Table of contents

I. INTRODUCTION

The quality of microscopy images is of importance when these images are aimed at being processed by analysis algorithms allowing to extract quantitative parameters. Therefore, particular care must be taken during the acquisition and the saving of microscopy images.

Considerations regarding image acquisition precautions and image properties are described in this documentation, to help users of Argolight and non-Argolight products acquiring and correctly saving their images, so that they can easily be processed with Daybook Analysis.

II. EXTRINSIC PARAMETERS AFFECTING THE QUALITY OF AN IMAGE

1. SAMPLE DIRTINESS

Description:

Traces of grease, fibers, or other dust at the sample surface(s) can add an undesired background or undesired features in the image (*cf.* Figure 1). Therefore, the sample should be cleaned before imaging. If the sample is a microscope slide, it is recommended cleaning both surfaces (top and bottom).

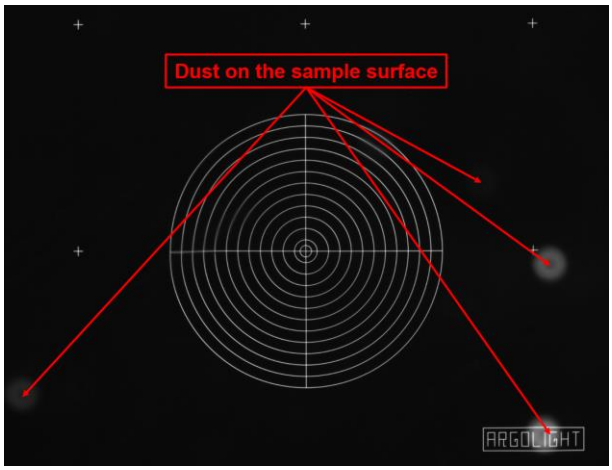


Figure 1: Image example of a sample with dust at the surface, inducing blurry spots in the image.

In Daybook Analysis:

If there is an undesired object in the image (dust for instance), Daybook Analysis allows cropping the image (*cf.* Figure 2).

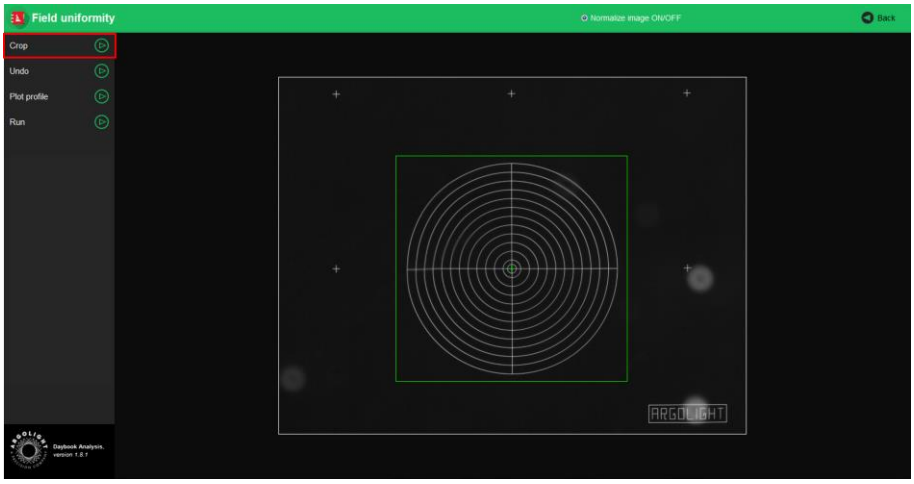


Figure 2: Cropping window in Daybook Analysis to select a ROI free from blurry spot.

2. MICROSCOPE DIRTINESS

Description:

Grease traces, fibers, or other dust can add an undesired background in the image (*cf.* Figure 3). Therefore, the microscope should be cleaned before imaging.

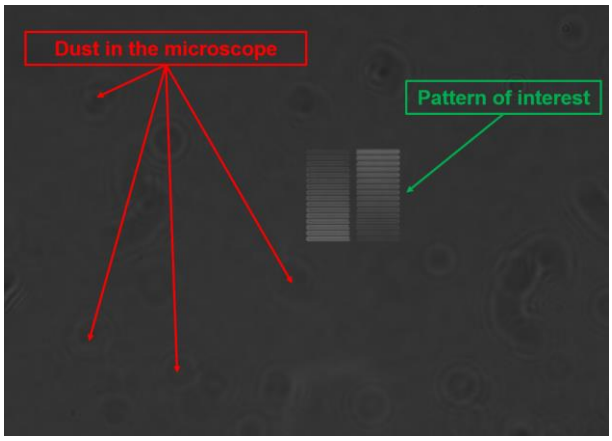


Figure 3: Image example of an object (a "2×16 intensity gradation" pattern) with blurry spots induced by dust inside the microscope.

