AutoQuant Deconvolution White Paper

Introduction

Epi-Fluorescence microscopy, in which fluorophores, when illuminated with a specific wavelength of light emit light of a longer wavelength (and therefore of a different color) has proved to be a powerful and exciting tool for biologists. It has transformed microscopy from an observational science into a dynamic and productive empirical science (Yuste, 2005).

Proteins of interest can be labelled with great specificity in fixed specimens thanks to antibodies and antibody derived probes (such as Fab and F(ab')2 fragments) to which fluorescent molecules can be attached. Advances in molecular biology have allowed biologists to directly tag proteins of interest with fluorescent proteins from jellyfish and corals (and their rainbow of mutants and variants) allowing the observation and tracking of proteins in living cells and organisms (Kremers, et al., 2011).

Both the resolution and contrast of widefield fluorescence microscopes are constrained by four independent phenomena: noise, scatter, glare, and blur (see Wallace, et al., 2001 for a concise description of each). Blur is particularly prevalent when observing specimens with significant depth. To observe a single focal plane of interest, the whole specimen, both above and below the plane of interest is illuminated. Blur results in fluorescence emission from above and below the optical focal plane contaminating the entire image with background haze, significantly degrading the image (Lee, et al., 2014; Sabarita, 2005; Wallace, et al., 2001).

Various solutions, each with their own pros and cons exist. Some are purely optical solutions, some are a combination of optics and mathematics, whilst some are purely mathematical.

Of the optical solutions, the most well-known and widely used is the laser scanning confocal microscope (LSCM). In this case a single bright diffraction limited point illuminates the sample, and a pinhole is used to only allow light emitted from the focal point to reach the detector. By scanning the bright spot in a raster across the sample an image of the plane of interest can be reconstructed. By acquiring a series of focal planes, a blur free volume of the specimen can be reconstructed (Bayguinov, et al., 2018).

Solutions that utilize a combination of optics and mathematics include structured illumination systems that project a sinusoidal pattern onto the sample. Varying the position of the pattern on the sample generates a series of raw images from which a final, mathematically processed image lacking out of focus light can be extracted (Neil, et al., 1997).

Wholly mathematical solutions include iterative deconvolution of widefield microscope images, as utilized by AutoQuant, which is the focus of this document. Deconvolution requires knowledge of the point spread function (PSF) of the microscope. The point spread function is a description of how a single, sub-resolution point of light is convolved in the microscope, including its appearance whilst in focus, and the way light from that point spreads both above and below the focal plane. The knowledge of the point spread function allows the appearance of the sample to be calculated in its natural state, before it was convolved (and therefore degraded) by the microscope optics. Deconvolution is an iterative process, with improvement in the accuracy of the appearance of the sample added at each iteration until the calculation reaches a satisfactory final image of the sample in which pre-determined stopping criteria are met.

The shape of a PSF is dependent on several factors including the numerical aperture of the imaging objective lens, the wavelength of light emitted by the fluorophore being imaged, and the refractive index of the medium between the objective lens and the cover glass (see detail panel 2). Different PSFs are therefore required for each channel of a multi-channel image set. Accurate determination of the PSF is essential for high quality deconvolution. PSFs can be determined both theoretically (calculated based on the prior knowledge of the imaging conditions) or empirically (measured on the imaging instrument), and consideration must be paid to this step.

During deconvolution of wide field microscope images, out of focus light is not simply removed from the focal plane of interest, but it is reassigned to its point of origin within the 3D volume based on knowledge of the PSF. When contrasted

with LSCM in which out of focus light is excluded from the plane of interest and lost, deconvolved wide field volumes have a significant advantage in that all fluorescent light can be collected and used (Lee, et al., 2014, Sabarita, 2005, Wallace, et al., 2001).

Deconvolution is most used to correct blur, but it also significantly improves noise (see Wallace et al., 2001 for a helpful definition of noise). This increases the versatility of the technique and makes it applicable not just to wide-field microscopy, but also LSCM, and other common imaging techniques including spinning disk confocal microscopy and Stimulated Emission Depletion (STED) confocal microscopy.

AutoQuant Deconvolution in Detail

AutoQuant deconvolution is an iterative method that is rooted in the Expectation-Maximization algorithm (Dempster, et al., 1977). It uses several additional accelerations and constraints that vary depending on the specific configuration of the input data and parameters used. The specifics of those adjustments are discussed in the following sections.

Terminology used in the detail panels is shown in detail panel 1.

Detail Panel 1. Common Term Definitions

IMG_{orig}: the pre-processed (background subtracted, flicker-corrected) observed image

$IMG_{est}{}^{(0)}$: the initial image estimate

$PSF_{est}{}^{(0)}$: the initial PSF estimate

$IMG_{est}{}^{(k)}$: the image estimate after k iterations

$PSF_{est}{}^{(k)}$: the PSF estimate after k iterations

$\mathcal{F}(f)$: Fourier transform of image f

$f \cdot g$: pointwise product of image f and image g

$f * g$: convolution operator ($\mathcal{F}^{-1}[\mathcal{F}(f) \cdot \mathcal{F}(g)]$)

$f \star g$: correlation operator ($\mathcal{F}^{-1}[\mathcal{F}(f) \cdot \mathcal{F}(g)^*]$)

3D Deconvolution

3D deconvolution is dependent upon knowledge of the sample as a 3D structure so that out of focus light can be reassigned to its origins, reducing blur, and improving the signal to background ratio of deconvolved datasets.

