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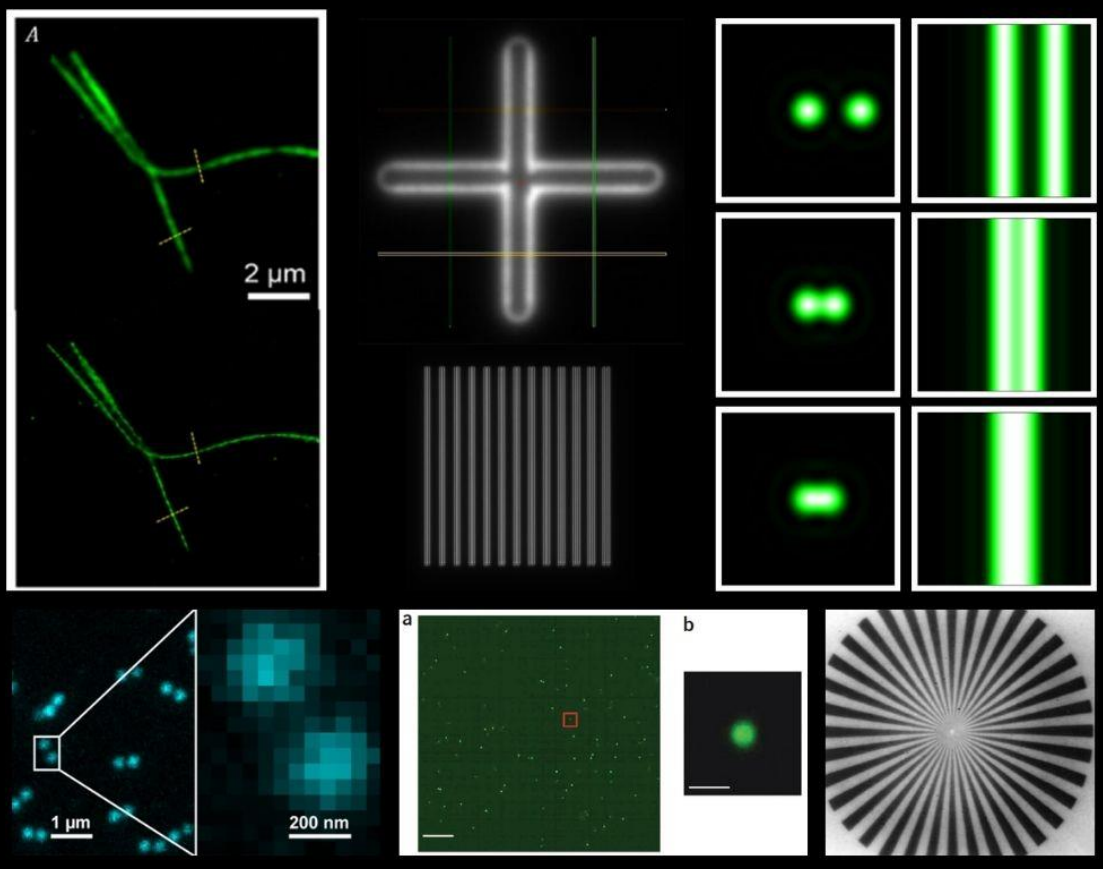
WHITE PAPER

The different approaches to measure **lateral resolution**



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1. Introduction

The term “resolution” is present everywhere in the field of microscopy because it is a determining factor of image quality. The resolution allows to see features with good contrast, as emphasized in the microscope manufacturers’ taglines: *“seeing is believing,” “make it visible,” “now we see,”* and *“seeing is solving”*; but also to count objects, define the scale in colocalization experiments, and so on.

The sales and marketing arguments from the microscope manufacturers usually focus on this feature, which is in turn sought by most microscope users. That is why resolution has been (and is still) one of the main metrics (if not the main) featured for image quality, and why it is a major parameter to measure for microscope performance assessment and monitoring [ISO2019] [QUAREP2021]. Despite this state of fact, its absolute and generic definition, other than “the capacity of a microscope for imaging fine detail,” remains floating because of the many approaches that can be used to define it, and the many methods and tools that are available to measure it.

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). This approach is known as the single-point resolution approach. However, there are other approaches to measuring spatial resolution: the two-point, the single-line, the two-lines, and the optical transfer function approaches can also be used, each one having its own advantages and drawbacks.

This white paper aims to present an up-to-date overview to contribute to the ongoing discussion and community endeavor to standardize the resolution definition and measurement. In particular:

- The original definition of spatial resolution will be reminded.
- The different approaches to measure spatial resolution will be reviewed, and their relationship will be described.
- The experimental parameters that influence spatial resolution will be presented.
- The Argolight’s approach and remarkable results obtained with it will be highlighted.

Unless otherwise specified, this paper deals with the ***lateral spatial resolution of fluorescence imaging systems***, as opposed to spectral resolution, which applies to spectrometers, or to axial spatial resolution, which applies to the Z-dimension.

The content of this document was presented by the author of this white paper in two workshops during the ELMI meeting in Noordwijkerhout and the EMBO practical course organized in Prague, both in June 2023.

2. What exactly is resolution? A brief literature review

It is not the goal of this section to review all the articles dealing with the theory of resolution, from Lord Rayleigh and Ernst Abbe to the recent Nobel laureates for super-resolution, but rather to have a look at how the community defines and describes resolution in modern fluorescence microscopy through the literature. Many articles clarify and review the different definitions of resolution [Jon2003] [Lau2012] [Cre2013] [Hor2016] [Sch2021]. From these articles, three main common approaches can be extracted.

The two-point approach

The original definition of resolution refers to the ability of an imaging system to discriminate two close point-like objects from one to another. In this approach, ***resolution is defined as the lateral minimum separation distance measurable between the two point-like objects***. Because defining this minimum separation distance can be subjective and ambiguous, the concept of "contrast" is necessary to quantify resolution. It is important to note that any contrast between 0 and 100% can be used to quantify resolution in this approach.

The contrast transfer function approach

Another definition of resolution refers to the ability of an imaging system to discriminate the finest lines composing a grating. In this approach, ***resolution is defined as the smallest line-to-line lateral distance that can still display contrast (i.e., the highest spatial frequency that can be captured by the imaging system)***. However, since the imaging system can transmit a finite amount of spatial frequencies up to the cut-off frequency, it is more complete and convenient to describe its ability to transfer contrast from the specimen to the intermediate image plane at a specific distance via the so-called "contrast transfer function (CTF)," rather than providing a single value.

The single-point approach

The third definition of resolution, unlike the previous two, relies on an isolated object. It does not refer to any ability of the imaging system to discriminate two objects close to one another, nor to transmit contrast for a given spatial frequency. Rather, ***it is based on the measurement of the lateral FWHM of the PSF from a single point-like object***.

Comparison of these three approaches

The fact that it is possible to achieve higher resolution (i.e., super-resolution) than the limit imposed by the diffraction barrier suggests that resolution is not an inherent property of a fluorescence microscope. Instead, the imaging system has the capacity to transmit a finite amount of information [Sch2021]. This suggests that a single number such as an FWHM or a minimum separation distance is not sufficient to describe resolution, unlike the CTF, which contains much more information.

Other approaches

On top of the single-point, the two-point, and the CTF approaches described above, the single- and the two-line approaches can also be used to quantify resolution [Cor1996], in a similar way as the single- and the two-point approaches.

In short, there is no unique approach to quantify resolution. Among the five different approaches, which one is the best? It depends on the application; for example, whether single molecules, filaments, or cells are aimed to be imaged.