In Daybook Analysis:

If there is an undesired object in the image (dust for instance), Daybook Analysis allows cropping the image (*cf.* Figure 4).

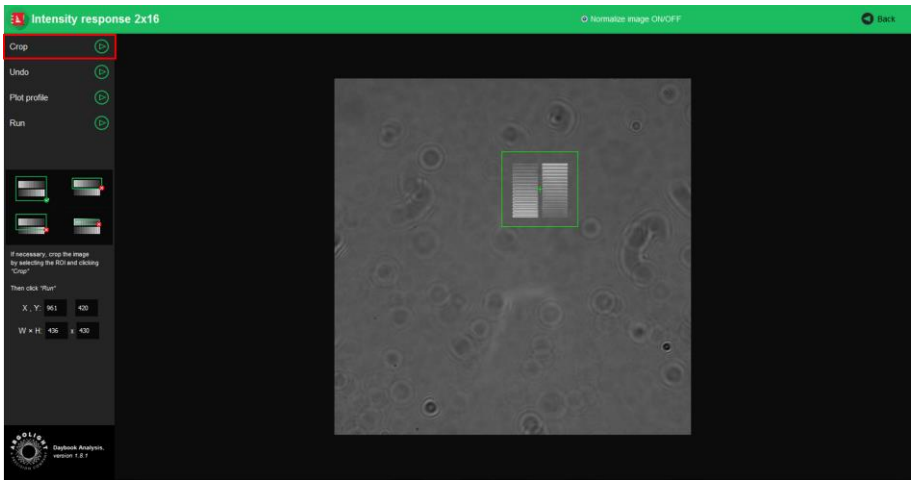


Figure 4: Cropping window in Daybook Analysis to select a ROI free from any blurry spot.

3. MICROSCOPE MISALIGNMENT

Description:

The detector orientation and/or the scanning should be correctly aligned with respect to the XY translation stage. Otherwise, the objects of interest in the images will appear rotated (*cf.* Figure 5).

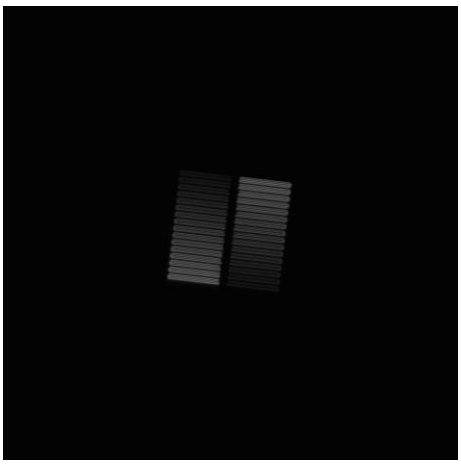


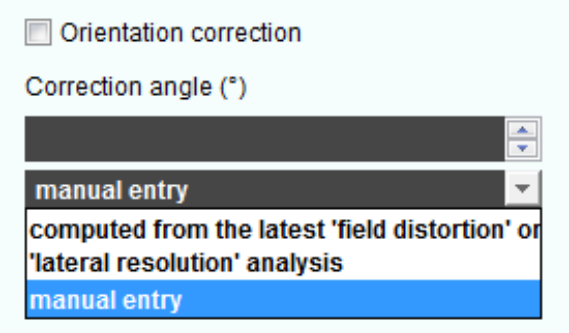
Figure 5: Image example of a rotated object (a "2×16 intensity gradation" pattern) acquired with a wide-field microscope, due to a misoriented camera with respect to the sample holder and stage.

In Daybook Analysis:

If a pattern looks rotated in an image, Daybook Analysis can correct a slight rotation (up to a few degrees).

Some analyses correct it automatically: field uniformity, field distortion, lateral resolution, intensity response, accuracy of 3D reconstruction.

Tick the "Orientation correction" button to enable this option. The correction angle is computed from other analyses (*field distortion* or *lateral resolution*). It can also be set manually.