3D Fixed PSF Deconvolution

3D Fixed PSF Deconvolution, also known as 3D Non-Blind Deconvolution, uses prior knowledge of the PSF which remains static throughout the iterative process. Only the image of the sample is updated during each iteration.

Prior knowledge of the PSF can be gathered empirically, by acquiring Z stacks of sub-resolution fluorescent objects (usually fluorescent beads), under the same conditions that the image of the sample will be acquired. These Z stacks are commonly known as '**Measured PSFs**'. Alternatively, PSFs can be calculated based on the knowledge of the imaging

conditions (defined in detail panel 2) that will be used to image the sample. Calculated PSFs are commonly known as "'**Theoretical PSFs'**.

Detail Panel 2. Optical Characteristics Required to calculate a Theoretical PSF

Imaging Technique (e.g., Wide-field or LSCM)

Objective Lens Numerical Aperture (NA)

Refractive Index of the Medium between the Objective Lens and Cover Glass

The wavelength of Light Emitted by the Sample

Sampling frequency in X, Y and Z (voxel size in X, Y and Z)

The use of accurate measured PSFs generates exceptionally reliable deconvolution data (Wallace, et al., 2001) and they can be used with imaging modalities for which AutoQuant is not able to generate a theoretical PSF.

The exact optical conditions of the microscope, including any potential aberrations are included in the measurement and are therefore corrected in the deconvolved data. The drawback to using measured PSFs is that they are difficult to acquire. Sub-resolution beads are by definition impossible to see as individual objects, so preparing them as solutions of well spread individual objects in the correct focal plane is challenging. Additionally, the use of poorly acquired PSFs that do not accurately reflect the optics of the acquiring microscope can introduce errors to deconvolved data (Sibarita, 2005, Lee, et al., 2014). Some microscopists will place beads onto the same slide as their sample to best match collection conditions. If the bead is well-isolated from the sample, to remain away from as much blurred light from the sample as possible, this can be a very effective approach. Given that PSFs are required for every objective, and every wavelength used for imaging, many users of AutoQuant opt for the much less challenging **theoretical** PSF.
The use of Theoretical PSFs has the advantage of being extremely easy and free of noise (Sibarita, 2005). Whilst theoretical PSFs lack the ability to detect and correct optical aberrations not predicted by theory, they are nonetheless of great value for estimating expected values in ideal experimental conditions (Sabarita, 2005).

The process begins with the establishment of the initial conditions.

1. Prepare the initial PSF estimate. This can be theoretically calculated from information about the optics, or it can be an image of a single sub-resolution bead, as described above. Such an image does not need to be the same size as the sample; it will be resized as needed internally and will also be pre-processed to ensure that it conforms to known theoretical limits for the sample's modality.
2. Prepare an initial estimate of the deconvolved image. For non-widefield data, this will generally be the original observed image (that is, start by estimating that there is no blur at all). For widefield data, that "original image" estimate is improved by applying a filter that provides a quick, though imperfect, inversion of initial PSF's effects.

An optional detailed description of this step is shown in detail panel 3.

Detail Panel 3 - Establishment of Initial Conditions - 3D Fixed PSF Deconvolution

1. The PSF estimate (PSF_{est}) is determined in one of the following ways:
    a. For a Theoretical PSF, apply these steps:
        i. Generate a collection of Bessel functions for the component frequencies of the PSF, as determined by the optics of the microscope.
        ii. For each depth plane of the PSF, form a 1D PSF array by aggregating the Bessel functions and applying a defocus term determined by Z offset and optical path difference terms (Gibson & Lanni, 1991).
            1. For confocal PSFs, square each value in the PSF array
            2. For STED PSFs, apply a depletion term for each element of the confocal PSF array (Harke, et al., 2008).
        iii. Circularly sweep the calculated 1D PSF array to create a radially symmetric 2D PSF for each depth plane, ultimately constructing a 3D PSF.
    b. For a measured PSF image (PSF_{orig}), apply these steps:
        i. Apply frequency limit constraint
        ii. Apply radial averaging
        iii. Subtract background intensity level
        iv. Apply spatial constraint
        v. Enforce attenuation of PSF along Z
        vi. Normalize PSF sum to 1.0

2. The first estimate of the deconvolved image ($IMG_{est}^{(0)}$) can be one of the following:

    a. Observed image IMG_{orig} (default for non-widefield data)
    b. IMG_{orig} with a Wiener filter applied using PSF_{est}

3. The iteration number is initialized: $k = 0$

The process continues with an iterative loop. The steps in the iterative loop are:

1. Obtain a new image estimate using an algorithm called "Expectation Maximization" (EM). This is a statistical approach that, with each iteration, increases the likelihood that the observed image has come from a convolution of the deconvolved image estimate with the PSF estimate.
2. Apply an acceleration to the new image estimate. One drawback of the EM algorithm is that it can take many iterations (in the order of 1000 or more) to converge to a near-maximum likelihood. To reduce the number of iterations needed to around 10-20, this acceleration takes the changes made between the old and new estimates and boosts them until the effect is no longer helpful (i.e., likelihood no longer increases).
3. Apply noise smoothing to the new image estimate.
4. Repeat from step 1 until the requested number of iterations has been completed.