3. Two-point resolution

3.1. Concept

The concept of the two-point resolution approach relies on considering two point-like objects mutually emitting the same amount of fluorescence, separated by a small distance. A point-like object is a spherical object with a size that is about one-third of the expected theoretical resolution of the fluorescence imaging system. A small distance is a distance corresponding roughly to the expected practical resolution.

Within this approach, the two points are considered resolved if they can be separated by a clear dip in intensity when imaged by a specific microscope. However, a clear dip is quite a subjective notion. This is why the concept of "contrast" needs to be introduced and associated with the separation measurement. The contrast is defined as the difference between the average peak intensity from the two points and the valley intensity between the two points, normalized to the average peak intensity. It is expressed in % and is given by the following equation:

$$Contrast = 100 \times \frac{\overline{I_{max}} - I_{min}}{\overline{I_{max}}}$$

The theoretical spacing value between two equally incoherent point sources is given by the following equation:

$$\Delta = K \times \frac{\lambda_{em}}{NA}$$

where K is a factor depending on the chosen contrast criterion, λ_{em} is the emission wavelength, and NA is the numerical aperture of the objective.

Although, conceptually, any contrast between 0 and 100% can be used to quantify resolution, some contrast criteria are more meaningful than others because they rely on physical basics. That is why the humble author of this white paper will spare the reader from the "Royon criterion."

Rayleigh criterion

A common choice is the ***Rayleigh criterion***, which was proposed in 1874. The Rayleigh resolution criterion states that two equally incoherent point sources are barely resolved by a diffraction-limited system with a circular pupil when the center of the Airy intensity pattern generated by one point source falls exactly on the first zero of the Airy pattern generated by the second point source. In this case, the associated theoretical contrast is 26.5% and the K factor is 0.610 (Figures 1b and 1e). Rayleigh's choice was made at a time when the human eye, which cannot resolve arbitrarily small intensity differences, was the common photodetector.

Schuster criterion

A second, yet less common, choice, is the ***Schuster criterion***, which was proposed in 1904. The Schuster criterion states that two equally incoherent point sources are called resolved when there is no overlap between the Airy intensity patterns generated by the two point sources. In this case, the associated theoretical contrast is 100% and the *K* factor is 1.220 (Figures 1a and 1d). Schuster's choice was justified by the fact that there is something arbitrary in the Rayleigh criterion because the dip in intensity necessary to indicate resolution is a physiological phenomenon (i.e., it depends on the ability of the observer's eye to perceive a weak intensity dip).

Sparrow criterion

A third choice, also less common, is the ***Sparrow criterion***, which was proposed in 1916. The Sparrow resolution criterion states that two equally incoherent point sources are barely resolved when their separation is the maximum separation for which the image of the pair of points shows no dip at the midpoint. In this case, the associated theoretical contrast is almost 0% and the *K* factor is 0.474 (Figures 1c and 1f). This is the ultimate limit for photodetectors (replacing the naked eye) that can resolve arbitrary small intensity differences.

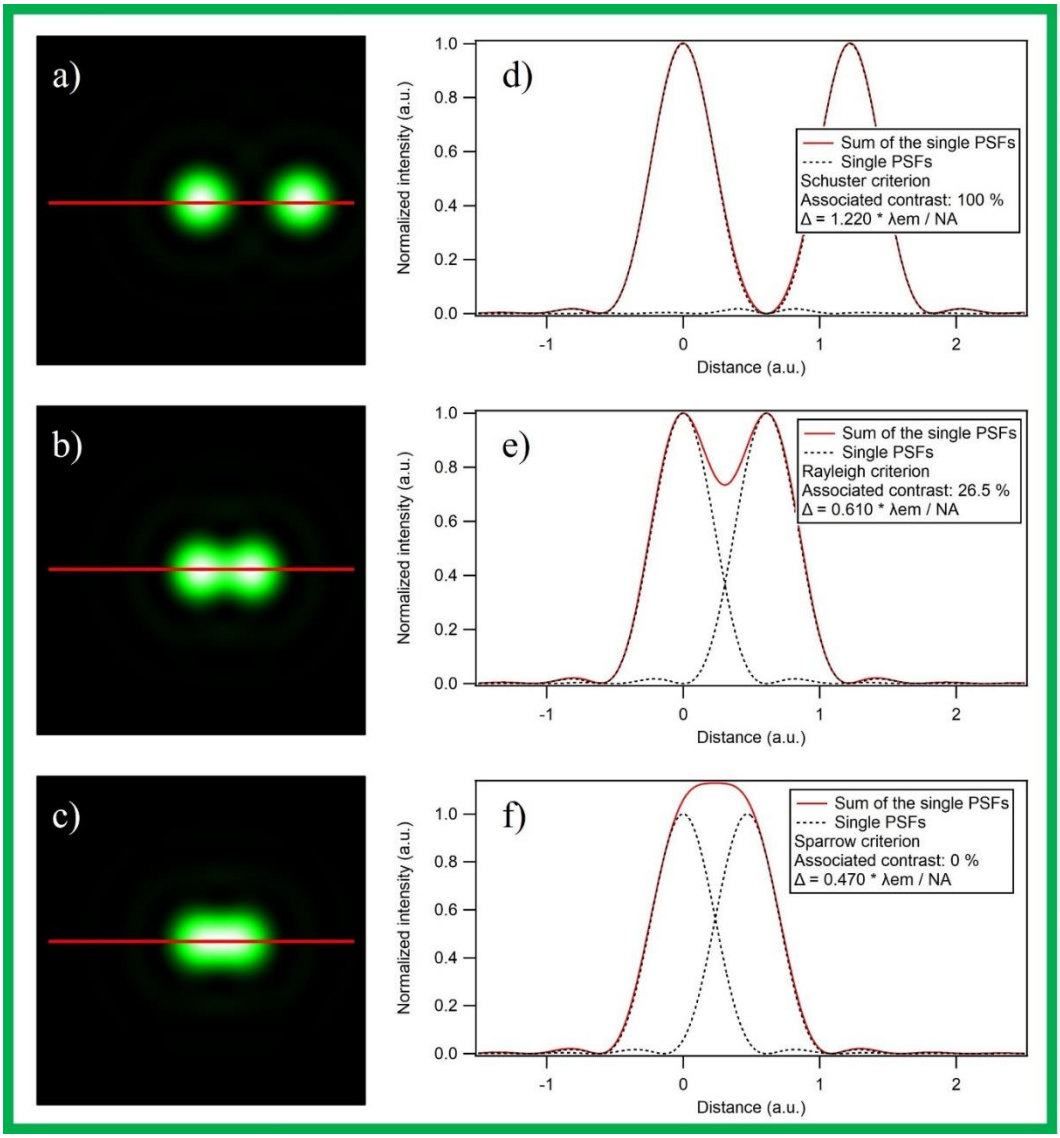

Figure 1: Simulated images of two point-like objects separated by a distance corresponding to the (a) Schuster, (b) Rayleigh, and (c) Sparrow criteria, and their associated intensity line profiles (d, e, f).

3.2. Measurement

In theory, the measurement of the lateral resolution within the two-point approach can be performed with any sample containing pairs of fluorescent point-like objects close to one another, ideally with different spacings. The lateral resolution is therefore the ***measured distance*** for which the two close point-like objects are distinguishable, ***for a given contrast***.

In practice, the measurement of the lateral resolution within the two-point approach can be carried out using DNA nanorulers, from Gattaquant [Gattawebsite], for instance (Figure 2a). These nanorulers consist of pairs of point-like objects separated by a controlled distance that can eventually be adjusted. The rulers carry two fluorescent marks out of multiple high quantum yield dye molecules, available in four colors (red, yellow, green, and blue). An example of blue nanorulers with a 350 nm spacing, imaged with a confocal laser scanning microscope, is shown in Figure 2b. An intensity line profile drawn along one nanoruler allows users to validate that the two point-like objects separated by 350 nm can be well discriminated, with an associated contrast of about 50% (Figure 2c).