4. STAGE OR SAMPLE TILT

Description:

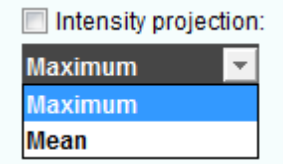
The XY stage or the sample may not be perfectly perpendicular to the optical axis of the microscope, *i.e.* usually not perfectly horizontal. As a result, thin objects located in the same sample plane may appear blurry in a region of the image, while looking distinct in another one (*cf.* Figure 6).

The stage or sample tilt is often confused with field non-uniformity, as they may lead to similar observations in images. The first one can be fixed by adjusting screws, while the second one can be optimized, but remains an intrinsic feature of the microscope.

Figure 6: Image example of an object (a “target” pattern) appearing distinct in a region (on the left).and blurry in another one (on the right), due to sample tilt.

In Daybook Analysis:

If a pattern looks both distinct and blurry in an image, due to XY stage or sample tilt, Daybook Analysis can perform an intensity projection (maximum or mean) on a Z-stack before processing the pattern image.
Tick the “Intensity projection” button to enable this option and choose between “Maximum” and “Mean”.



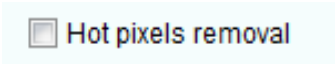
5. CAMERA PIXEL ARTIFACTS

Description:

Damaged pixels on camera chips lead to cold pixels (with null intensity value). Pixels with an unusually high dark current can lead to hot pixels (with maximum intensity value). The first artifact is not so problematic, while the last one can lead to a biased image analysis. The intensity value of a hot pixel should therefore be set to zero or replaced by the average values of its neighbors.

In Daybook Analysis:

If there are hot pixels in the image, Daybook Analysis can perform a “hot pixels removal” to remove the very intense pixels (*i.e.* hot) that may cause analysis issues.
Tick the “Hot pixels removal” button to enable this option.



III. INTRINSIC PARAMETERS DETERMINING THE QUALITY OF AN IMAGE

A “2×16 intensity gradation” pattern of an Argo-HM slide was imaged with a wide-field microscope, at low illumination power and low exposure time, to illustrate the notions of signal, noise and background.

Figure 7 displays an image of the “2×16 intensity gradation” pattern, as well as the intensity profile along the red line. From the manufacturing process of this pattern, it is expected that the intensity line profile follows a linear evolution; the raw data are therefore fitted with a linear curve, representing the signal, in black. The fluctuations in red around the signal represent the noise. The background, in green, is the offset on top of the signal and the noise.
In this example, because both the illumination power and the exposure time are low, the noise and the background are important compared to the signal. Post-processing analyses of this image might therefore be complicated, for example in Daybook Analysis.

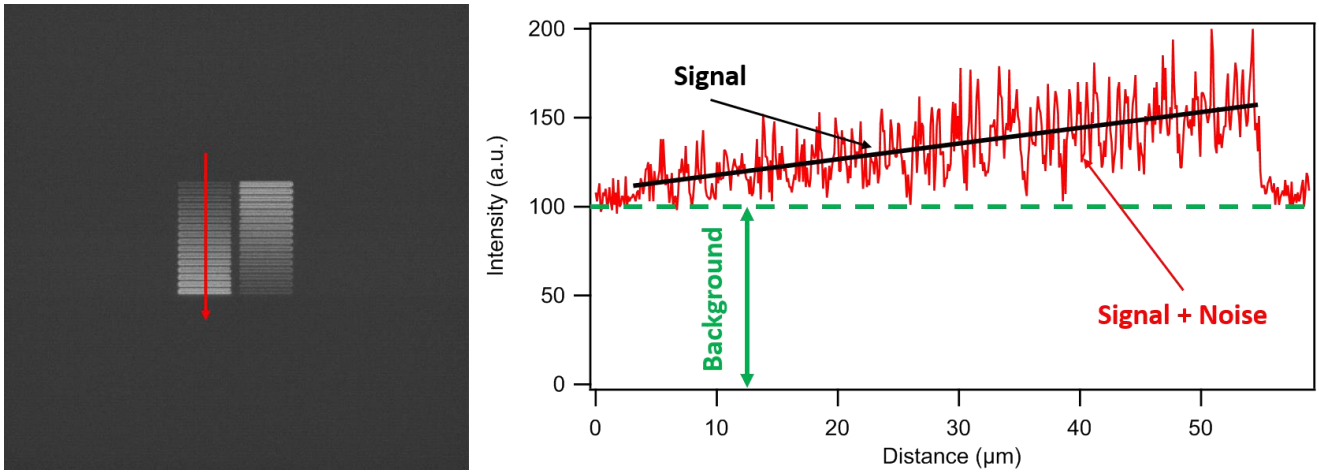


Figure 7: Images of a “2×16 intensity gradation” pattern (left) acquired at low illumination power and exposure time, and intensity profile (right) corresponding to the red line showing the signal, noise and background.