A detailed description of the iterative loop is shown in detail panel 4.

Detail Panel 4. Iterative loop - 3D Fixed PSF Deconvolution

The steps below are repeated while $k < number\ of\ iterations$:

1. Use Expectation-Maximization step to obtain a new "pre-estimate" (i.e., prior to acceleration and/or constraint application) for the image (Holmes, 1992):

$$IMG_{est}{}^{(k+1)} = \left[\frac{IMG_{orig}}{IMG_{est}{}^{(k)} * PSF_{est}} \star PSF_{est}\right] \cdot IMG_{est}{}^{(k)}$$

2. Apply line-search image estimate acceleration (Holmes & Liu, 1991):
   a. Determine unaccelerated change in log-likelihood (Δl) from previous estimate:

$$IMG_{reblur} = IMG_{est}{}^{(k)} * PSF_{est}$$

$$\Delta IMG_{reblur} = \left(IMG_{est}{}^{(k+1)} * PSF_{est}\right) - \left(IMG_{est}{}^{(k)} * PSF_{est}\right)$$

$$\Delta l = \sum_{xyz \in W} \left\{IMG_{orig} \cdot \left[\ln\left(\frac{\Delta IMG_{reblur}}{IMG_{reblur}} + 1.0\right)\right]\right\} - \Delta IMG_{reblur}$$

$xyz \in W$: *coordinates within the image region that excludes the guardband*

   b. Initialize acceleration factor, $\alpha = 2$
   c. Determine accelerated change in log-likelihood (Δl_a)

$$\Delta l_a = \sum_{xyz \in W} \left\{IMG_{orig} \cdot \left[\ln\left(\frac{\alpha \Delta IMG_{reblur}}{IMG_{reblur}} + 1.0\right)\right]\right\} - \alpha \Delta IMG_{reblur}$$

   d. While $\Delta l_a > \Delta l$
      i. $\Delta l = \Delta l_a$
      ii. $\alpha = \alpha \times 2$
      iii. Recalculate Δl_a with new α

   e. When likelihood no longer increases, revert to last α (i.e., $\alpha = \alpha/2$)
   f. Replace new image estimate with accelerated estimate

$$IMG_{est}{}^{(k+1)} = \alpha\left(IMG_{est}{}^{(k+1)} - IMG_{est}{}^{(k)}\right) + IMG_{est}{}^{(k)}$$

3. Apply Good's roughness penalty to $IMG_{est}{}^{(k+1)}$ (Good & Gaskins, 1971)

4. $k = k + 1$

3D Adaptive PSF Deconvolution

It's possible to vary the 3D deconvolution algorithm so that not just the estimate of the deconvolved image is updated at each iteration, but that the PSF is similarly updated at each iteration. The process is intended to improve the outcome by adapting the PSF to better fit the observed data. This type of algorithm has mixed terminology, sometimes being known as 'Blind' deconvolution, sometimes being known as 'Adaptive' deconvolution.

Adaptive deconvolution was developed with the intention of improving the accuracy of theoretical PSFs on systems with minor optical aberrations (Holmes, et al., 2006) or where knowledge of the PSF is imprecise (Holmes, 1992).

The process begins with the establishment of the initial conditions.

1. Prepare the initial PSF estimate. This can be theoretically calculated from information about the optics, or it can be an image of a single sub-resolution bead. Such an image does not need to be the same size as the sample; it will be resized as needed internally and will also be pre-processed to ensure that it conforms to known theoretical limits for the sample's modality.
2. Prepare an initial estimate of the deconvolved image. For non-widefield data, this will generally be the original observed image (that is, start by estimating that there is no blur at all). For widefield data, that "original image" estimate is improved by applying a filter that provides a quick, though imperfect, inversion of initial PSF's effects.

A detailed description of this step is shown in detail panel 5.

Detail Panel 5 - Establishment of Initial Conditions - 3D Adaptive PSF Deconvolution

1. The PSF estimate (PSF_{est}) is determined in one of the following ways:
    a. For a Theoretical PSF, apply these steps:
        i. Generate a collection of Bessel functions for the component frequencies of the PSF, as determined by the optics of the microscope.
        ii. For each depth plane of the PSF, form a 1D PSF array by aggregating the Bessel functions and applying a defocus term determined by Z offset and optical path difference terms (Gibson & Lanni, 1991).
            1. For confocal PSFs, square each value in the PSF array
            2. For STED PSFs, apply a depletion term for each element of the confocal PSF array (Harke, et al., 2008).
        iii. Circularly sweep the calculated 1D PSF array to create a radially symmetric 2D PSF for each depth plane, ultimately constructing a 3D PSF.
    b. For a measured PSF image (PSF_{orig}), apply these steps:
        i. Apply frequency limit constraint
        ii. Apply radial averaging
        iii. Subtract background intensity level
        iv. Apply spatial constraint
        v. Enforce attenuation of PSF along Z
        vi. Normalize PSF sum to 1.0

2. The first estimate of the deconvolved image (${IMG_{est}}^{(0)}$) can be one of the following:
    a. Observed image IMG_{orig} (default for non-widefield data)
    b. IMG_{orig} with a Wiener filter applied using ${PSF_{est}}^{(0)}$

3. The iteration number is initialized: $k = 0$

The process continues with an iterative loop. The steps in the iterative loop are:

1. Obtain a new image estimate using an algorithm called "Expectation Maximization" (EM). This is a statistical approach that, with each iteration, increases the likelihood that the observed image has come from a convolution of the deconvolved image estimate with the PSF estimate.
2. Obtain a new PSF estimate using the EM algorithm. This effectively does the same thing as step 1 but swaps the PSF estimate and image estimate in the EM equations.
3. Apply an acceleration to the new image estimate. One drawback of the EM algorithm is that it can take many iterations (in the order of 1000 or more) to converge to a near-maximum likelihood. To reduce the number of iterations needed to around 10-20, this acceleration takes the changes made between the old and new estimates and boosts them until the effect is no longer helpful (i.e., likelihood no longer increases).
4. Apply noise smoothing to the new image estimate.
5. Constrain the new PSF estimate to a set of theoretical limits. This prevents the shape of the PSF from going "out of bounds" while we're modifying two variables (the image and the PSF) in tandem.
6. Repeat from step 1 until the requested number of iterations has been completed.

A detailed description of the iterative loop is shown in detail panel 6.

Detail Panel 6 - Iterative loop - 3D Adaptive PSF Deconvolution

The steps below are repeated while $k < number\ of\ iterations$:

1. Use Expectation-Maximization step to obtain new "pre-estimates" (i.e., estimates before acceleration and/or constraint application) for both image and PSF (Holmes, 1992)

$$IMG_{est}{}^{(k+1)} = \left[\frac{IMG_{orig}}{IMG_{est}{}^{(k)} * PSF_{est}{}^{(k)}} \star PSF_{est}{}^{(k)}\right] \cdot IMG_{est}{}^{(k)}$$

$$PSF_{est}{}^{(k+1)} = \left[\frac{IMG_{orig}}{IMG_{est}{}^{(k)} * PSF_{est}{}^{(k)}} \star IMG_{est}{}^{(k)}\right] \cdot PSF_{est}{}^{(k)}$$

2. Apply line-search image estimate acceleration (Holmes & Liu, 1991)
   a. Determine unaccelerated change in log-likelihood (Δl) from previous estimate:

$$IMG_{reblur} = IMG_{est}{}^{(k)} * PSF_{est}{}^{(k)}$$

$$\Delta IMG_{reblur} = \left(IMG_{est}{}^{(k+1)} * PSF_{est}{}^{(k)}\right) - \left(IMG_{est}{}^{(k)} * PSF_{est}{}^{(k)}\right)$$

$$\Delta l = \sum_{xyz \in W} \left\{IMG_{orig} \cdot \left[\ln\left(\frac{\Delta IMG_{reblur}}{IMG_{reblur}} + 1.0\right)\right]\right\} - \Delta IMG_{reblur}$$

   $xyz \in W$: *coordinates within the image region that excludes the guardband*

   b. Initialize acceleration factor, $\alpha = 2$
   c. Determine accelerated change in log-likelihood (Δl_a)

$$\Delta l_a = \sum_{xyz \in W} \left\{IMG_{orig} \cdot \left[\ln\left(\frac{\alpha \Delta IMG_{reblur}}{IMG_{reblur}} + 1.0\right)\right]\right\} - \alpha \Delta IMG_{reblur}$$

   d. While $\Delta l_a > \Delta l$
      i. $\Delta l = \Delta l_a$
      ii. $\alpha = \alpha \times 2$
      iii. Recalculate Δl_a with new α

   e. When likelihood no longer increases, revert to last α (i.e., $\alpha = \alpha/2$)
   f. Replace new image estimate with accelerated estimate

$$IMG_{est}{}^{(k+1)} = \alpha\left(IMG_{est}{}^{(k+1)} - IMG_{est}{}^{(k)}\right) + IMG_{est}{}^{(k)}$$

3. Apply Good's roughness penalty to $IMG_{est}{}^{(k+1)}$ (Good & Gaskins, 1971)

4. Apply constraints to $PSF_{est}{}^{(k+1)}$
   a. Apply spatial constraint with Lagrange multiplier optimization
   b. Apply frequency limit constraint
   c. Normalize PSF sum to 1.0

5. $k = k + 1$

2D Deconvolution

The 3D iterative deconvolution methods described above use knowledge of the sample in 3 dimensions to remove out of focus light and restore it to its point of origin.
Even without knowledge of planes above and below the plane of interest, image restoration is possible in the form of 2D deconvolution.

2D Fixed PSF Deconvolution

The 2D Fixed PSF Deconvolution technique, also known as 2D Non-Blind Deconvolution, updates only the image estimate during each iteration, but still allows for image sub-iterations (set by the AutoQuant input parameter "Image Correction Factor") to be used. A setting greater than 1 for this field causes noise smoothing to be applied only at intervals, instead of during every image estimate update. The "Image Correction Factor" is always set to 1 in the AutoQuant module of Image-Pro 11 and cannot be adjusted.

The process begins with the establishment of the initial conditions.

1. Prepare the initial PSF estimate. This can be theoretically calculated from information about optics, or it can be an image of a single sub-resolution bead. Such an image does not need to be the same size as the sample; it will be resized as needed internally.
2. Prepare an initial estimate at the deconvolved image. For 2D data, this will be the original observed image (that is, start by estimating that there is no blur at all).

A detailed description of this step is shown in detail panel 7.

