} 6012.89 75.4975 2 fwhm 1026.68 92.209 3 skew -0.0488328 0.0299356 4 I_{\text{total}} 90.6395 4.68024$
Sectioned image characterization with thin uniform fluorescent layers

Comparing confocal systems, system 2

<table>
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<tr>
<td>2</td>
<td>fwhm</td>
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<td>skew</td>
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<tr>
<td>4</td>
<td>l_total</td>
<td>81.7096</td>
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SIP Chart

Confocal System 2

Obj. 63x, oil, 1.4 NA pinhole 1 Airy

CLSM 04-05-04 no4 Bin 8 from 512*512

Fluorescence Int. (10^3)

Fwhm resolution: Zmax axial position of maximum

Axial PSF: \( s = \frac{a - b}{a + b} \)
Sectioned image characterization with thin uniform fluorescent layers

Comparing confocal systems

Comparison resolution

System 1

Avg = 1026.68
SD = 92.209

fwhm

System 2

Avg = 1194.62
SD = 66.1558

fwhm
Sectioned image characterization with thin uniform fluorescent layers

**Comparison of lenses:**
Overview from 4 SIPcharts each taken with a different 63x objective

Conclusion: easy to spot differences between lenses: quality control!
4 Objectives: resolution at pinhole 1 Airy equiv.

**objective 1**
63x oil immersion
170204 fred010

**objective 2** phase contrast
63x oil immersion
170204 fred012

**objective 3**
63x oil immersion
170204 fred013

**objective 4**
100 x oil immersion
170204 fred014
Confocal course with partial beam block

Leica TCS NA= 1.4 63X with partial beam block
Confocal course without partial beam block

Leica TCS
NA= 1.4
63X
Without partial beam block

www.SIPchart.org

Description: Leica TCS SL new dataset 11.14.2007
Processed on C2P7 10/11/03 09:17 by SIP 1.0 (version of 25 May 2002 09:47)
Run time parameters: NA=1.4, A=0.0944, F=0.0511, Bin factor: 1

Average Std. dev.

Imax 13.334 13.336
Zmax 3.717 0.215
FWHM 0.951 0.003
Skew -0.142 0.154
Total 0.176 0.340

Image intensity

with partial block removed
Leica SP2-2
NA= 1.2
Confocal course function NA

Leica SP2-4
NA= 1.4
100X
Sipchart_after_cleaning objective

www.SIPchart.org

Description: Zeiss ism510 63x 1.40 na schoonmaak
Processed on 2009-09-23 10:21:03 (UTC) by SIP 1.0 (version of 14-Sep-2009 23:39)
Runtime parameters: NA=1.4, λex=488, λem=565, Bin factor=8

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<td>Z_max</td>
<td>3.987</td>
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<td>FWHM</td>
<td>0.795</td>
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<td>Skew</td>
<td>0.085</td>
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Background: 1.8

Recorded fluorescence intensity

Z-profiles
Spinning Disk -1.1.pdf

www.SIPchart.org

Description: PerkinElmer1-512-2
Processed on 2007-10-11 09:16:02 (UTC) by SIP 1.0 (version of 29-Nov-2006 16:47)
Runtime parameters: NA=1.4, λex=488, λem=560, Bin factor=8

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<th></th>
<th>Average</th>
<th>Std. dev.</th>
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<tbody>
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<td>Zmax</td>
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<tr>
<td>FWHM</td>
<td>1.744</td>
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<td>Skew</td>
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<tr>
<td>Itotal</td>
<td>72.912</td>
<td>35.269</td>
</tr>
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</table>

Z-profiles

Recorded fluorescence intensity

Background: 6198.2
Dependence of imaging on proper immersion oil.

At temperatures for live cell imaging –37 C- regular oils provide not optimal imaging.

Sipchart representations show: Total photon yield is about 23% higher in the DF2 oil for 37 C then for the regular DF23 and Leica oils.