Both the background and the noise depend on the image acquisition settings. They are therefore an intrinsic property of the images, rather than of the imaging systems. It can even be more complex if the signal is not spatially uniform, as it is the case in Figure 7, the signal-to-background ratio and the signal-to-noise ratio will have different values, depending on the region of interest in the image.

1. SIGNAL-TO-BACKGROUND RATIO

Definition:

The signal-to-background ratio (SBR) represents the ratio of the measured light signal to the background (*i.e.* the offset), which consists of undesirable signal components arising from any undesired light source: dust on the sample, mounting medium, light leakage from the microscope, ambient light, etc..

Practical tip:

The signal-to-background ratio can usually be improved by:

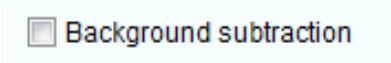
- Cleaning the sample.
- Cleaning the microscope.
- Sealing the light leakage.
- Suppressing any source of external light.
- Optimizing the image acquisition settings (illumination power, exposure time, etc.).

Recommendation:

Images should be acquired with enough contrast between the pattern and the background. A ***signal-to-background ratio higher than 2:1*** is recommended.

In Daybook Analysis:

If the signal-to-background ratio in the image is too low and cannot be improved, Daybook Analysis can perform a “background subtraction” to get rid of it.
Tick the “Background subtraction” button to enable this option.



It requires acquiring an image of an area where there is no fluorescent pattern (*i.e.* a background image) with the same imaging settings (channel, illumination power, exposure time, etc.) as the image of the pattern to be analyzed. For multi-channel analyses, a background image for each channel is required.

2. SIGNAL-TO-NOISE RATIO

Definition:

The signal-to-noise ratio (SNR) represents the ratio of the measured light signal to the combined noise, which consists of undesirable signal components arising in the electronic acquisition system, and inherent natural variation of the incident photon flux.

Practical tip:

The signal-to-noise ratio can usually be improved by optimizing the image acquisition settings:

- In wide-field and confocal spinning disk microscopes:
  - Increase the illumination power and/or the exposure time.
- In confocal laser scanning microscopes:
  - Increase the pixel dwell time (*i.e.* reduce the scan speed).
  - Perform averaging (line averaging, frame averaging or integration).
  - Increase the laser power
  - Increase the pinhole size (but at the detriment of optical sectioning).

Recommendation:

Images should be acquired with enough contrast between the pattern and the noise. A ***signal-to-noise ratio higher than 10:1*** is recommended.

In Daybook Analysis:

In the analyses where parameters such as ROI width, interpolation factor or smoothing factor are involved (lateral resolution, line spread function, ring spread function, point spread function), Daybook Analysis allows to increase the value of these parameters to compensate too low SNR values.

3. IMAGE INTENSITY

Images should be acquired ***within the linear response range of the detector***, that is well above the detection limit and well below the saturation limit. If available in the acquisition software, the color-coded pixels should be used to adjust properly the image intensity. The image histogram (*i.e.* the number of intensity occurrences versus the pixels intensity values) provides a quick visual way to ensure the intensity values are within the right response range of the detector.

Figure 8 displays three images of a "2×16 intensity gradation" pattern, acquired with the same illumination power and different exposure times, as well as their associated histograms:

- Too low exposure time (*cf.* Figure 8a): the histogram (*cf.* Figure 8d) shows that most pixels have 0 values, *i.e.* intensities around or below the detection limit.
- Appropriate exposure time (*cf.* Figure 8b, auto exposure mode): the histogram (*cf.* Figure 8e) shows that the pixels are evenly spread, *i.e.* intensities well above the detection limit and well below the saturation limit.
- Too high exposure time (*cf.* Figure 8c): the histogram (*cf.* Figure 8f) shows that most pixels have 65535 values, *i.e.* intensities around or above the saturation limit.