Detail Panel 7 - Establishment of Initial Conditions – 2D Fixed PSF Deconvolution

1. The PSF estimate (PSF_{est}) is determined in one of the following ways:
    a. For a Theoretical PSF, apply these steps:
        i. Generate a collection of Bessel functions for the component frequencies of the PSF, as determined by the optics of the microscope.
        ii. Form a 1D PSF array by aggregating the Bessel functions and applying a defocus term determined by Z offset and optical path difference terms (Gibson & Lanni, 1991).
            1. For confocal PSFs, square each value in the PSF array
            2. For STED PSFs, apply a depletion term for each element of the confocal PSF array (Harke, et al., 2008).
        iii. Circularly sweep the calculated 1D PSF array to create a radially symmetric 2D PSF.
    b. A measured PSF image (PSF_{orig})

2. The first estimate of the deconvolved image (${IMG_{est}}^{(0)}$) will typically be the observed image IMG_{orig}.

3. Normalize ${IMG_{est}}^{(0)}$ sum to 1.0
4. Initialize iteration number: $k = 0$

5. Define overall image iteration total: $k_{img} = 0$

The process continues with an iterative loop, which contains an inner loop. The steps in the iterative loop are:

1. Obtain a new image estimate using an algorithm called "Expectation Maximization" (EM). This is a statistical approach that, with each iteration, increases the likelihood that the observed image has come from a convolution of the deconvolved image estimate with the PSF estimate. In a general (but not exact) sense, this means the deconvolved image estimate keeps improving.
2. Apply an acceleration to the new image estimate. One drawback of the EM algorithm is that it can take many iterations (in the order of 1000 or more) to converge to a near-maximum likelihood. To reduce the number of iterations needed to around 20, the acceleration scheme used in the 2D deconvolution tracks changes made over the course of the previous iterations to determine how much of a boost it can apply to the most recent

change. It offers speed and accuracy advantages over the scheme used in 3D deconvolution, but is considerably more memory-intensive, and so is currently only used for 2D deconvolution.

3. Repeat from step 1 for the number of times indicated by the "Image Correction Factor", which is only 1 by default (and is always 1 for the AutoQuant module of Image-Pro).
4. Apply noise smoothing to the new image estimate.
5. Repeat from step 1 until the requested number of iterations has been completed.

A detailed description of the iterative loop is shown in detail panel 8.

Detail Panel 8 - Iterative loop - 2D Fixed PSF Deconvolution

The steps below are repeated while $k < number\ of\ iterations$:

1. Image sub-iterations, 0 to "Image Correction Factor" - 1:

   a. Expectation-Maximization step to obtain new pre-acceleration estimate for image

$$IMG_{preaccel}{}^{(k_{img})} = \left[\frac{IMG_{orig}}{IMG_{est}{}^{(k_{img})} * PSF_{est}{}^{(k_{psf})}} \star PSF_{est}{}^{(k_{psf})}\right] \cdot IMG_{est}{}^{(k_{img})}$$

   b. Apply vector extrapolation acceleration (Biggs, 1998):

$$For\ k_{img} = 0:$$

$$\nabla IMG^{(0)} = \{0\}$$

$$IMG_{est}{}^{(k_{img}+1)} = IMG_{preaccel}{}^{(k_{img})}$$

$$For\ k_{img} > 0:$$

$$\nabla IMG^{(k_{img})} = \ln\left(\frac{IMG_{orig}}{IMG_{est}{}^{(k_{img})} * PSF_{est}{}^{(k_{psf})}} \star PSF_{est}{}^{(k_{psf})}\right) \cdot IMG_{preaccel}{}^{(k_{img})}$$

$$\alpha^{(k_{img}+1)} = \frac{\sum\left(\nabla IMG^{(k_{img})} \cdot \nabla IMG^{(k_{img}-1)}\right)}{\sum\left(\nabla IMG^{(k_{img}-1)} \cdot \nabla IMG^{(k_{img}-1)}\right)}, 0 \leq \alpha^{(k_{img}+1)} \leq 1$$

$$IMG_{est}{}^{(k_{img}+1)} = IMG_{preaccel}{}^{(k_{img})} \cdot \left(\frac{IMG_{preaccel}{}^{(k_{img})}}{IMG_{preaccel}{}^{(k_{img}-1)}}\right)^{\alpha^{(k_{img}+1)}}$$

   c. $k_{img} = k_{img} + 1$

2. Apply Good's roughness penalty to $IMG_{est}{}^{(k+1)}$ (Good & Gaskins, 1971)
3. $k = k + 1$

2D Adaptive PSF Deconvolution

In common with 3D adaptive deconvolution, both the estimate of the original image and the estimate of PSF are updated in 2D adaptive PSF deconvolution. This process is also known as 2D Blind Deconvolution.
The process uses asymmetric iterations (Biggs, 1998) to focus more on updating either the PSF or the image. Each deconvolution iteration performs a pre-defined number of PSF sub-iterations (set by input parameter "PSF Correction Factor"), followed by a pre-defined number of image sub-iterations (set by input parameter "Image Correction Factor").

In common with the other deconvolution methods in AutoQuant, the initial PSF estimate can be based on a **measured** or **theoretical** PSF as well as a third possibility, a **derived** PSF. A derived PSF is generated through autocorrelation of the original image. Autocorrelation of real-world captured images tends to take the general form of a PSF, being clustered near the origin, and outwardly decaying. Working with a derived PSF is extremely easy as no prior knowledge of the optical conditions under which the original image was acquired is required.