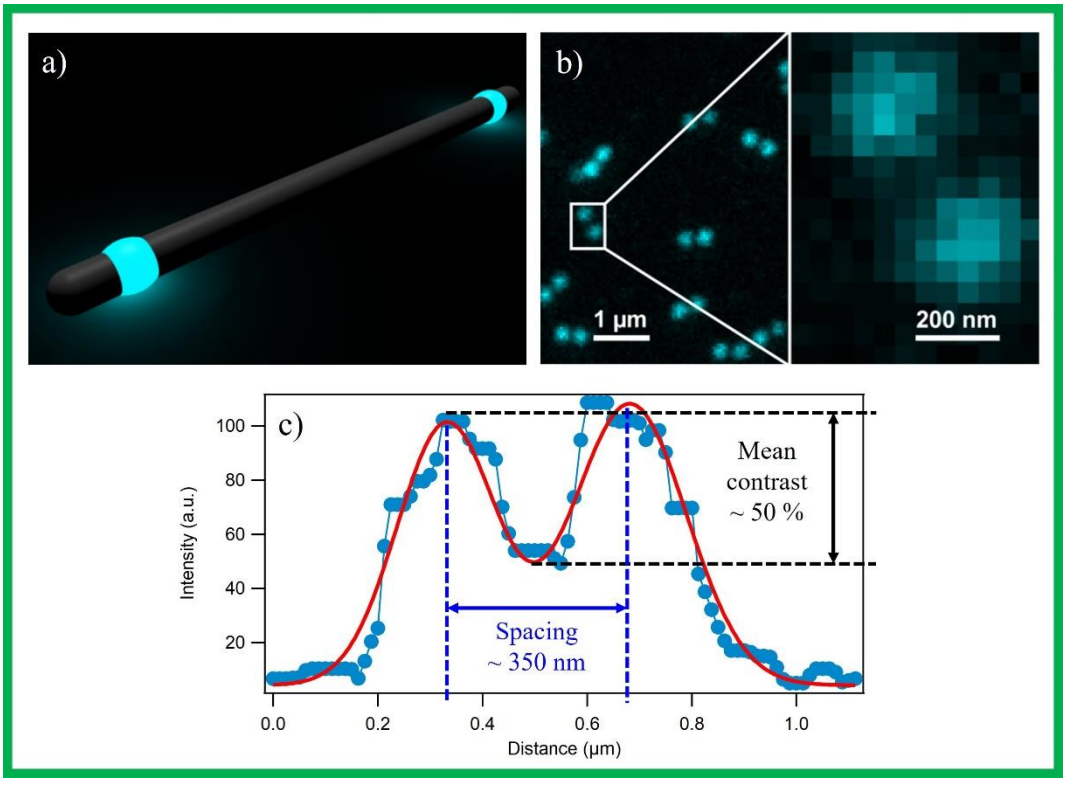

Figure 2: (a) Conceptual illustration of a DNA nanoruler from Gattaquant; (b) image of nanorulers with a 350 nm spacing acquired with a confocal laser-scanning microscope; and (c) intensity line profile drawn along one nanoruler, fitted with a sum of two Gaussian functions.

3.3. Advantages and limitations

DNA nanorulers are samples that are close to what the theory considers describing the two-point approach: they are composed of true point-like objects, emitting almost the same amount of fluorescence.

However, it is important to note that, for this kind of sample, the resolution (i.e., the measured spacing between the points) is valid only for a single contrast and does not necessarily allow users to measure the best achievable resolution. If one wants to have access to more spacing and contrast measurements, more samples with different spacings are required.

4. Single-point resolution

4.1. Concept

The concept of the single-point resolution approach relies on considering a single point-like object emitting fluorescence. A point-like object is a spherical object with a size that is about one-third of the expected theoretical resolution of the fluorescence imaging system. The image of such an object is the PSF of the microscope, which describes how the light spreads from the point-like object.

The ***Houston criterion***, proposed in 1927, uses the FWHM of the PSF to quantify resolution. This criterion is widely used because it is, in contrast to the Rayleigh criterion, also applicable to diffraction patterns that do not fall off to zero, as in the case of a Gaussian or Lorentzian profile.

The PSF is theoretically described by a first-order Bessel function (Figures 3a and 3b), whose FWHM is given by the following equation:

$$FWHM_{PSF} = 0.515 \times \frac{\lambda_{em}}{NA}$$

where λ_{em} is the emission wavelength and NA is the numerical aperture of the objective.

Although it does not describe any diffraction phenomenon, a Gaussian function is a very good approximation to model the PSF (Figure 3b).

If two point-like objects were separated by a distance equivalent to the FWHM of their PSF, the associated contrast would be 3.9% (Figures 3c and 3d).

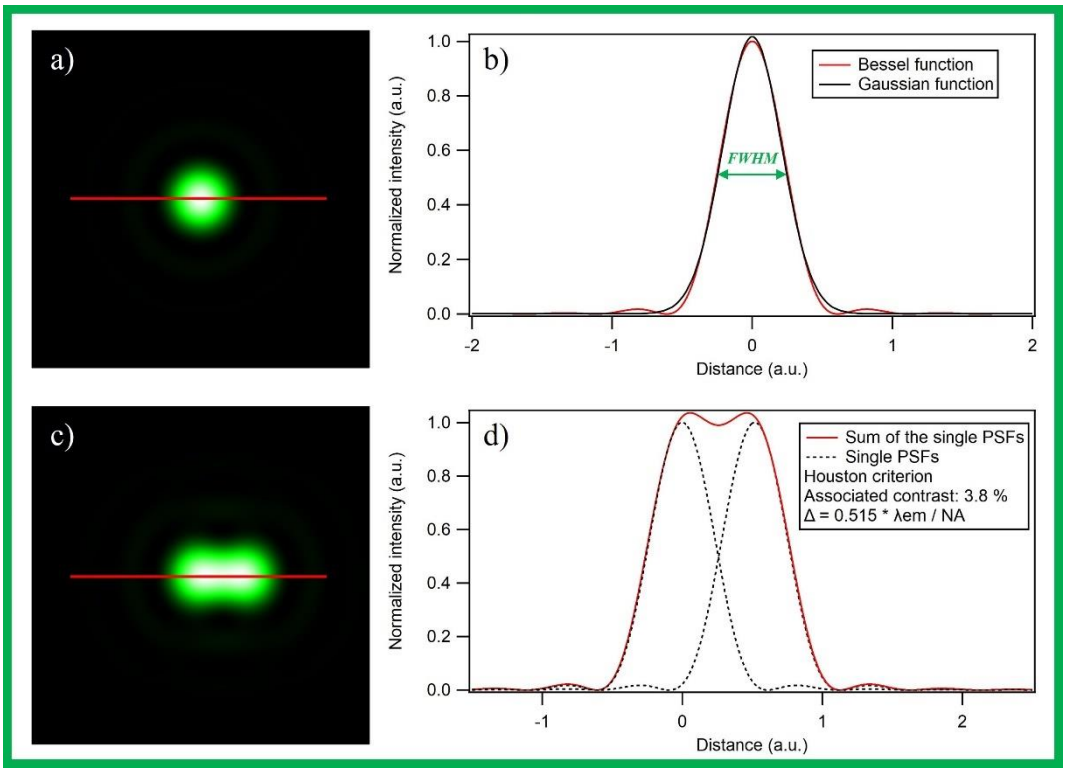

Figure 3: Simulated image of a single point-like object (a) and its associated intensity line profile (b). Simulated image of two point-like objects separated by a distance corresponding to the Houston criterion (c) and its associated intensity line profile (d).

