ARGOLIGHT
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# TECHNICAL NOTE

# The Don’ts and Do’s to **measure lateral resolution with Argolight's solutions**


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# 1. Introduction

Lateral resolution is usually the main feature that is sought by fluorescence microscope users. In a general sense, lateral resolution is defined as the minimum separation between two-point objects, such that they can still be distinguished. Historically, due to the commercial availability of fluorescent beads, the resolution limit of fluorescence microscopes has been associated with the measurement of the full-width at half maximum (FWHM) of the point spread function (PSF).

However, there are other approaches to measure spatial resolution. The fact that it is possible to do better than the resolution limit (i.e., super-resolution) suggests that resolution is not an inherent property of an optical system. Instead, the optical system has the capacity to transmit a finite amount of information.

Argolight suggests an approach to quantify lateral resolution based on the "gradually spaced lines" patterns. Their analysis provides the contrast transfer function (i.e., the contrast versus the spacing between the lines). The contrast transfer function translates the capacity of the imaging system to transmit a finite amount of information.

In this technical note, we provide information about the specifications and manufacturing tolerance of the "gradually spaced lines" patterns as well as some guidelines for best performance.

This manuscript aims to:

1. Remind the user of the specifications and manufacturing tolerance of the "gradually spaced lines" patterns.
2. Inform the user about the wrong approaches to measure the lateral resolution from the "gradually spaced lines" patterns alone.
3. Provide a description and justification of the necessity to use Daybook, the companion software of Argolight's slides.

# 2. Specifications of the “gradually spaced lines”

## 2.1. Reminder

The “gradually spaced lines” pattern consists of pairs of lines whose spacing gradually increases. It is offered into four orientations (Figure 1): one horizontal, one vertical, one descending (–45°) and one ascending (+45°).

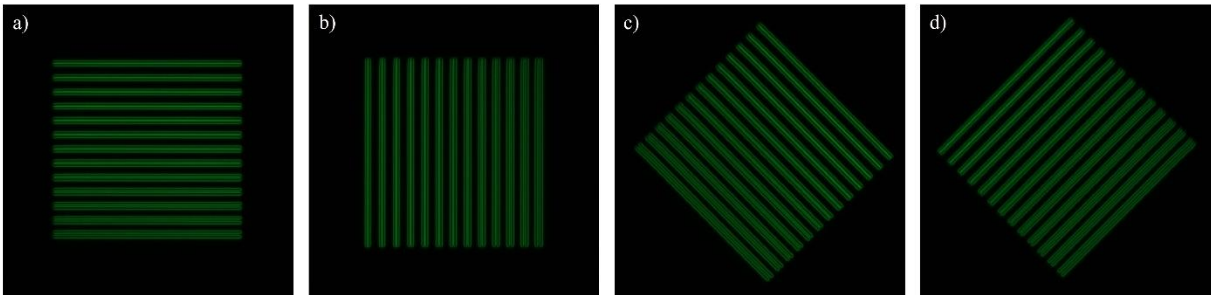


**Figure 1:** Images of the “gradually spaced lines” for the four orientations: (a) horizontal, (b) vertical, (c) descending, and (d) ascending.

At the date of issue of this document, two slide models contain the “gradually spaced lines” patterns: the Argo-HM and the Argo-SIM slides (and their respective Argo-POWER product range).

For the Argo-HM slide, the lines are 50-µm long. The spacing between the two inner lines of each group gradually increases from 100 to 700 nm, with a step of 50 nm (Figure 2a).

For the Argo-SIM slide, the lines are 36-µm long. The spacing between the two inner lines of each group gradually increases from 0 to 390 nm, with a step of 30 nm (Figure 2b).