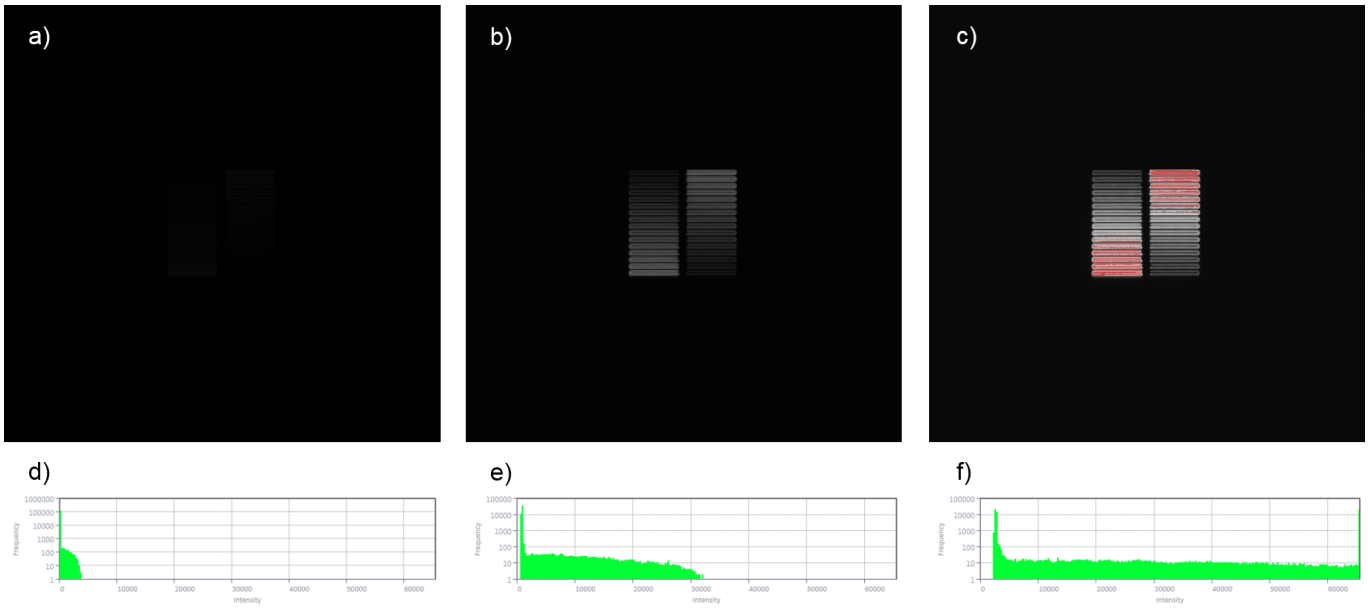


Figure 8: Images of a "2×16 intensity gradation" pattern acquired with the same illumination power and different exposure times, as well as their associated histograms (logarithmic scale). The exposure time increases from left to right; it was set too low on the left, appropriate in the middle and too high on the right. The saturated pixels are shown in red in the image on the right.

4. IMAGE DYNAMIC RANGE

Daybook Analysis can only process 8- or 16-bits images. Therefore, images should be saved with an ***8- or 16-bit dynamic range***.
If the detector captures raw data with a bit depth different from 8 or 16 bits (like 10,12 or 14 bits), the images should be saved into 8- or 16-bit dynamic range without distorting or losing any information.
For example, information contained within a 12-bit depth should not be saved as an 8-bit image.

Note:
The "image dynamic range" should not be confused with the "detector bit depth", nor with the "detector dynamic range" or the "pattern dynamic range":

- The ***image dynamic range*** is a computer feature. It can take only three values: 8, 16 or 32 bits, *i.e.* 1-, 2- or 3-byte chunks, respectively.
- The ***detector bit depth*** is the range on which the information is coded by the detector. It can take the most common 8- or 16-bit values, but also 10-, 12-, or 14-bit values. Therefore, a 16-bit dynamic range image can contain only 14-bit depth of useful information; the values between 14 and 16 bits are simply zeroes (*cf.* Figure 9).
- The ***detector dynamic range*** is the ratio between the highest to the lowest light fluxes the detector can measure. For example, it can take a value of 10000:1.
- The ***pattern dynamic range*** is the ratio between the highest to the lowest measured intensities of the pattern of interest. For example, it can take a value of 10:1.