4.2. Measurement

In theory, the measurement of the lateral resolution within the single-point approach can be performed with any sample containing isolated fluorescent point-like objects. The lateral resolution is therefore the ***FWHM*** of the fitted intensity line profile in the XY plane, usually with a Gaussian function.

In practice, the measurement of the lateral resolution within the single-point approach can be carried out using fluorescent microbeads, from Thermo-Fischer Scientific [Thermowebsite], for instance (Figure 4a). These microbeads are available in different sizes (0.1, 0.2, 0.5, 1.0, and 4.0 µm). It is often necessary to compromise on their sizes. The beads must have both a sufficiently small size to be considered as point-like objects and a sufficient emission of fluorescence, to allow the acquisition of an image with a decent signal-to-noise ratio (SNR) and no photobleaching. An example of beads with a 100 nm size, imaged with a confocal laser scanning microscope, is shown in Figure 4b. An intensity line profile drawn along the X and Y axes, fitted with a Gaussian function, shows an FWHM of about 250 nm (Figures 4c and 4d) [Col2011].

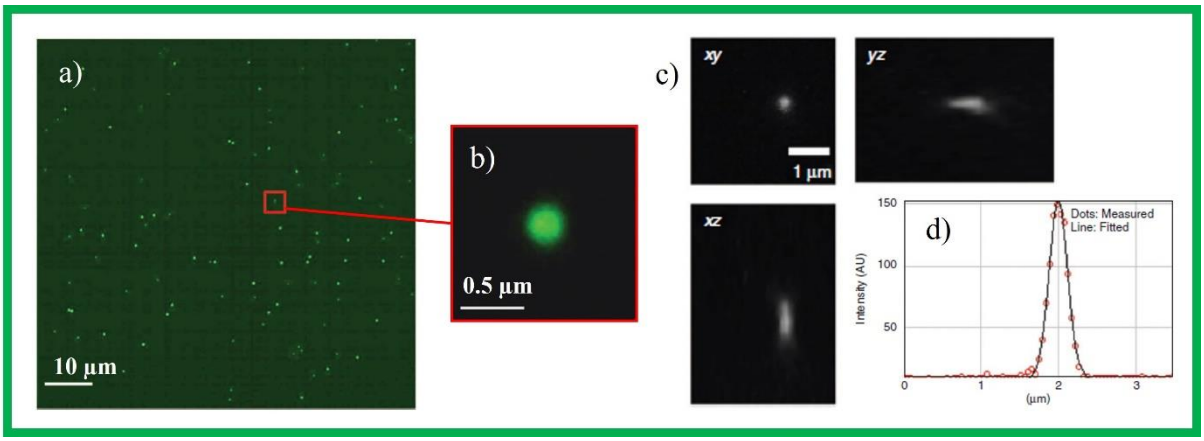

Figure 4: (a) Fluorescence confocal image of a field of fluorescent microbeads; (b) zoom showing an isolated microbead; (c) XY, YZ, and XZ orthogonal views of the selected microbead; and (d) intensity line profile along the X-direction, fitted with a Gaussian function.

While most microscopists perform a single measurement on an isolated bead in the center of the field of view, it is also possible to obtain a statistical measurement based on many beads spread out in the field of view.

Although this measurement protocol looks simple, there are many practical aspects to look into carefully to obtain a reliable PSF FWHM measurement:

- The centroid position of the bead should accurately be determined because the X and Y intensity line profiles are drawn through it.
- The choice of the fitting function (symmetric Gaussian vs. asymmetric Gaussian for instance) should be wisely chosen.
- The number of dimensions that are fitted at the same time influences the results. Fitting the X and the Y profiles separately with a 1D function will not provide the same results as fitting the bead in the image with a 2D function.
- The fitting method should be adequately chosen: a least squares or a maximum likelihood estimation approach will provide different results in the FWHM measurements.
- Last but not least, the preparation of the sample and the acquisition of the images are very important. The size of the beads, the mounting medium, the preparation, and the imaging protocols greatly influence the results. Beads aggregation and mounting medium background are well-known and common issues.

4.3. Advantages and limitations

Fluorescent microbeads are samples that are exactly what the theory considers describing the single-point approach: they are true point-like objects, as long as their size is small enough compared to the expected practical resolution.

This approach does not associate the concept of contrast with the PSF FWHM measurement, although contrast is just as essential as a “good PSF” in the production of “well-resolved” images.

However, the main limitation of this approach relies on the ability to obtain reliable and reproducible results. The number of involved user steps, from sample preparation to image acquisition to analysis, is prone to errors, despite the availability of shared and spread protocols [Col2011]. A study performed on a large scale at an international level concluded that the largest source of errors across all tests was user error, which could be subdivided between failure to follow provided protocols and improper use of the microscope [Col2013].

5. Two-line resolution

5.1. Concept

The concept of the two-line resolution approach relies on considering two thin lines mutually emitting the same amount of fluorescence, separated by a small distance. A thin line is a one-dimensional object with a size that is about one-third of the expected theoretical resolution of the fluorescence imaging system. A small distance is a distance corresponding roughly to the expected practical resolution.

Similar to the two-point approach, the Schuster, Rayleigh, and Sparrow criteria can be used within the double-line approach. However, slight differences in the associated contrast values and the theoretical separation distances are present, due to the different mathematical functions used to describe the line spread function (LSF) (Struve function) and the PSF (Bessel function).