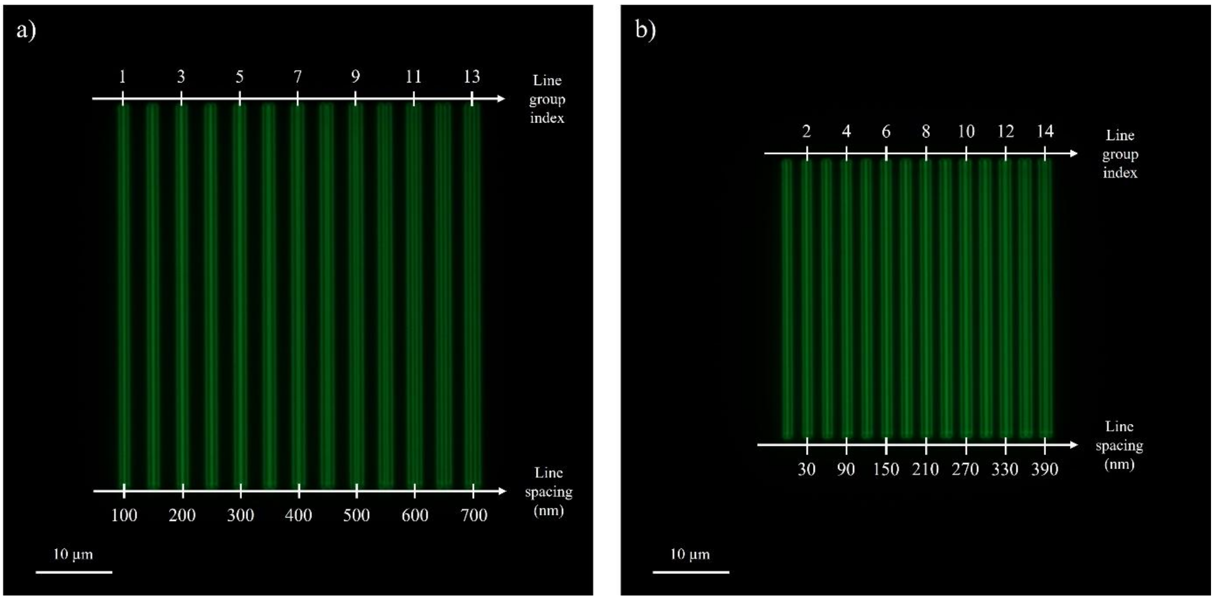


**Figure 2:** Images of the vertical “gradually spaced lines” of (a) an Argo-HM and (b) an Argo-SIM acquired using a wide-field microscope, 100×/1.49 objective, GFP channel. The specified spacings between the two inner lines of each group are indicated for both slide models.

The specifications of this pattern are provided in the user guides of the products: the line spacings are given in the section "Gradually spaced lines" (Table 1) and the lateral positioning error (i.e., the manufacturing tolerance) in the section "Patterns overview" (*"the constituents within each individual pattern are positioned with a maximum relative error of ± 0.11 µm in XY and ± 0.15 µm in Z"*).

**Argo-HM** Step increase between lines: 50 nm

| Line # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spacing (nm) | 100 | 150 | 200 | 250 | 300 | 350 | 400 | 450 | 500 | 550 | 600 | 650 | 700 |

**Argo-SIM** Step increase between lines: 30 nm

| Line # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spacing (nm) | 0 | 30 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 270 | 300 | 330 | 360 | 390 |

**Table 1:** Specifications of the "gradually spaced lines" provided in the user guides of the Argo-HM and Argo-SIM slides. The manufacturing tolerance on the spacings is ± 110 nm.

## 2.2. Spatial distribution

Argolight uses a photolithography process to engrave fluorescent patterns inside the Argoglass®. The elementary pattern that can be engraved is a ring. From this ring, any pattern (straight lines, curved lines, etc.) can be engraved, with the specificity that two lines are always engraved at the same time.

The fluorescent patterns in the Argolight slides are composed of two fluorescent species. These fluorescent species, put together, are responsible for the remarkable spectral features of the patterns (broad excitation and emission ranges, nanosecond-range lifetime and photostability).

It happens that these two fluorescent inorganic species are not exactly located at the same place in the patterns. One species (let's call it species X), excitable in the UV, is located in the inner part of the ring, while the other one (let's call it species Y), excitable in the visible (from blue to red), is located in the outer part of the ring (Figure 3a). This is inherent to Argolight's manufacturing process and cannot be avoided.

The fluorescent species composing a pair of lines have a similar spatial distribution than those composing a ring. The species X, excitable in the UV, is located inside the pair of lines, while the species Y, excitable in the visible, is located outside the pair of lines (Figure 3b).

In the "gradually spaced lines" pattern, a group of lines is composed of two pairs of lines. The measured spacing between the lines of interest (i.e., the two inner lines), depends therefore on the excitation wavelength. The spacing measured for a UV excitation is larger than the one measured for a visible excitation (Figure 3c).

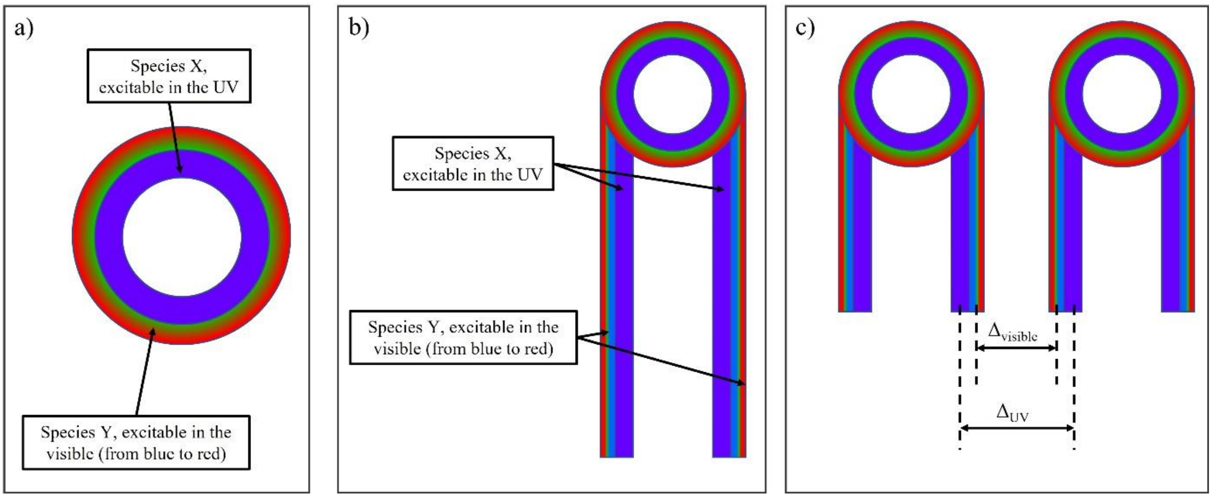


**Figure 3:** Spatial distribution of the fluorescent species X (excitable in the UV) and Y (excitable in the visible) for (a) a ring elementary pattern, (b) a pair of lines, and (c) a group of lines of the "gradually spaced lines" pattern. In (c), the spacing between the two inner lines measured for a UV excitation is larger than the one measured for a visible excitation.