The process begins with the establishment of the initial conditions.

1. Prepare the initial PSF estimate. For Adaptive 2D Deconvolution, this will be a derived PSF by default. Alternatively, the initial PSF can be theoretically calculated from information about the optics, or it can be an image of a single sub-resolution bead. Such an image does not need to be the same size as the sample; it will be resized as needed internally.
2. Prepare an initial estimate of the deconvolved image. For 2D data, this will be the original observed image (that is, start by estimating that there is no blur at all).

A detailed description of this step is shown in optional detail panel 9.

Detail Panel 9 - Establishment of Initial Conditions – 2D Adaptive Deconvolution

1. The PSF estimate (PSF_{est}) is calculated from either:
    a. **Measured** PSF
    b. **Theoretical PSF** (PSF_{orig})
        i. Generate a collection of Bessel functions for the component frequencies of the PSF, as determined by the optics of the microscope.
        ii. Form a 1D PSF array by aggregating the Bessel functions and applying a defocus term determined by Z offset and optical path difference terms (Gibson & Lanni, 1991).
            1. For confocal PSFs, square each value in the PSF array
            2. For STED PSFs, apply a depletion term for each element of the confocal PSF array (Harke, et al., 2008).
        iii. Circularly sweep the calculated 1D PSF array to create a radially symmetric 2D PSF.
    c. **Derived PSF** – Autocorrelation of IMG_{orig}: ${PSF_{est}}^{(0)} = \left(IMG_{orig} \star IMG_{orig}\right)$

2. The first estimate of the deconvolved image (${IMG_{est}}^{(0)}$) will typically be the observed image IMG_{orig}.

3. Normalize ${IMG_{est}}^{(0)}$ sum to 1.0
4. Normalize ${PSF_{est}}^{(0)}$ sum to 1.0
5. Initialize iteration number : $k = 0$

6. Define overall PSF iteration total: $k_{psf} = 0$

7. Define overall image iteration total: $k_{img} = 0$

The process continues with two iterative loops within an outer iterative loop, which is executed as many times as specified by the user in the "Total Iterations" setting. The first inner loop updates the PSF, and the second updates the image. The steps in the outer iterative loop are:

1. Obtain a new PSF estimate using an algorithm called "Expectation Maximization" (EM). This is a statistical approach that, with each iteration, increases the likelihood that the observed image has come from a convolution of the deconvolved image estimate with the PSF estimate. While this generally indicates that the PSF is improving, it is possible in the 2D Deconvolution – especially with a derived initial PSF – to reach suboptimal solutions if too many iterations are performed.
2. Apply an acceleration to the new PSF estimate. One drawback of the EM algorithm is that it can take many iterations (on the order of 1000 or more) to converge to a near-maximum likelihood. To reduce the number of iterations needed, the acceleration scheme used in the 2D deconvolution tracks changes made over the course of the previous iterations to determine how much of a boost it can apply to the most recent change. It offers speed and accuracy advantages over the scheme used in 3D deconvolution, but is considerably more memory-intensive, and so is currently only used for 2D deconvolution.
3. Repeat from step 1 for the number of times indicated by the "PSF Correction Factor", which is 2 by default and is always 2 in the AutoQuant module of Image-Pro.
4. Obtain a new image estimate using the EM algorithm. This effectively does the same thing as step 1 but swaps the PSF estimate and image estimate in the EM equations.
5. Apply the 2D deconvolution acceleration scheme to the new image estimate. This is the same algorithm described in step 2 but applies to the image estimate this time.
6. Repeat from step 4 for the number of times indicated by the "Image Correction Factor", which is 1 by default and is always 1 in the AutoQuant module of Image-Pro.
7. Apply noise suppression to the new image estimate.
8. Repeat from step 1 until the requested total number of iterations has been completed.

A detailed description of the iterative loop is shown in detail panel 10.

Detail Panel 10 - Iterative loop - 2D Adaptive PSF Deconvolution

The steps below are repeated while $k < number\ of\ iterations$:

1. PSF sub-iterations, 0 to "PSF Correction Factor" - 1:
   a. Expectation-Maximization step to obtain new pre-acceleration estimate for PSF

$$PSF_{preaccel}{}^{(k_{psf})} = \left[\frac{IMG_{orig}}{IMG_{est}{}^{(k_{img})} * PSF_{est}{}^{(k_{psf})}} \star IMG_{est}{}^{(k_{img})}\right] \cdot PSF_{est}{}^{(k_{psf})}$$

   b. Apply vector extrapolation acceleration (Biggs, 1998):

$$For\ k_{psf} = 0:$$

$$\nabla PSF^{(0)} = \{0\}$$

$$PSF_{est}{}^{(k_{psf}+1)} = PSF_{preaccel}{}^{(k_{psf})}$$

$$For\ k_{psf} > 0:$$

$$\nabla PSF^{(k_{psf})} = \ln\left(\frac{IMG_{orig}}{IMG_{est}{}^{(k_{img})} * PSF_{est}{}^{(k_{psf})}} \star IMG_{est}{}^{(k_{img})}\right) \cdot PSF_{preaccel}{}^{(k_{psf})}$$