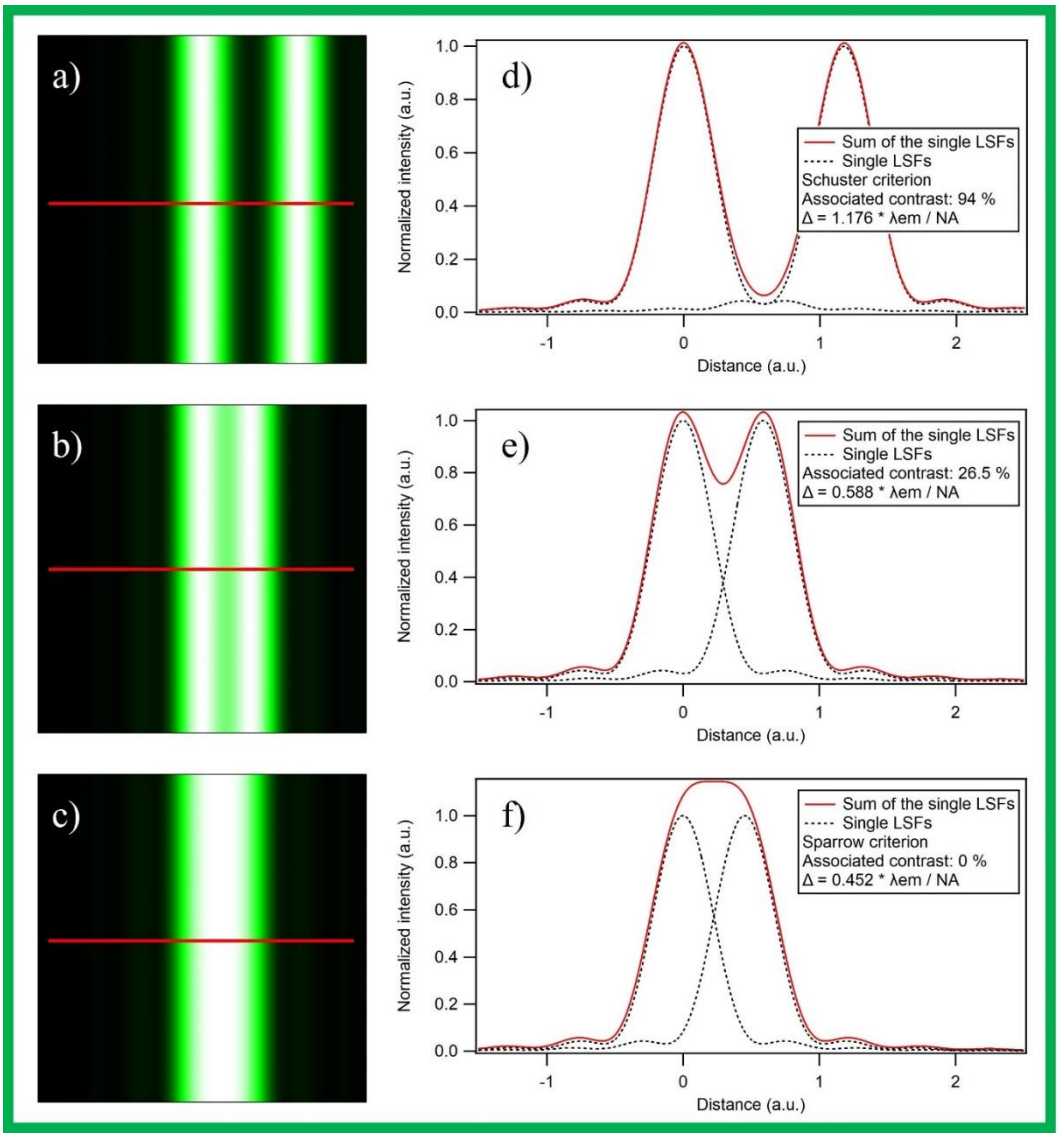

Figure 5: Simulated images of two thin lines separated by a distance corresponding to the (a) Schuster, (b) Rayleigh, and (c) Sparrow criteria, and their associated intensity line profiles (d, e, f).

5.2. Measurement

In theory, the measurement of the lateral resolution within the two-line approach can be performed with any sample containing pairs of fluorescent thin line objects close to one another, ideally with different spacings. The lateral resolution is therefore the ***measured distance*** for which the two close point-like objects are distinguishable, ***for a given contrast***.

In practice, the measurement of the lateral resolution within the two-line approach can be carried out with vertical and horizontal pairs of lines, featuring a cross-like pattern, that is available in quality control and quality assurance (QC/QA) slides, from Argolight, for instance [Argowebsite]. Two pairs of intensity line profiles drawn along the vertical and horizontal directions allow users to validate that the vertical and horizontal pairs of lines can be well discriminated, with a spacing of about 670 nm and an associated contrast of about 52% (Figures 6b and 6c).

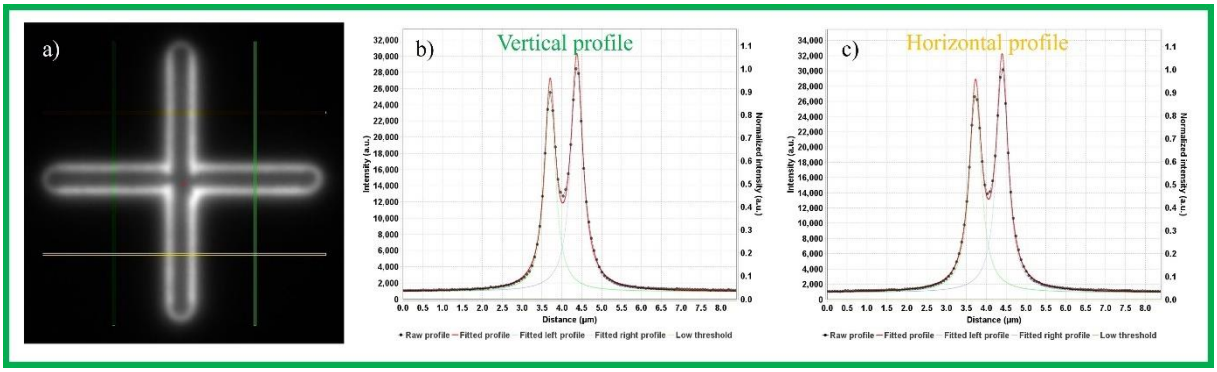

Figure 6: (a) Fluorescence wide-field image of a "cross" pattern from an Argolight slide, composed of pairs of lines separated by a fixed distance; (b) averaged vertical intensity line profile fitted with a double Lorentzian function; and (c) averaged horizontal intensity line profile fitted with a double Lorentzian function. The results are obtained from Daybook Analysis, version 1.10.0.

5.3. Advantages and limitations

Vertical and horizontal pairs of lines, featuring the "cross" pattern, are close to what the theory considers describing the two-line approach: they are composed of thin lines, emitting almost the same amount of fluorescence.

However, because the "cross" pattern has a finite axial extension, out-of-focus light adds background, which decreases the contrast and therefore increases the minimum resolvable spacing.

Besides, it is important to note that, for this kind of sample, the resolution (i.e., the measured spacing between the lines) is valid only for a single contrast, and does not necessarily allow users to measure the best achievable resolution. If one wants to have access to more spacing and contrast measurements, more samples with different spacings are required.

6. Single-line resolution

6.1. Concept

The concept of the single-line resolution approach relies on considering a single thin line emitting fluorescence. A thin line is a one-dimensional object with a size that is about one-third of the expected theoretical resolution of the fluorescence imaging system. The image of such a line is the LSF of the microscope, which describes how the light spreads from the thin line.

The LSF is theoretically described by a first-order Struve function, whose FWHM is given by the following equation:

$$FWHM_{LSF} = 0.501 \times \frac{\lambda_{em}}{NA}$$

where λ_{em} is the emission wavelength and NA is the numerical aperture of the objective.

Although it does not describe any diffraction phenomenon, a Gaussian function is a very good approximation to model the LSF (Figure 7b).

If two thin line objects were separated by a distance equivalent to the FWHM of their LSF, the associated contrast would be 5.5% (Figures 7c and 7d).