Figure 4 shows an example of the measured line spacings for the same "gradually spaced lines" pattern of an Argo-HM slide, imaged with the GFP (blue excitation at 488 nm) and the DAPI (UV excitation at 405 nm) channels. The spacings measured on the image acquired with the GFP channel are smaller than those measured on the image acquired with the DAPI channel. In this case, the difference is an average of about 90 nm. This spectral dependency of the spatial distribution of the species composing the "gradually spaced lines" pattern is taken into account in the aforementioned ± 110 nm manufacturing tolerance.

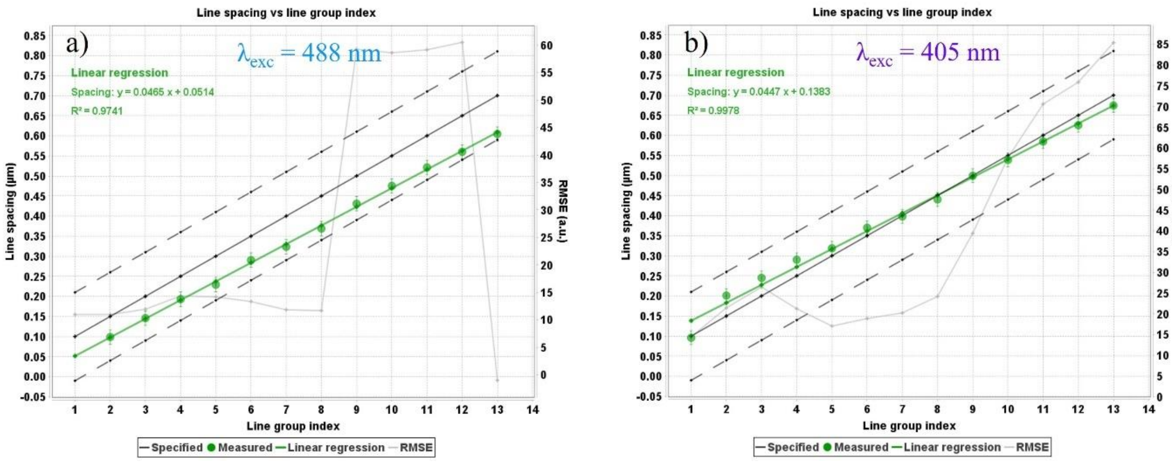


**Figure 4:** Evolution of the measured line spacing versus the line group index, for the same "gradually spaced lines" pattern of an Argo-HM slide, acquired with (a) blue and (b) UV excitations. The measured spacings (green dots) were fitted using a linear regression (green line). The specified spacings (100, 150, 200, …, 700 nm) and the manufacturing tolerance tunnel (± 110 nm) are indicated in the black continuous line and black dashed lines, respectively.

## 2.3. Manufacturing variability

Similar to any manufacturing process, there is some variability between the slides that are produced at Argolight. After fabrication, each slide is systematically inspected; there is no statistical control.

For the “gradually spaced lines” patterns, for both UV and blue excitations, the inspection is considered as successful when:

- The measured line spacings are within the manufacturing tolerance tunnel (i.e., the specified line spacings are ± 110 nm).
- The spacing step (i.e., the slope of the linear regression curve) is within (50 ± 10) nm for the Argo-HM or (30 ± 10) nm for the Argo-SIM.

Figure 5 shows an example of the measured line spacings for the same “gradually spaced lines” pattern of two different Argo-HM slides, imaged with the GFP channel (blue excitation at 488 nm). The spacings measured on the images fulfills the two inspection criteria described above: the measured line spacings (green dots in the graphs) are within the manufacturing tolerance tunnel (black dashed lines in the graphs) and the spacing steps (i.e., the slope of the linear regression curves [6.8 and 46.5 nm]), are within (50 ± 10) nm. The manufacturing variability is taken into account in the aforementioned ± 110 nm manufacturing tolerance.

