Confocal microscopy, deconvolution and image processing

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The Point Spread Function (PSF)
The image of a point object

2D PSF for different defocus

Z=+2µm
Z=0
Z=-2µm

3D PSF

Calculated
Measured
- How can we eliminate out-of-focus photons from images?

- How can we increase resolution by excluding the blur from the PSF?
The concept of confocal microscopy was developed by Marvin Minsky at MIT in the middle of the 1950’s.
The Confocal microscope

Lasers used: 405 nm (violet), 488 nm (green), 543 nm (yellow), 633 nm (red)

Widefield versus confocal fluorescence imaging

- Widefield illumination produces a larger cone of light on the specimen compared to the point illumination of the confocal
- As a consequence, widefield illumination stimulates more fluorophores outside of the focal plane ➔ creates more blur
Laser scanning confocal microscope

Lasers and fluorophores

Since the illumination wavelengths available are often limited the selection of matching fluorochromes is very important.
What do you get with a confocal?

Better resolution
(not by increasing optical resolution but by decreasing the background)

Better estimation of colocalization
(z-sections are narrower, better axial resolution)
What do you get with a confocal?

Optical sections of cells or tissues

Detectors - PMTs

- Must be fast - confocal beam spends only a few ms on each pixel
  - Photomultiplier tubes

  - Pulse width for single photon: ~ 10-100ns
  - Very linear
  - Very high gain
  - ~ 0 read noise
  - Low quantum efficiency:
    - 10% for old PMTs, 40% new GaAsP PMTs
Detectors - PMTs

The confocal microscope

- Slow (~1 sec to acquire an image)
- Low light efficiency (due to use of PMT as detector)
- Photobleaching

Solution:
Use multiple pinholes and a camera
A Solution: Spinning Disk Confocal

Image with many pinholes at once, so fast
Use CCD as detector, so much higher QE

Comparison of images from different kinds of confocal microscopes

Signal to noise is best with the LSCM, but SDCM has other advantages: faster, less photobleaching

Images collected by JWS at the Nikon Imaging Center at Harvard Medical School
Pros/Cons of spinning disk

- Fast - multiple points are illuminated at once
- Photon efficient - high QE of CCD
- Gentler on live samples - usually lower laser power

- Fixed pinhole - except in swept-field
- Small field of view (usually)
- Crosstalk through adjacent pinholes limits sample thickness

Deconvolution
Widefield image restoration

The Object

The Microscope Image

What went wrong ???????

The PSF - Point Spread Function

A PSF can be determined empirically by imaging a sub-resolution fluorescent bead. This best performed by adding sub-resolution beads (200 nm) to your experimental set-up.

However, theoretical PSF often better as it is averaged and corrected

PSF = a measure of the convolution caused by the microscope optics
What does the PSF do?

- An object is a collection of point sources
- Mathematical operation called a convolution
- The microscope is a convolution operator

The PSF and deconvolution

- Detector (camera) noise prevents a return to the original image
**Convolution and Deconvolution**

Convolution = the way the microscope optics “distort” the observed image of an object

What do you need for image deconvolution?

The components are much simpler consisting of a conventional fluorescence microscope, focus motor, CCD camera and computer with software.
Much like a confocal, a deconvolution system collects images in a Z-stack by repeatedly sampling the specimen at different focal planes.

Different Classes of Deconvolution

- Eliminate out-of-focus photons
  - Confocal microscopy
  - Nearest Neighbours deconvolution

- Bring back photons to the focal point
  - Constrained Iterative deconvolution
Different Classes of Deconvolution

- Nearest Neighbours
  - Not true deconvolution, subtractive (throws away light)
  - Quick and easy

  Assumes loss of contrast due to light from planes immediately above and below focal plane (uses only 2D data)

  Works by subtracting a fraction of the PSF-blurred versions of the non focal planes from the focal plane.

  Good for real time deconvolution, as it is quick

  Limited effectiveness because its subtractive and does nothing about noise.

  Only used for qualitative studies

- No Neighbours
  - Same but planes above and below are considered identical to image plane

Nearest-neighbors subtraction

+ Very fast, little memory required
- Ignores cross-talk between distant optical slices
Nearest Neighbours deconvolution

- Remove the blur from images

Xenopus cells with stained microtubules

Different Classes of Deconvolution

**Image restoration**: non-linear constrained iterative algorithms

- True deconvolution, light is re-assigned to its point of origin.
- Attempts to deal with noise - different approaches
- Can use measured (non-blind), theoretical or derived (blind) PSF

- Work with the whole 3D image of the specimen, and not plane by plane

- Noise results in negative pixel values (impossible) so any negative values are immediately set to zero. The next iteration fixes any errors that this clipping has introduced.

- The algorithm should then iterate its way to a non-negative solution which should be a close approximation to the real image.
Non-Blind Deconvolution

- Needs a measured or theoretical PSF
- Iteratively improves image
- Constrains image to prevent negative values
- Requires optimized microscope system (e.g., Deltavision)

**Step 1**
Initial Image Guess (usually original image) \[ \rightarrow \text{Convolve guess with PSF} = \text{Blurred Guess} \]

**Step 2**
Blurred Guess compared to the original image \[ \rightarrow \text{Comparison used to update the initial guess in Step 1} \]

This comparison is used to compute an error criterion that represents how similar the blurred estimate is to the original image. This error criterion is then used to alter the initial image guess in such a way that the error is reduced.

**Step 3**
Non-Negativity Constraint \[ \rightarrow \text{Any negative pixel values are set to 0} \]

**Step 4**
Iteration number increased and process repeated until a stable guess is produced or user stops it
Comparison of deconvolution techniques

- Images from Nearest Neighbor and constraint iterative deconvolution look quite similar
- The signal-to-noise ratio in the restored image is better than in the one generated by Nearest Neighbor

Blind Deconvolution

- An extension of Non-Blind.
- PSF and image guess derived from original data
- 3 main differences:
  1. Initial guess performed with a guessed PSF (Guess actually derived PSF based on wavelength, NA, pixel size, etc)
  2. Update performed using maximum likelihood
  3. Step 3 has non-negativity restraint but PSF is constrained to lie within a calculated PSF based on wavelength, etc.
**DeltaVision Deconvolution System:**

*Schizosaccharomyces pombe*
Deconvolution and pixel brightness

- Deblurring causes a decrease in pixel intensity over the whole image
- Iterative restoration result in increased pixel intensity in areas of the specimen

Confocal vs Widefield Deconvolution

Confocal (optical configuration)
- Discards out of focus light using a pinhole in the light path
- Less sensitive - throws away light, generally poorer signal to noise
- Deals well with strong but diffuse signal with a lot of out of focus light (low contrast)
- More convenient - immediate high contrast images, even with single Z sections
- Confocal images can be deconvolved as well

Widefield Deconvolution (processing)
- Reassigns out of focus light to its point of origin
- More sensitive (and quantitative) Better signal to noise ratio
- Better for point sources of light and weak signals
- Less convenient - requires time consuming calculations on expensive computers, best with multiple Z sections.
Softwares for deconvolution

Free software
- DeconvolutionLab, a plugin in ImageJ
- XCOSM (Washington University)
- IVE (UCSF)

Commercial softwares
- AutoQuant (Bitplane): all types of deconvolution
- Huygens (SVI): all types of deconvolution
- DeltaVision (Applied Precision): all types of deconvolution