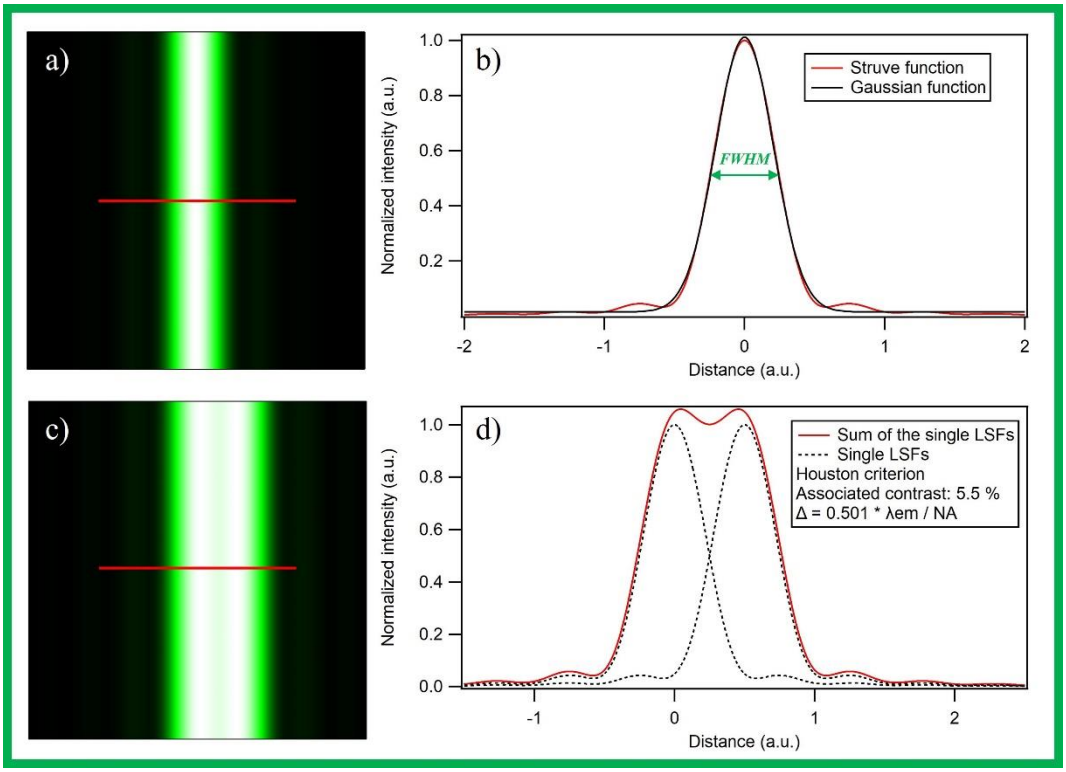

Figure 7: Simulated image of a single thin line object (a) and its associated intensity line profile (b). Simulated image of two thin line objects separated by a distance corresponding to the Houston criterion (c) and its associated intensity line profile (d).

6.2. Measurement

In theory, the measurement of the lateral resolution within the single-line approach can be performed with any sample containing isolated fluorescent thin lines. The lateral resolution is therefore the ***FWHM*** of the fitted intensity line profile perpendicular to the line in the XY plane, usually with a Gaussian function.

In practice, to the best of the author's knowledge, there is no artificial sample (commercially available) that features single lines. Biological samples, such as microtubules, could be used to measure the LSF, but special care should be taken to select a single and isolated filament, rather than aggregated ones. This makes the measurement with conventional fluorescence microscopes (i.e., not super-resolution microscopes) prone to mistakes. An example of microtubules, imaged with a wide-field field microscope and a Structured Illumination Microscope (SIM), is shown in Figures 8a and 8b, respectively. Intensity profiles drawn along lines perpendicular to the microtubules show an FWHM ranging from 223 to 245 nm (Figures 8c and 8d) [Wan2021]. Variability in the microtubules' thickness induces variability in the FWHM measurement.

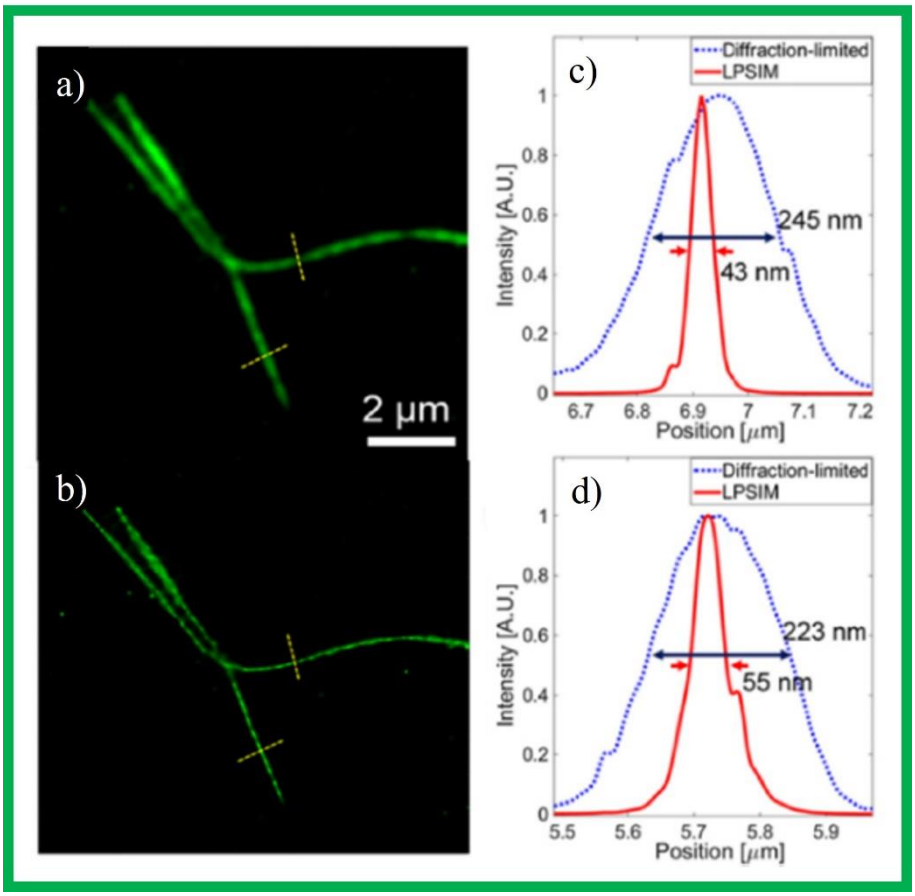

Figure 8: Fluorescence images of green microtubes imaged with a wide-field microscope (a) (i.e., diffraction-limited) and a type of SIM microscope (b) (i.e., a super-resolution microscope). Normalized intensity profiles are along the dashed yellow lines, perpendicular to the microtubules (c, d). For the wide-field microscope, the measured FWHM ranges from 223 nm to 245 nm (blue curves).

6.3. Advantages and limitations

Some biological samples, such as microtubules, are best described by lines rather than by points. As such, the single-line approach is more suited than the single-point approach to measure resolution.

The main drawback of this approach is the lack of an artificial sample (commercially available) featuring single lines that could be used to measure the resolution within the single-line approach. Instead, one could use microtubules, but should be aware of the pitfalls inherent to biological samples (ageing, photobleaching, size variability, etc.).

7. Contrast transfer function

7.1. Concept

Behind the concept of the CTF there is the fact that ***contrast is just as essential to the production of an image as what is usually called "resolution."*** Indeed, the two concepts can only be thought of in terms of each other. They are linked by the CTF (or power spectrum), which is the most fundamental and useful measure for characterizing the information transmission capability of any optical imaging system. Quite simply, it is a graph that plots the contrast produced by features in the image as a function of their size, or rather of the inverse of their size: their spatial frequency [Paw2006, chapter 4].

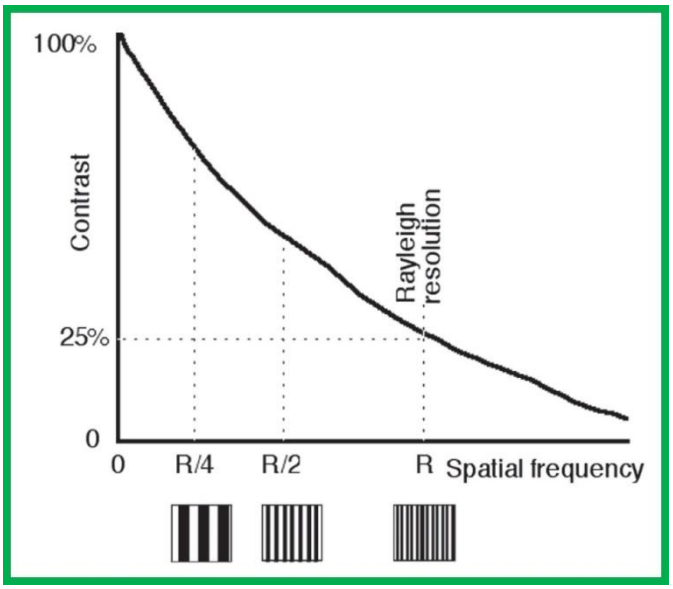

Figure 9: Conceptual graph representing a typical CTF of a fluorescence imaging system.