$$\alpha^{(k_{psf}+1)} = \frac{\sum\left(\nabla PSF^{(k_{psf})} \cdot \nabla PSF^{(k_{psf}-1)}\right)}{\sum\left(\nabla PSF^{(k_{psf}-1)} \cdot \nabla PSF^{(k_{psf}-1)}\right)}, 0 \le \alpha^{(k_{psf}+1)} \le 1$$

$$PSF_{est}{}^{(k_{psf}+1)} = PSF_{preaccel}{}^{(k_{psf})} \cdot \left(\frac{PSF_{preaccel}{}^{(k_{psf})}}{PSF_{preaccel}{}^{(k_{psf}-1)}}\right)^{\alpha^{(k_{psf}+1)}}$$

   c. $k_{psf} = k_{psf} + 1$

2. Image sub-iterations, 0 to "Image Correction Factor":
   a. Expectation-Maximization step to obtain new pre-acceleration estimate for image

$$IMG_{preaccel}{}^{(k_{img})} = \left[\frac{IMG_{orig}}{IMG_{est}{}^{(k_{img})} * PSF_{est}{}^{(k_{psf})}} \star PSF_{est}{}^{(k_{psf})}\right] \cdot IMG_{est}{}^{(k_{img})}$$

   b. Apply vector extrapolation acceleration, per (Biggs, 1998):

$$For\ k_{img} = 0:$$

$$\nabla IMG^{(0)} = \{0\}$$

$$IMG_{est}{}^{(k_{img}+1)} = IMG_{preaccel}{}^{(k_{img})}$$

$$For\ k_{img} > 0:$$

$$\nabla IMG^{(k_{img})} = \ln\left(\frac{IMG_{orig}}{IMG_{est}{}^{(k_{img})} * PSF_{est}{}^{(k_{psf})}} \star PSF_{est}{}^{(k_{psf})}\right) \cdot IMG_{preaccel}{}^{(k_{img})}$$

$$\alpha^{(k_{img}+1)} = \frac{\sum\left(\nabla IMG^{(k_{img})} \cdot \nabla IMG^{(k_{img}-1)}\right)}{\sum\left(\nabla IMG^{(k_{img}-1)} \cdot \nabla IMG^{(k_{img}-1)}\right)}, 0 \le \alpha^{(k_{img}+1)} \le 1$$

$$IMG_{est}{}^{(k_{img}+1)} = IMG_{preaccel}{}^{(k_{img})} \cdot \left(\frac{IMG_{preaccel}{}^{(k_{img})}}{IMG_{preaccel}{}^{(k_{img}-1)}}\right)^{\alpha^{(k_{img}+1)}}$$

   c. $k_{img} = k_{img} + 1$
3. Apply Good's roughness penalty to $IMG_{est}{}^{(k+1)}$ (Good & Gaskins, 1971)
4. $k = k + 1$

AutoQuant Deconvolution in the Literature

3D Fixed PSF Deconvolution

AutoQuant 3D fixed deconvolution software was assessed in a study of several deconvolution packages conducted by a microscopy and optics core facility for biological research. Several deconvolution tests were performed on several widefield datasets. Test images consisted of a synthetic dataset (for which a ground truth is known) convolved with a synthetic dataset and corrupted with noise, a volume of synthetic bead, and multi-channel volume of a *Caenorhabditis elegans* embryo (Griffa, et al., 2010).

AutoQuant deconvolution was found to be robust to noise, the authors noted that contrast enhancement was particularly significant in AutoQuant, both for the bead and *C.elegans* images. Visual judges of the deconvolution of the *C.elegans* embryo judged AutoQuant data to be 'high level', with AutoQuant scoring the highest of all the packages under consideration on a qualitative scale (Griffa, et al., 2010).

3D Adaptive PSF Deconvolution

Sibarita compared deconvolution results obtained with four different algorithms, including AutoQuant blind deconvolution, applied to image stacks acquired with different exposure times. All the results obtained with the blind deconvolution algorithm were described as 'good', although it was noted that some artifacts began to appear with noisy data (Sibarita, 2005).

Lee et al. conducted an analysis using widefield Z stacks of Inspeck beads which were deconvolved using AutoQuant. The study was designed to assess whether wide-field microscopes are quantitative and whether deconvolution algorithms maintain that quantitative intensity relationship in 3D image stacks of fluorescent calibration microspheres (Lee, et al., 2014). 3D fixed and adaptive PSF deconvolution were compared. Adaptive deconvolution was found to improve image contrast and to decrease out of focus blur. The intensity of the microspheres increased fivefold following blind deconvolution, thanks to out of focus light being reassigned to its origin. The perceived volumes of the deconvolved microspheres was 70% lower than the perceived volumes of the widefield data, bringing the volume closer to the known volumes of the objects. Measurement of the relative intensities of the beads following deconvolution showed that the AutoQuant adaptive deconvolution algorithm preserved the relative quantitative intensity data. The authors found no significant difference between fixed PSF and adaptive PSF deconvolution. The authors concluded that it is not surprising given that the PSF for these images is very close to the theoretical, with images collected of microspheres near the coverslip in a thin sample (and therefore not subject to spherical aberration). The calibration procedure outlined by the Lee et al. (which should be considered by all microscopists using deconvolution for quantitative procedures) left the authors confident that the use of AutoQuant is appropriate and accurate for quantitative fluorescence imaging (Lee, et al., 2014).