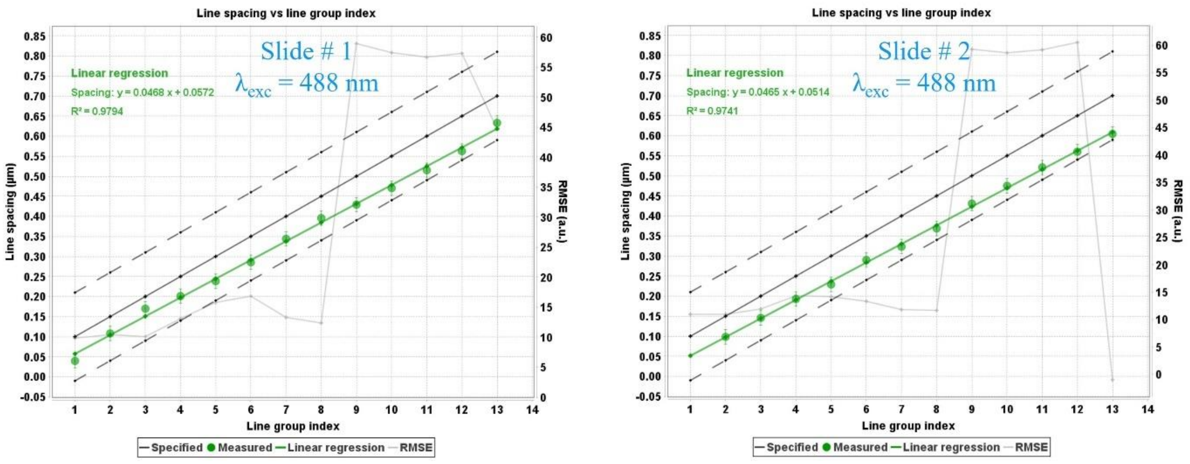


**Figure 5:** Evolution of the measured line spacing versus the line group index, for the same “gradually spaced lines” pattern of two different Argo-HM slides, acquired with blue excitation. The measured spacings (green dots) were fitted using a linear regression (green line). The specified spacings (100, 150, 200, …, 700 nm) and the manufacturing tolerance tunnel (± 110 nm) are indicated in the black continuous line and black dashed lines, respectively.

## 2.4. Summary

To summarize, the manufacturing tolerance of ± 110 nm actually takes into account many parameters:

- The positioning uncertainty of the lines due to the manufacturing tools.
- The spectral dependency of the spatial distribution of the fluorescent species composing the lines.
- The slide-to-slide manufacturing variability.

The specifications and manufacturing tolerance on the line spacings and the spacing steps provided in the user guides could be alternatively seen as presented in Table 2: the offset $x_0$ has a positioning uncertainty of ± 110 nm while the spacing step has a variability of ± 10 nm.

**Argo-HM**

| **Line #** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Spacing (nm)** | $x_0$ + 100 | $x_0$ + 150 | $x_0$ + 200 | $x_0$ + 250 | $x_0$ + 300 | $x_0$ + 350 | $x_0$ + 400 | $x_0$ + 450 | $x_0$ + 500 | $x_0$ + 550 | $x_0$ + 600 | $x_0$ + 650 | $x_0$ + 700 |

- Offset: $x_0 = (0 \pm 110)$ nm
- Spacing step between lines: $\Delta = (50 \pm 10)$ nm

**Argo-SIM**

| **Line #** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Spacing (nm)** | $x_0$ + 0 | $x_0$ + 30 | $x_0$ + 60 | $x_0$ + 90 | $x_0$ + 120 | $x_0$ + 150 | $x_0$ + 180 | $x_0$ + 210 | $x_0$ + 240 | $x_0$ + 270 | $x_0$ + 300 | $x_0$ + 330 | $x_0$ + 360 | $x_0$ + 390 |

- Offset: $x_0 = (0 \pm 110)$ nm
- Spacing step between lines: $\Delta = (30 \pm 10)$ nm

**Table 2:** An alternative way to describe the specifications and manufacturing tolerance of the “gradually spaced lines” of the Argo-HM and Argo-SIM slides.

## 3. Do not use the “gradually spaced lines” as a ruler

### 3.1. Description of the approach

This approach consists in looking at the group of lines for which the two inner lines can visually be resolved, and claiming a lateral resolution value corresponding to the specified spacing.

Figure 6 shows an image of the vertical “gradually spaced lines” of an Argo-HM slide. The zoom inside the pattern indicates that the achievable lateral resolution of the used microscope ranges between 300 and 400 nm, depending on the reader.

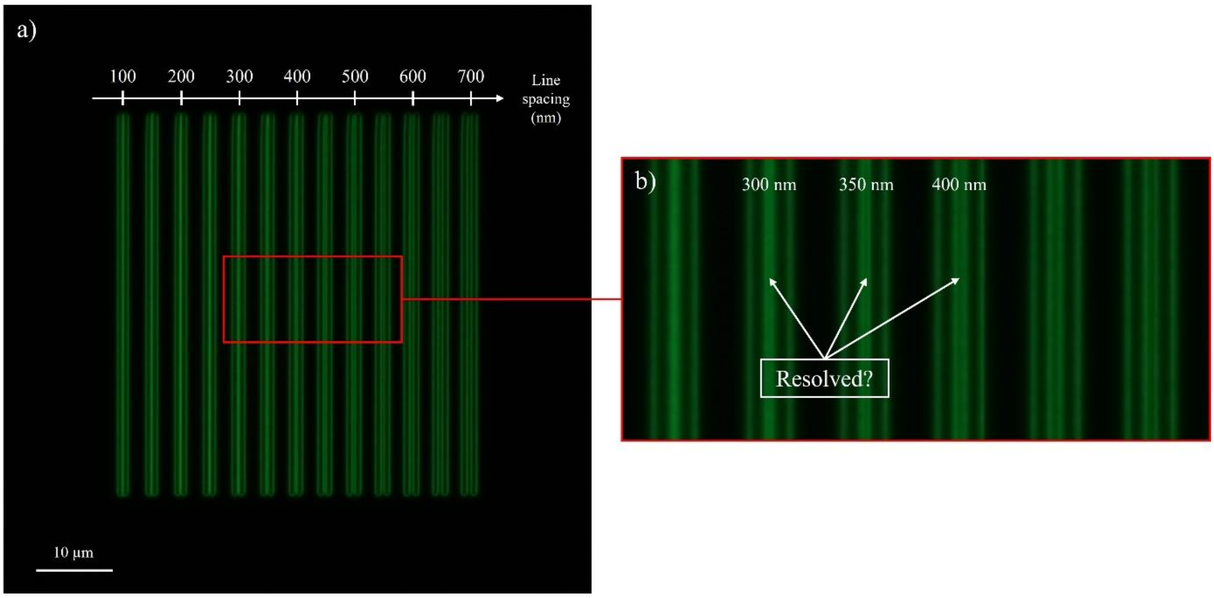


**Figure 6:** (a) Image of the vertical “gradually spaced lines” of an Argo-HM slide. The specified spacings between the two inner lines of each group are indicated. (b) Zoom inside the pattern, showing the subjectivity of claiming a lateral resolution of 300, 350, or 400 nm.