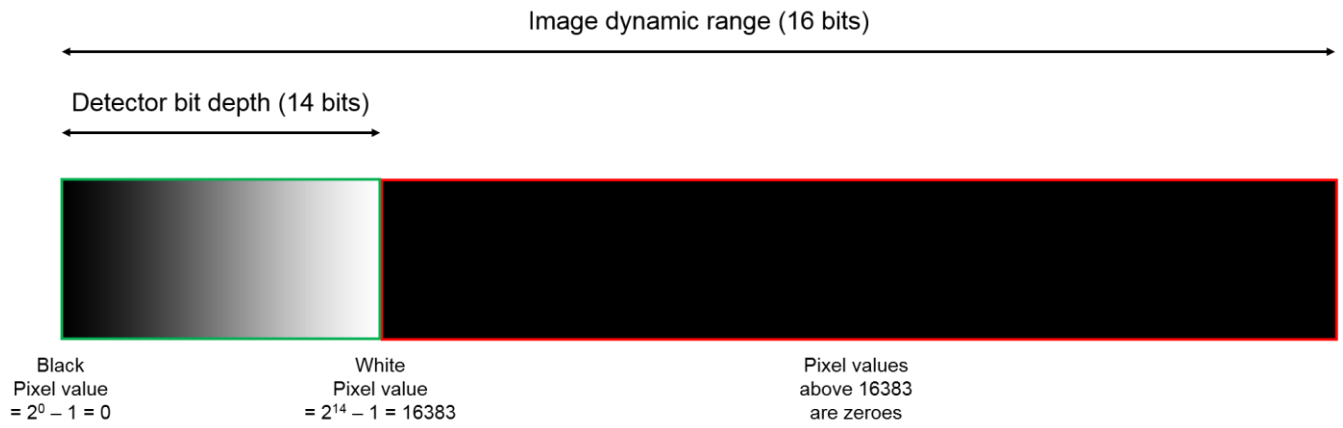


Figure 9: A 14-bit grayscale ranging from black (with the pixel value at 0) to white (with the pixel value at 16383). All the pixel values above 16383 are zeroes, to fill out the image 16-bit container. The useful part of the information in the image dynamic range is in the green rectangle, while the non-useful part is in the red one.

How to recognize them?
When opened in an image player (ImageJ for instance), the histogram of an 8-bit image should range from 0 to 255, and from 0 to 65535 for a 16-bit image.

5. IMAGE SAMPLING RATE

The sampling rate of images should fulfill the Nyquist criterion: the image lateral pixel size should be lower or equal to the half of the theoretical lateral resolution limit. However, if possible, adjusting the image lateral pixel size to ***one-third of the theoretical lateral resolution limit*** is recommended.

The same applies for Z-stacks: the image axial pixel size (the distance between two slices) should be lower or equal to the half of the theoretical axial resolution limit. However, if possible, adjusting the image axial pixel size to ***one-third of the theoretical axial resolution limit*** is recommended.

Theoretical values calculation:

The theoretical resolution limits, both lateral (XY) and axial (Z), are given by the following formula, based on the FWHM measurement of the PSF.

- For fluorescence wide-field microscopes:

$$FWHM_{xy} = \frac{0.515\lambda_{em}}{NA}$$

$$FWHM_{z} = \frac{1.772n\lambda_{em}}{NA^2}$$

- For fluorescence confocal, both laser scanning and spinning disk, microscopes (for a pinhole size of 1 Airy unit):

$$FWHM_{xy} = \frac{0.515\lambda_{exc}}{NA}$$

$$FWHM_{z} = \frac{0.886\lambda_{exc}}{n - \sqrt{n^2 - NA^2}}$$

Where λ_{exc} and λ_{em} are the excitation and emission wavelengths, respectively, NA the numerical aperture of the objective and n the refractive index of the immersion medium.

- In wide-field and confocal spinning disk microscopes, equipped with cameras, the lateral pixel size cannot easily be modified: it can only be adjusted by switching the objective (changing the objective magnification) or the camera (changing the chip pixel size).
- In confocal laser scanning microscopes, equipped with point detectors, the lateral pixel size can easily be modified: either by adjusting the zoom or the image pixels number (512×512, 1024×1024, 2048×2048, etc.).
- In wide-field and confocal microscopes, the axial pixel size can be modified by adjusting the step of the Z-stage between two slices of the Z-stack.

Example:

A “gradually spaced lines” pattern of an Argo-HM slide was imaged with a wide-field microscope, equipped with a 2048×2048 pixels sCMOS camera, whose chip pixel size is 6.5 µm. Two objectives were used: a 40×/0.95 dry objective and a 100×/1.46 oil objective.

Table 1 below shows the theoretical lateral resolution limit, the image pixel size, *i.e.* the actual sampling rate, which is compared to the required sampling rate (equal to one third or the theoretical lateral resolution limit) for these two objectives.