With proper immersion oil: resolution is improved together with imaging field uniformity.
Dependence of imaging on proper immersion oil. Imaging at 37C with standard immersion oil.
Dependence of imaging on proper immersion oil. Imaging at 37C with immersion oil for 37 C.
Sectioned image characterization with thin uniform fluorescent layers

**Axial image plane dependence on wavelength settings**

**Imaging wavelengths:**
- Ext: \(\lambda\) 488, detection: \(\lambda\) bp 505-530
- Excitation: \(\lambda\) 543, detection: \(\lambda\) 560-615

**Shift axial image plane with wavelength settings**

Important for co-localization and FRET etc. studies!
Multi-excitation, multi-detector confocal microscope

detection channels (PMT)

variable pinhole

dichroics

computer
display

lasers

dichroic

pinhole

scan mirror

objective
Sectioned image characterization with thin uniform fluorescent layers

3D characterization reference:

Characterization of sectioning fluorescence microscopy with thin uniform fluorescent layers: Sectioned Imaging Property or SIPcharts.

Next:

Sectioning microscopy image calibration
• SIPchart based relative and absolute image calibration

  - Relative image calibration
    • uniform sensitivity of the lateral image field (= shading compensation)
    • images can be related by expressing their intensities in
      fluorescence layer units (FLU’s)

Calibrated image $P_c(x,y)$

$$
\begin{align*}
P_c(x,y) &= \frac{P_s(x,y)}{P_r(x,y)} \cdot \frac{F(x,y) \cdot PSF(x,y)}{F_r \cdot PSF(x,y)} \\
&= \frac{F(x,y)}{F_r} \quad (FLU’s)
\end{align*}
$$

- Examples:
  • fluorescent beads
  • shading correction BPAE cells
  \textbf{Bovine pulmonary artery endothelial cells.}
  • comparison of images taken with different magnifications
Shading correction, beads

I total
SIPchart
based
• SIPchart based relative and absolute image calibration
  – Using the SIPchart data to do correction of the total intensities of image stacks
  • proof of principle on fluorospheres
Calibration imaging with different magnifications / NA

Reference layer image $P_r(x,y)$

from I total SIPchart!

Before calibration

I total SIPchart’s

after calibration in FLU’s

NA 1.32
63x
Planapo

NA 1.25
40x
Planapo
Calibration imaging with between different microscopes

Calibration between two confocal microscopes:
- SP2 Leica system
- NT Leica system
  - 63x objectives (1.32 NA) (exc 488 nm)
  - Only detection filters were somewhat different: NT - lp530 nm
    SP2 - +530 nm

• After calibration (FLU intensity scales are comparable)

• Intensities within 30%! > Small differences in spectral filtering conditions
SIPchart based absolute image calibration

– object image:

\[ F_{obj}(x, y) = QE_{obj} \cdot \sigma_{ref} \cdot N_{obj}(x, y) \cdot P_{eff}(x, y) \]

fluorescence quantum yield
absorption cross section
number of molecules

eff, PSF

– reference layer image:

\[ F_{ref}(x, y) = QE_{ref} \cdot \sigma_{ref} \cdot N_{ref} \cdot P_{eff}(x, y) \]

– ratio image:

\[ \frac{F_{obj}(x, y)}{F_{ref}(x, y)} = \frac{QE_{obj}}{QE_{ref}} \cdot \frac{\sigma_{obj}}{\sigma_{ref}} \cdot \frac{N_{obj}(x, y)}{N_{ref}}. \]
SIPchart based absolute image calibration

SIPchart based absolute image calibration

\[
\frac{F_{obj}(x,y)}{F_{ref}(x,y)} = \frac{QE_{obj}}{QE_{ref}} \cdot \frac{\sigma_{obj}}{\sigma_{ref}} \cdot \frac{N_{obj}(x,y)}{N_{ref}}.
\]

then:

\[
N_{obj}(x,y) = 1.7 \times 10^4 \cdot \frac{F_{obj}(x,y)}{F_{ref}(x,y)} \text{ mol/μm}^2
\]