The inverse of the cut-off frequency (i.e., the highest spatial frequency that can be captured by the imaging system) is known as the Abbe limit and is given by the following equation:

$$Cutoff_{Abbe} = 0.500 \times \frac{\lambda_{em}}{NA}$$

where λ_{em} is the emission wavelength and NA is the numerical aperture of the objective.

Note: Contrast transfer function (CTF), modulation transfer function (MTF), and optical transfer function (OTF) are closely related, but not identical, as explained below.

The CTF and the MTF are only different from the way that contrast and modulation are calculated. The contrast is defined as the difference between the average peak intensity from two objects and the valley intensity between the two objects, normalized to the average peak intensity, while the modulation is defined as the difference between the average peak intensity from two objects and the valley intensity between the two objects, divided by their sum. They are expressed in % and are given by the following equation:

$$Contrast = 100 \times \frac{\overline{I_{max}} - I_{min}}{\overline{I_{max}}}$$

$$Modulation = 100 \times \frac{\overline{I_{max}} - I_{min}}{\overline{I_{max}} + I_{min}}$$

The MTF is the modulus of the OTF, a complex quantity that can be described with a modulus and a phase.

The OTF in the spatial frequency domain is the Fourier transform of the PSF in the real space domain.

In practice, the CTF is the most convenient to measure.

7.2. Measurement

To measure the CTF, a periodic structure, with a linear or circular symmetry with different spacings, is required. There are several tools corresponding to this description:

- A so-called "Siemens star," featuring an umbrella seen from the top.

- Gratings with different periods and orientations, similar as to those present in the so-called "USAF targets."

Siemens star

To extract the CTF from a Siemens star, one measures the contrast along several circle arc lengths, corresponding to several spacings [Hor2016]. In the example shown in Figure 10, a cut-off spatial frequency of 5 μm^{-1} is measured, corresponding to a resolution limit of 0.2 µm [Old1996, Chapter 8].

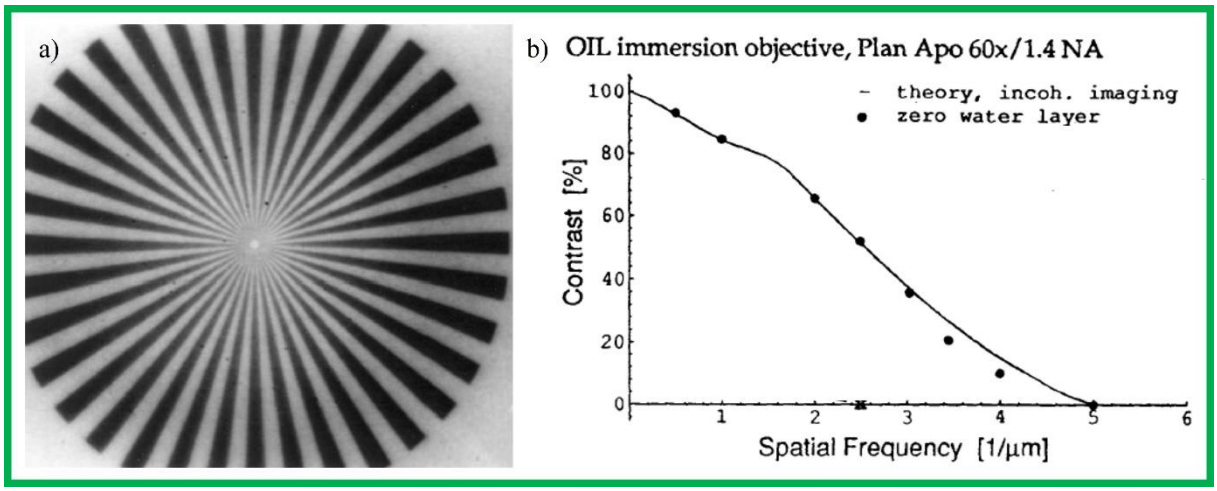

Figure 10: Example of a Siemens star imaged with a Plan-Apo 60×/1.4 oil immersion objective (a), from which the CTF is measured (b). The cut-off spatial frequency is about 5 μm^{-1}, corresponding to a resolution limit of 0.2 µm.

Gratings

To extract the CTF from different gratings of different periods and orientations, one measures the contrast of each grating having a given spacing. To measure the resolution limit along different directions, the previous step has to be repeated for each available grating orientation. In the example shown in Figure 11, a 500 nm spacing can be resolved with an associated contrast of about 45%, while a 100 nm cannot [Ike2007].

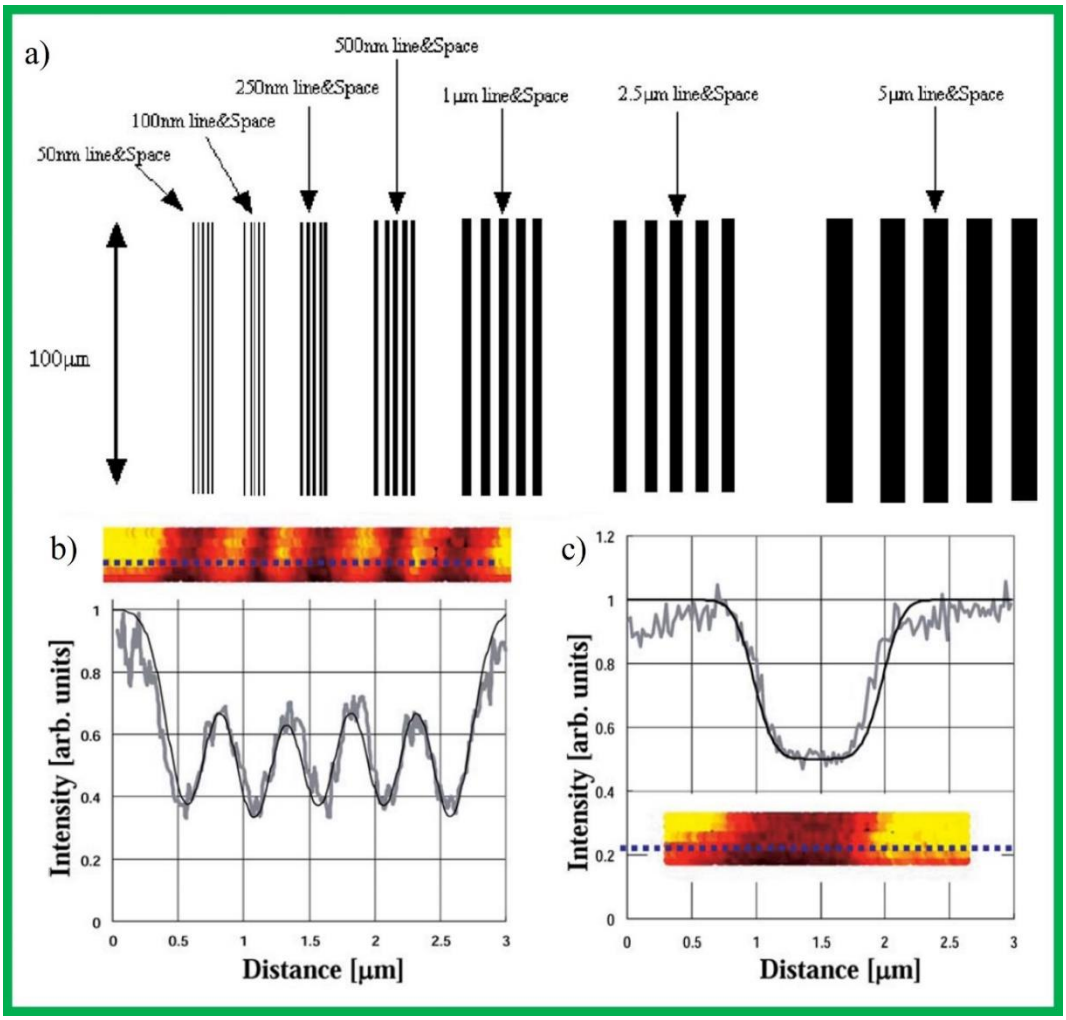

Figure 11: Vertical gratings of different periods (a). Image acquired with a 0.9 NA objective and intensity line profile perpendicular to the gratings of period 250 nm (b) and 100 nm (c). A 500 nm spacing can be resolved with an associated contrast of 45%, while a 100 nm cannot.