Further investigation was done in an unpublished study by Media Cybernetics (https://mediacy.com/wp-content/uploads/2023/03/An-evaluation-of-the-efficacy-of-adaptive-and-fixed-PSF-deconvolution-using-the-AutoQuant-module-of-Image-Pro-version-11.pdf). The study compared deconvolution of volumes of sub-resolution beads that were acquired both under ideal optical conditions (with beads close to the coverslip) and under conditions likely to lead to significant spherical aberration (with beads far from the coverslip in refractive index mismatched medium). Beads were imaged with several modalities, wide-field, laser scanning confocal, and spinning disc confocal. Both fixed and adaptive PSFs were found to always improve the deconvolved data considerably relative to the raw data. Circumstances in which adaptive deconvolution conferred significant improvement relative to fixed PSF deconvolution were not identified, moreover, measurable difference between datasets deconvolved with fixed and adaptive PSFs was not found. These observations are consistent with the published studies of adaptive deconvolution, in that whilst consistently good deconvolution data was observed, no compelling reason to apply adaptive deconvolution in preference to fixed deconvolution was found. For this reason, adaptive 3D deconvolution is not featured in the most recent iteration of AutoQuant, as a module of Image-Pro 11. This decision was made to improve the simplicity and the usability of the AutoQuant module of Image-Pro 11. Adaptive deconvolution remains of interest to Media Cybernetics and further work to characterize and improve the technique is scheduled.

AutoQuant Deconvolution Best Practices

Microscopy

Good sample preparation and the use of a well-designed imaging system are essential for achieving good deconvolution outcomes. You should ensure that your instrument is spatially calibrated, that you acquire data at the Nyquist frequency in X,Y and Z, and that noise is minimized (Sage, et al., 2017).

Spatial Calibration

Spatial calibration is the calculation of the size of pixels in acquired images, this is essential for the correct measurement of features in images and for deconvolution. A rough (and often sufficient) method to calculate image pixel size is to divide the actual dimensions of the camera sensor pixels by the total magnification of the imaging system. Pixel sensor size can invariably be found in the camera manufacturer's documentation. A more accurate method, that doesn't require any prior knowledge about the pixel size is to acquire an image of a stage micrometer, and to use software to divide the size of a line of known length (based on the scale of the micrometer) by the number of pixels along the length of the line. High Quality image analysis software such as Image-Pro makes this calculation easy.

Sampling at Nyquist Frequency

When imaging with systems that use a digital camera for image acquisition, it's important to ensure that the pixel size of your camera is small enough to meet the Nyquist criterion during image acquisition. The Nyqusit criterion states that the sampling frequency should be at least 2.3 times higher than the highest frequency in the data (Pawley, 1995), which in the case of widefield microscopy means that the image pixel size much be 2.3 times smaller than the resolution of the objective.

Check detail panel 11 for steps to ensure that your system samples at Nyquist frequency in X and Y. To ensure that you are sampling at Nyquist frequency in X and Y you should ensure that the use of optovars and camera adapters are appropriate for your acquisition objectives when designing your imaging system.

Detail Panel 11. Sampling in X and Y at Nyqist Frequency in the case of wide-field epifluorescence

Step 1. Calculate the resolution of the imaging objective.

$$Resolution = \frac{1.22\,\lambda}{2\mathrm{NA}}$$

λ = The wavelength of Light Emitted by the Sample
NA = Numerical Aperture of the Imaging Objective

Step 2. Calculate the Nyquist Sampling Frequency.

$$Nyquist\ Sampling\ Frequency = \frac{Resolution}{2.3}$$

Step 2. Calculate the total magnification of the system.

$$Total\ Magnification = Objective\ lens\ magnificatiob * Additional\ Magnification$$

Step 3. Calculate the Image Pixel Size.

$$Image\ Pixel\ Size = \frac{Camera\ Pixel\ Size}{Total\ Magnification}$$

Step 4. Ensure the Image Pixel Size <= Nyquist Sampling Frequency.

The sampling frequency in the Z axis should also be considered to ensure that you are acquiring data at the Nyqusit frequency. Unlike the sampling frequency in X and Y, which is difficult to adjust after assembly of the system, the sampling rate in the Z axis is easily controlled as the acquisition Z step. The correct procedure for calculating the sampling frequency in Z is described in detail panel 12.

Detail Panel 12. Sampling in Z at the Nyquist Frequency

Step 1. Calculate the axial (z) resolution of the imaging objective

$$Axial\ Resolution = \frac{2\lambda\eta}{NA^2} \qquad \text{(Inoué, 1995)}$$

λ = The wavelength of Light Emitted by the Sample
η = Refractive Index of the Immersion Medium
NA = Numerical Aperture of the Imaging Objective

Step 2. Divide the Axial resolution by 2.3

$$Nyquist\ Sampling\ Frequency\ Z = \frac{Axial\ Resolution}{2.3}$$

Noise Minimization

The noise of digital cameras can generally be minimized by reducing gain, increasing the brightness of the signal (by increasing the intensity of the excitation illumination) or by increasing the exposure time.

All the microscopy best practices should be implemented pragmatically with compromises made when necessary. There is little point in rigidly applying the Nyquist Z sampling frequency if in the process the sample becomes degraded by photobleaching or killed by phototoxicity (in the case of live specimens). Sometimes sacrificing the optimum settings for deconvolution is worthwhile to achieve your broader experimental aims.

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