Used parameter values:
QE (BodipyFL) = 0.8
QE (dtbpd ref layer) = 0.95
dtbpd (488 nm): \(\varepsilon = 84000 \text{ l.mol}^{-1}\text{cm}^{-1}\) \(\Rightarrow \sigma = 3.2 \times 10^{-16} \text{ cm}^2\)
bodipyFL (488 nm): \(\varepsilon = 60000 \text{ l.mol}^{-1}\text{cm}^{-1}\) \(\Rightarrow \sigma = 2.3 \times 10^{-16} \text{ cm}^2\)
\((\sigma = \varepsilon \times \ln 10 / N_A = 3.825 \times 10^{-21} \times \varepsilon)\)

\[N_{ref} = 1.0 \times 10^4 \text{ molecules/μm}^2\]
SIPchart based absolute image calibration

\[ N_{obj}(x,y) = 1.7 \times 10^4 \frac{F_{obj}(x,y)}{F_{ref}(x,y)} \text{ mol} / \text{µm}^2 \]

Total number of BodipyFl fluorophores in the cell: \(4.7 \times 10^7\)
Sectioned image characterization with thin uniform fluorescent layers

Comparing confocal systems

Comparison resolution
How to make a SIPchart?
**Sipchart creation**

Get a calibration layer. Collect 3D data set from this calibration layer.

Go to:
- [www.SIPchart.org](http://www.SIPchart.org)
- [www.SIPchart.com](http://www.SIPchart.com)

Click restricted area:

- **Web-application**
  - Accepts large 3D data sets
  - Automatic generation of SIPcharts
  - SIPchart delivered to user via website:
    - [www.SIPchart.org](http://www.SIPchart.org)
    - [www.SIPchart.com](http://www.SIPchart.com)

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**Introduction:** The concept of SIPcharts has been developed because there is in our view not a simple method available to characterize sectioned imaging microscopes like fluorescence confocal and two-photon microscopes in an easy way. In our view method to characterize systems like these is highly useful for anyone pursuing qualitative measurements on such systems.

We have found that a convenient way to get information about the optical setup of a confocal microscope is to make a z-scan of a thin, uniformly fluorescing deposited layer on a microscope cover glass. Key point is that the thickness of this layer is small with respect to the resolution in the z direction. Originally similar layers were developed for wide field fluorescence microscopy calibration.
We-application:
- upload page:
• Webapplication
  – Return email:

Hello,

You submitted:

H:\Backup_CLSM\H0\test-CLSMs\2006\06_11_13-AOBS\061113_AOBS_SIP\061113_AOBS_SIP_63x.zip
[Recorded length: 13517420 bytes, logged as transaction: 20061113-161415-06672]

for SIPchart processing. Other data you supplied:

dataformat: TIFFPLANES
setup: 061113 AOBS 63x
na: 1.4
emission: 540
excitation: 488
xscanstep: 0.465
yscanstep: 0.465
zscanstep: 0.122
email: l.oomen@nki.nl

If SIPchart processing was succesful you can access the results at

Mark Savenije
--
www.SIPchart.org
• Webapplication:
  – SIPchart
Sipchart creation

You need a uniform fluorescence calibration layer

Send me an email at: g.j.brakenhoff@uva.nl
    I can then send you a layer. For free or else?

The “layer project” has ended sucessfully.
    No real grounds for a continuation grant.

If the layers are usefull for maintaining and calibrating microscope performance
    they should be of some value to people.

I want to keep the project going by inviting people to buy a few layers.
    comes with unlimited website access to produce SIPcharts.
    Euro 175 each?

Money goes to the University, to pay people etc.
Application:
Generation of local 3D PSF's
Correction of datasets acquired at different wavelengths for chromatic axial and lateral image shifts
Applications SIPcharts:

Testing and Evaluation, Optimization and alignment, Maintenance of confocal systems

of sectioned imaging systems

Co-localization studies
identify axial image plane shifts with spectral settings

Suitable basis for more detailed analysis

Correction of sectioned imaging
first order correction fluorescence intensity variations
optimization of de-convolution algorithms

Basis for:
absolute calibration of sectioned imaging

Email: g.j.brakenhoff@uva.nl
Close