### 3.2. Why this approach is not suited

As evidenced in Figure 6, the resolution measurement based on this approach is rather subjective. Indeed, it depends on the capacity of the reader to distinguish a contrasted dip between the two inner lines. **This comes down to evaluating a contrast of a few percent, but not measuring it.**

Note that the contrast is a quantitative parameter that can actually be measured. It can be defined as the difference between the intensity of the signal of interest (i.e., the mean peak intensity of the two inner lines) and the dip between the two inner lines.

Besides, this approach does not take into account the positioning uncertainty, the spectral dependency of the spatial distribution of the fluorescent species composing the lines, nor the manufacturing variability of the “gradually spaced lines.”

# 4. Do not measure the line spacings from the peaks of the intensity profile

## 4.1. Description of the approach

This approach consists in drawing an intensity profile perpendicular to the lines in a region of interest (ROI) with a width of a few pixels, measuring the smallest resolvable spacing (i.e., the peak-to-peak distance between the two inner lines) and claiming a lateral resolution value corresponding to this measured spacing.

Figure 7 shows an image of the vertical "gradually spaced lines" of an Argo-HM slide. An intensity profile was drawn as perpendicular as possible to the lines, with a width of a few pixels. The measurement of the smallest resolvable spacing indicates that the achievable lateral resolution of the used microscope is about 260 nm.

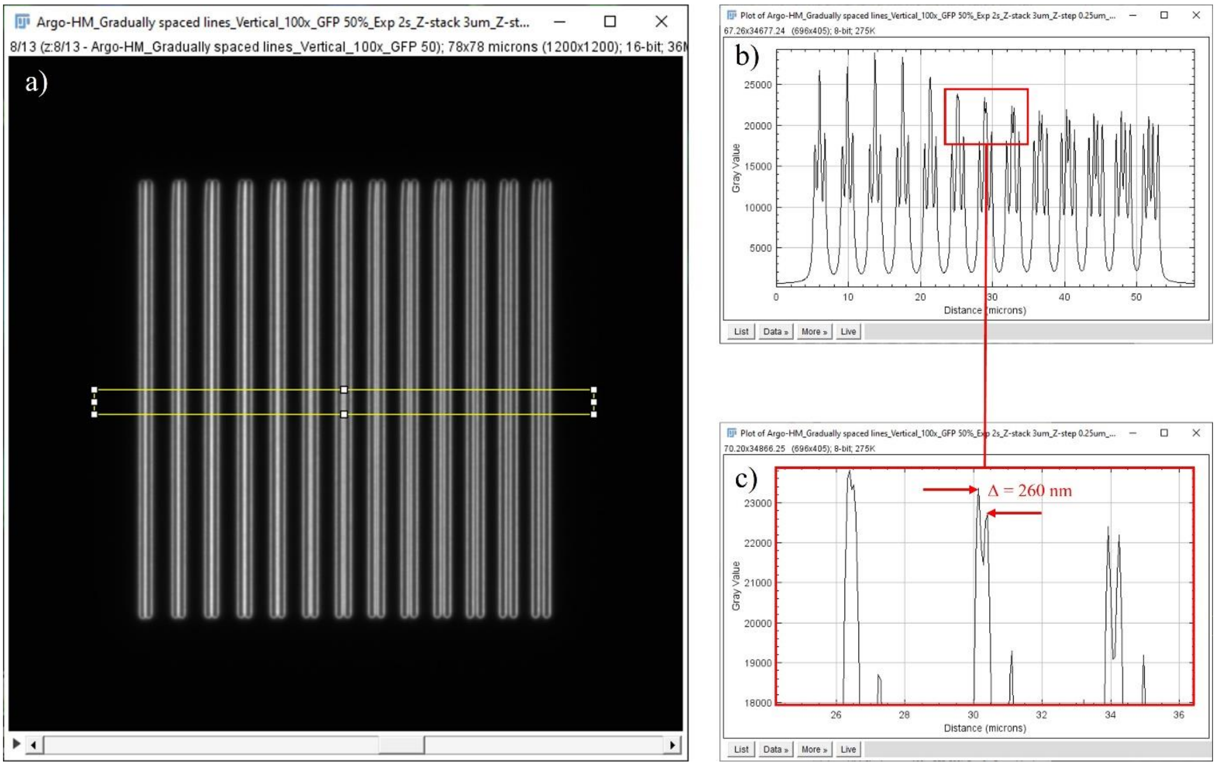


**Figure 7:** (a) Image of the vertical "gradually spaced lines" of an Argo-HM slide. (b) Intensity profile drawn the most perpendicular as possible to the lines, with a width of a few pixels. (c) Zoom inside the intensity profile showing the smallest resolvable spacing measured from the peak-to-peak distance between the two inner lines of interest.

## 4.2. Why this approach is not suited

Although this approach is more accurate than the previous one, because it can take into account the positioning uncertainty, the spectral dependency of the spatial distribution of the fluorescent species composing the lines, as well as the manufacturing variability, it nevertheless suffers from several drawbacks.