	40×/0.95 dry objective	100×/1.46 oil objective
Theoretical resolution limit (nm)	298	194
Image pixel size (nm)	163	65
Required sampling rate	99	65

Table 1: Lateral theoretical resolution limit, image pixel size and required sampling rate for the two used objectives.

Figure 10 displays the images of this “gradually spaced lines” pattern acquired with those two objectives, as well as the intensity line profile perpendicular to the lines in the green ROI. The line profiles in the green ROI show that the four lines are much better described with the 100×/1.46 oil objective, for which the sampling rate is suited, than with the 40×/0.95 dry objective, for which the sampling rate is too low. In particular, one can easily observe that the peak positions and magnitudes are more accurate for the 100×/1.46 oil objective, consequently the spacing and contrast measurements.

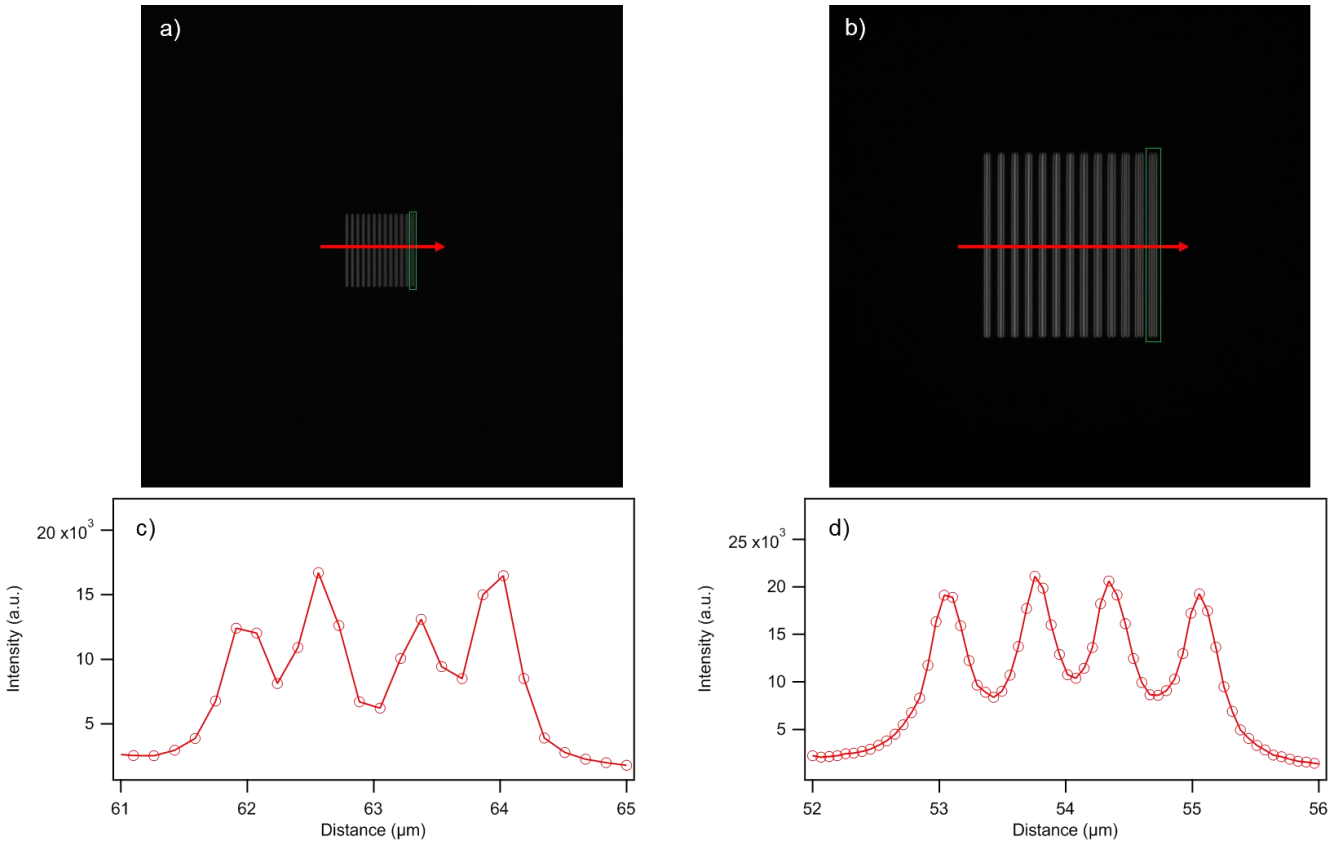


Figure 10: Images of a “gradually spaced lines” pattern acquired with the 40×/0.95 dry objective (a) and the 100×/1.46 oil objective (b), with their respective intensity line profiles perpendicular to the lines (c) and (d).