First, **the precision of the measurement depends on the lateral sampling rate** (i.e., the lateral pixel size). Therefore, there is a reading uncertainty (the same kind of uncertainty one would have when measuring distances with a ruler) associated to the image pixelation. This reading uncertainty is equal to ± one half of the lateral pixel size.

Second, **drawing an intensity profile manually** (with ImageJ for instance) **necessarily induces an uncertainty as per the line perpendicularity**.

Third, **when the inner lines come closer and closer, their real positions differ more and more from their associated intensity peak positions**. Simulating the signal superposition emitted by two lines that are close from one to another allows to confirm that the spacing between the lines becomes smaller than the spacing between their real positions (Figure 8). This behavior tends to improve the resolution measurement (i.e., to make it smaller).

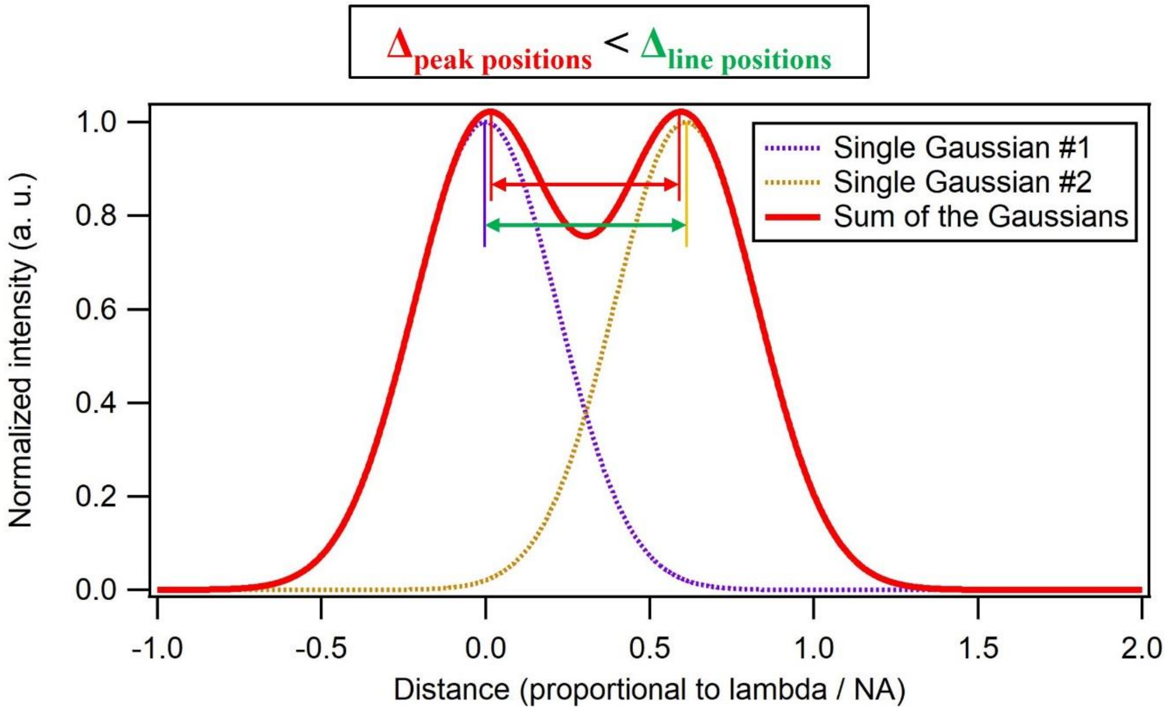


**Figure 8:** Simulated data showing the individual signals (in purple and orange) coming from two close lines, and their superposition (in red). The signal emitted by each line was supposed to be Gaussian. The red and the green arrows, representing the spacing measured from the peak positions and the object positions, show that the first one is smaller than the latter.

# 5. Do use Daybook

For reproducible and trustable results, we recommend analyzing the "gradually spaced lines" patterns using Daybook software.

## 5.1. Description of the approach

This approach consists in analyzing the images of the "gradually spaced lines" using the analysis "lateral resolution" in Daybook Analysis.

In short, the analysis works as follows:

- It plots a mean intensity profile perpendicular to the lines.
- It measures the contrast between the two inner lines for each group of lines.
- It fits the intensity profile of each group of lines with a sum of Gaussian or Lorentzian functions, from which it measures the spacing between the two inner lines of each group.
- From the contrast and the line spacing measurements, it plots the contrast versus the line spacing, to provide the so-called "contrast transfer function."
- It also measures the signal-to-noise ratio (SNR) and the signal-to-background ratio (SBR) of the analyzed image.

Figure 9 shows the results page of the analysis "lateral resolution" obtained from an image of the vertical "gradually spaced lines" of an Argo-HM slide.

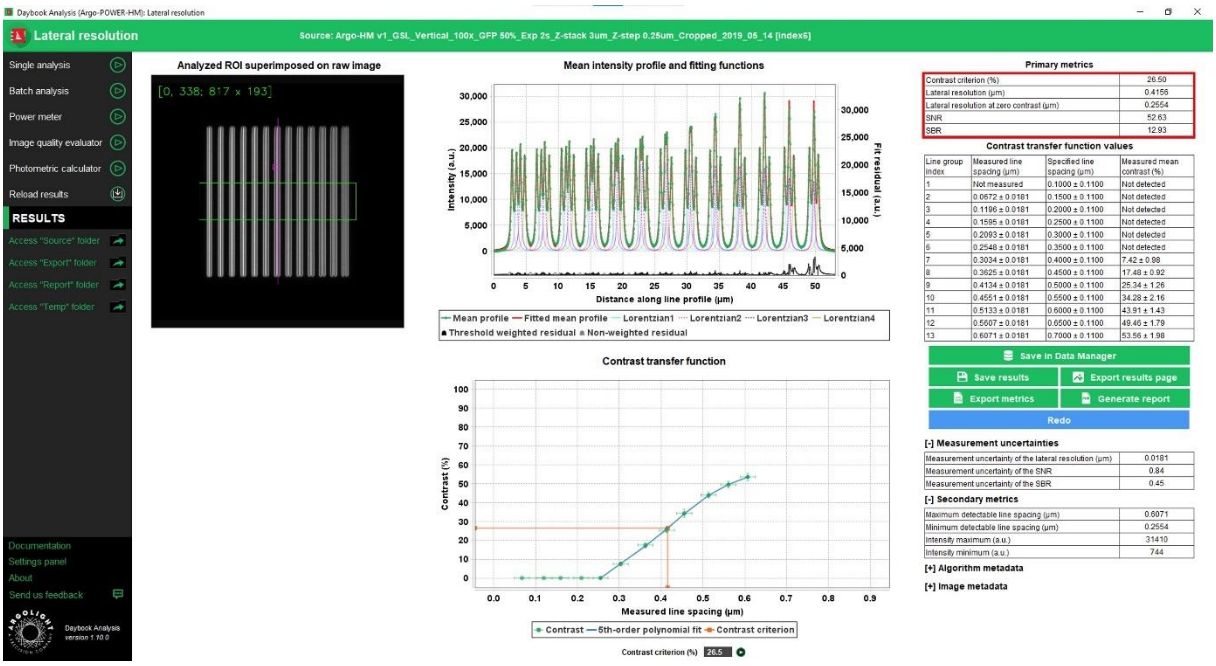


**Figure 9:** Results page of the analysis "lateral resolution" obtained from an image of the vertical "gradually spaced lines" of an Argo-HM slide. Top left: analyzed ROI superimposed on the image of the "gradually spaced lines." Top center: mean intensity profile, drawn perpendicularly to the lines. Bottom center: contrast transfer function (i.e., the measured contrast versus the measured line spacing). Right: different tables showing the primary metrics (red box) of the analysis, the contrast transfer function values, the measurement uncertainties, the secondary metrics, the algorithm metadata, and the image metadata.

The strength of this approach is that it provides the **distance that the imaging system can resolve for a given contrast, with an associated SNR and SBR**. It is very important to measure these four parameters together because they have an influence on one another.

A more detailed description about this analysis can be found in the associated software documentation.

## 5.2. Why this approach is suited

This approach is better suited than the two previous ones because **it takes into account all the aforementioned pitfalls.**

- The positioning uncertainty, the spectral dependency of the spatial distribution of the fluorescent species composing the lines, and the manufacturing variability are managed via the measurement of the line spacings obtained from the positions of the fitting functions.
- The actual spacing between the inner lines is measured, not the peak-to-peak distance from the intensity profile.
- The contrast is measured; it is not a subjective notion.
- The angle between the lines and the image axes is measured and a rotation correction is applied if this angle is different from 0, ± 45, or 90°. Thus, the drawn intensity profile is perfectly perpendicular to the "gradually spaced lines."
- In the case of a Z-stack, the most contrasted plane of the "gradually spaced lines" can automatically and reliably be selected via the "best focus selection" option, preventing some bias in the choice of the image to be analyzed.
- The lateral resolution is measured from the contrast transfer function, for a given contrast, SNR and SBR.

For the aforementioned reasons, it is strongly recommended to use Daybook with the slide.

## 5.3. Validation of this approach

To prove that this approach can deal with positioning uncertainty and manufacturing variability, seven simulated images of the "gradually spaced lines" of an Argo-SIM slide were generated. The spacings between the lines were shifted 5 nm from the specified values: 0, 30, 60, …, 390 nm; 5, 35, 65, …, 395 nm; 10, 40, 70, …, 400 nm; etc.

The seven images were processed in Daybook Analysis using the analysis "lateral resolution," resulting in seven contrast transfer functions. Figure 10 shows these contrast transfer functions on the same graph. Within the measurement uncertainties, the seven contrast transfer functions superimpose almost perfectly (except for the first spacing value having a 0% contrast) indicating that this approach is not sensitive to variabilities in the actual line spacings. In particular, the resolvable distance for a contrast of 26.5% (Rayleigh criterion) is the same for the seven images.