6. IMAGE FILE FORMAT

Images saved in lossy compressed formats, such as JPEG alter the intensity data in the images in a non-linear fashion, leading to unpredictable biases in intensity measurements.

Each time it is possible, images should be saved in the acquisition software proprietary format (*e.g.* Leica LIF, Nikon ND2, Olympus OIR, Zeiss CZI, etc.). Thus, the data are still raw, lossless, uncompressed, and the metadata are contained within the image file.

When it is not possible, images should be saved into a lossless compressed format, *e.g.* the lossless TIFF format. Be careful as the TIFF format can be lossy, depending on the technique chosen for storing the pixel data.
If saved into a lossless compressed format, the image file should have a dynamic range of 8 or 16 bits, the allowed image dynamic range for computers (1-byte and 2-byte chunks, respectively). Also, the metadata should be contained within the image file.

IV. NOISE, CONTRAST AND RESOLUTION ARE RELATED

1. IMPORTANT ASPECTS TO KEEP IN MIND

From E. Stelzer's [Ste1998] as well as J. Jonckman and co-workers' publications [Jon2003]:

- Contrast and resolution are related terms.
- Pixelation (*i.e.* insufficient sampling rate) reduces the contrast.
- Noise reduces the contrast.
- Dynamic range influences the cut-off frequency, *i.e.* the resolution.
- Decreasing the diameter of the point spread function increases the noise.

From A. Ferrand's and co-worker's publication [Fer2019]:

- Signal-to-noise ratio influences resolution, while signal-to-background ratio does not.

2. DIFFERENCE BETWEEN SYSTEM RESOLUTION AND IMAGE RESOLUTION

"System resolution" and "image resolution" are not identical:

- The ***system resolution*** is the ability of a microscope to discriminate (resolve) two point-objects separated by a minimal distance, in the absence of geometrical aberrations, and without the help of any post-processing algorithm. It is an intrinsic property of a microscope [Cre2013].
- The ***image resolution*** is the distance between two point-objects measured in an image, such that they are discernable as separate objects with a given contrast. It can be improved with post-processing algorithm, such as deconvolution [Jon2003].

3. HOW TO MEASURE RESOLUTION, CONTRAST AND SNR TOGETHER?

The "lateral resolution" analysis in Daybook Analysis, associated to the "gradually spaced lines" patterns, allows uniquely, to the best of our knowledge, to measure simultaneously, from the same image, the ***minimal resolvable distance*** between lines close to each other, for a given ***contrast*** value, with an associated ***signal-to-noise ratio*** (SNR) value.

This analysis was inspired by the work of K. Korobchevskaya and co-workers [Kor2017]. Their approach has been improved and automatized in Daybook Analysis. The detailed description about how this analysis works can be found in the documentation of the "lateral resolution" analysis.

V. REFERENCES

[STE98] E. H. K. Stelzer, *"Contrast, resolution, pixelation, dynamic range and signal-to-noise ratio: fundamental limits to resolution in fluorescence light microscopy,"* Journal of Microscopy **189**, 15–24 (1998)

[Jon2003] J. E. N. Jonkman, J. Swoger, H. Kress, A. Rohrbach and E. H. K. Stelzer, *"Resolution in optical microscopy,"* Biophotonics, Chapter 18, 416–446 (2003).

[Cre2013] C. Cremer and B. R. Masters, "Resolution enhancement techniques in microscopy," European Physical Journal H **38**, 281–344 (2013).

[Kor2017] K. Korobchevskaya, H. Colin-York, B. C. Lagerholm, and M. Fritzsche, *"Exploring the Potential of Airyscan Microscopy for Live Cell Imaging,"* Photonics **4**, 41 (2017).

[Fer2019] A. Ferrand, K. D. Schleicher, N. Ehrenfeuchter, W. Heusermann, and O. Biehlmaier, *"Using the NoiSee workflow to measure signal-to-noise ratios of confocal microscopes,"* Nature Scientific Reports **9**, Article number 1165 (2019).

Encountered an issue or a question when using Daybook Analysis?
Please send a screenshot and your issue description at:
customer@argolight.com