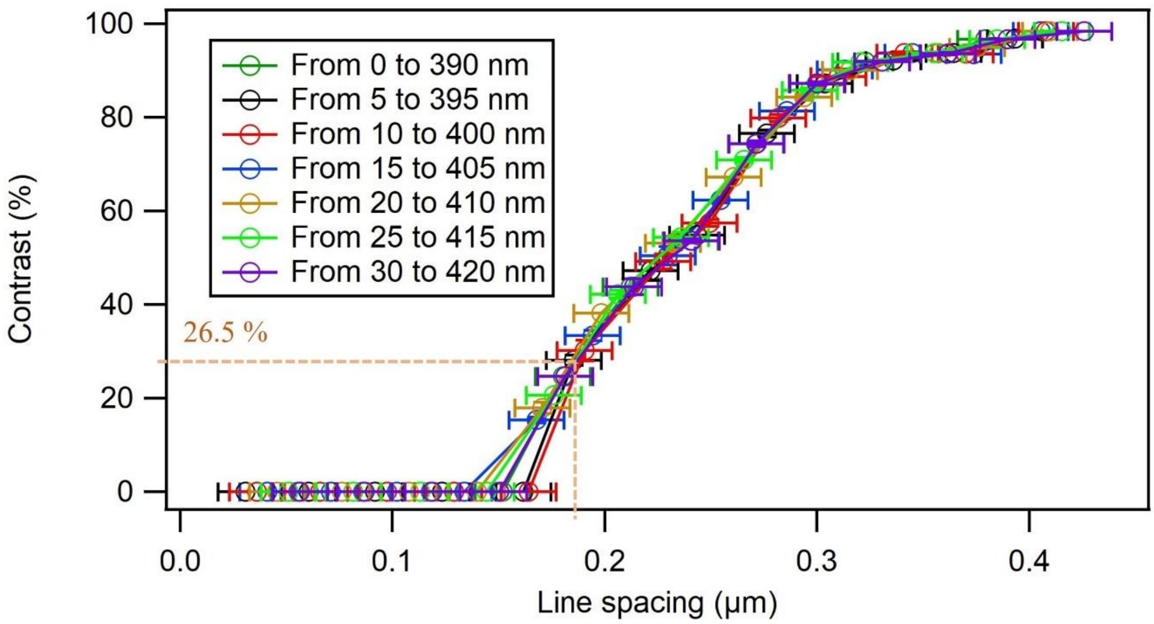


**Figure 10:** Contrast transfer functions obtained from seven simulated images of the "gradually spaced lines" of an Argo-SIM slide having spacings shifted by 5 nm, using the analysis "lateral resolution." Within the measurement uncertainties, the seven contrast transfer functions superimpose almost perfectly, except for the first spacing value having a 0% contrast.

### 5.4. How to analyze the pattern within Daybook software?

The detailed guide of the analysis "lateral resolution" can be found at Argolight Knowledge Center (https://argolight.notion.site) and within Daybook Analysis ("Documentation" menu on the bottom left of the interface).

## 6. Conclusion

In this technical note, we tried to answer the questions raised by some of our customers about the specifications and manufacturing tolerance of the "gradually spaced lines" patterns used to measure the lateral resolution.

We reminded the user of the specifications and manufacturing tolerance of the "gradually spaced lines" patterns. We described the wrong approaches to measure the lateral resolution from the "gradually spaced lines" patterns alone. We explained and justified the necessity to use Daybook, in association with the slides.

We hope that this note will provide the necessary information to answer all the interrogations and remove the misunderstanding about the specifications and manufacturing tolerance of "gradually spaced lines" patterns.

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**Note for readers**

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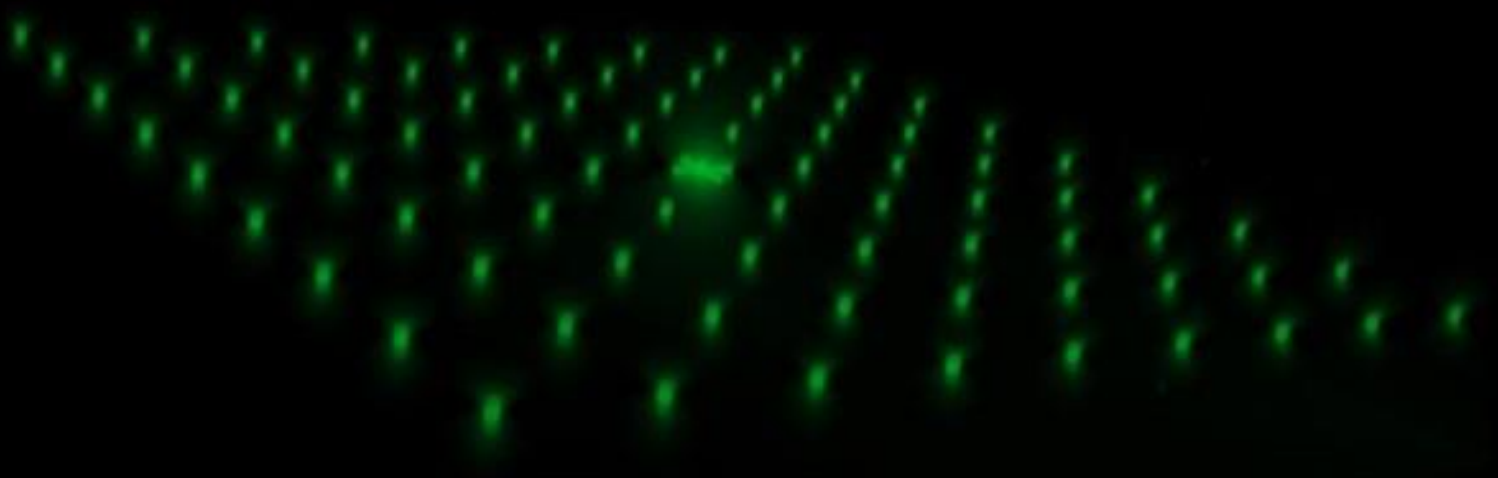

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