7.3. Advantages and limitations

To the best of the author's knowledge, there are no commercially available fluorescent gratings, nor Siemens stars, with suited spatial features (i.e., compatible with sub-micrometer resolution measurements), allowing to measure the lateral resolution within the CTF approach. The tools used in the two publications cited above were "homemade" and fabricated with extremely expensive techniques, such as electron beam lithography, mostly available in governmental facilities [Ike2007] [Old1996, Chapter 8].

8. How do these approaches relate?

8.1. Relationship between the different approaches

As described earlier, the PSF (within the single point approach) and the CTF approach are related via the Fourier transform (yellow arrow in Figure 12).

The single-point approach and the two-point approach can be related if one considers two point-like objects separated by a distance equivalent to the FWHM of the PSF (i.e., the Houston criterion) (red arrow in Figure 12).

The two-point approach and the CTF approach can be related if one considers sets of two point-like objects that are separated by different spacings, from which contrast and spacing can be measured, so that a CTF can be built (blue arrow in Figure 12).

However, these relationships are only valid for an ideal (i.e., perfect) fluorescence imaging system, which would be a microscope without any optical aberrations capturing noise-free images with an infinite sampling rate and dynamic range.

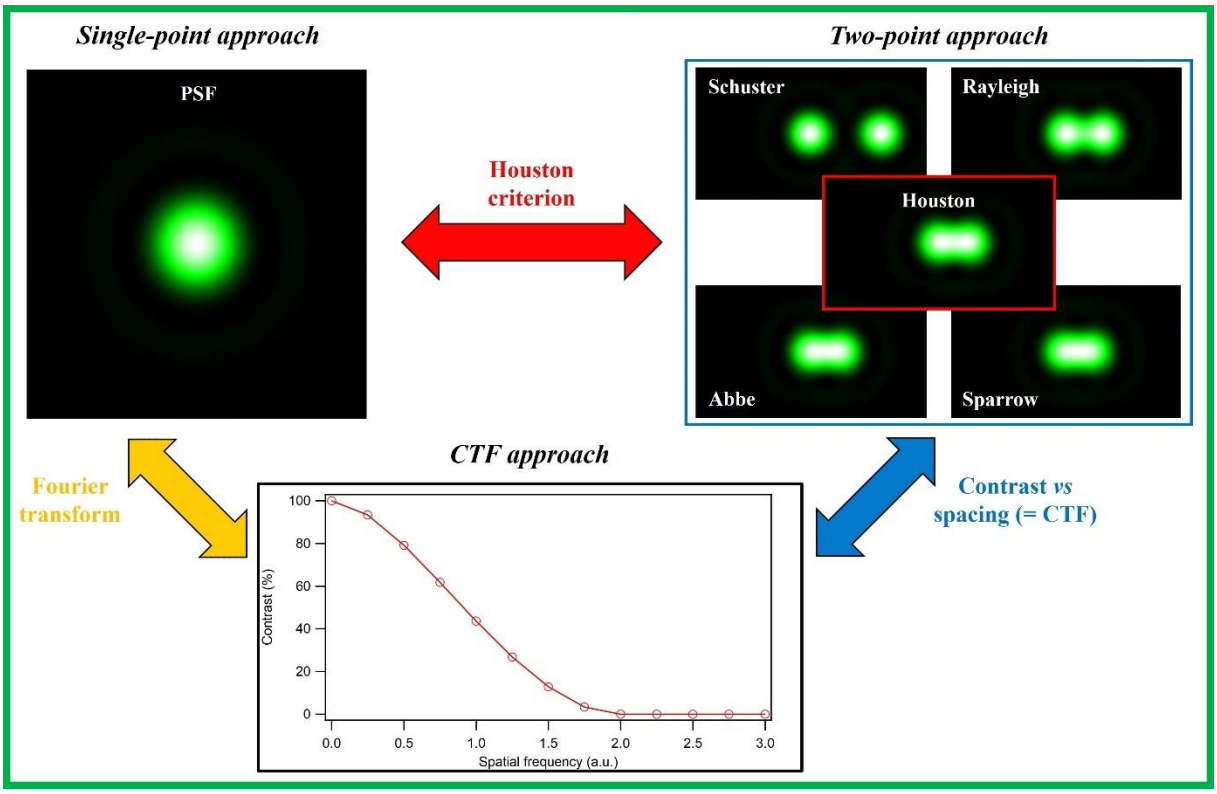

Figure 12: Relationship between the single-point, the two-point, and the CTF approaches.

8.2. Relationship between the different resolution criteria

Tables 1 to 4 recapitulate the different aforementioned contrast criteria within the different approaches, as well as their associated "theoretical resolutions" compared to the theoretical resolution of the Houston criterion (FWHM of the PSF/LSF), for both fluorescence wide-field and confocal microscopy.

Fluorescence wide-field microscopy

Criterion	Approach	Associated contrast	Theoretical "resolution"	Variation with regards to the Houston criterion
Schuster	Two-point	100%	$1.220 \times \frac{\lambda_{em}}{NA}$	+137%
Rayleigh	Two-point	26.5%	$0.610 \times \frac{\lambda_{em}}{NA}$	+18%
Houston (FWHM of the PSF)	Single-point	3.9%	$0.515 \times \frac{\lambda_{em}}{NA}$	0%
Abbe	Contrast transfer function	1.7%	$0.500 \times \frac{\lambda_{em}}{NA}$	–3%
Sparrow	Two-point	~0%	$0.474 \times \frac{\lambda_{em}}{NA}$	–8%

Table 1: The criteria within different approaches for a perfect fluorescence wide-field microscope, ranked by decreasing contrast magnitude. The contrast is assumed to arise from two fluorescent point-like objects. The theoretical "resolution" is compared with respect to the one defined within the Houston criterion.

Criterion	Approach	Associated contrast	Theoretical "resolution"	Variation with regards to the Houston criterion
Schuster	Two-line	94%	$1.176 \times \frac{\lambda_{em}}{NA}$	+135%
Rayleigh	Two-line	26.5%	$0.588 \times \frac{\lambda_{em}}{NA}$	+17%
Houston (FWHM of the LSF)	Single-line	5.5%	$0.501 \times \frac{\lambda_{em}}{NA}$	0%
Abbe	Contrast transfer function	5.3%	$0.500 \times \frac{\lambda_{em}}{NA}$	0%
Sparrow	Two-line	~0%	$0.452 \times \frac{\lambda_{em}}{NA}$	–10%

Table 2: The criteria within different approaches for a perfect fluorescence wide-field microscope, ranked by decreasing contrast magnitude. The contrast is assumed to arise from two fluorescent lines. The theoretical "resolution" is compared with respect to the one defined within the Houston criterion.

Fluorescence confocal microscopy

The theoretical PSF of a fluorescence confocal microscope is the square of the theoretical PSF of a fluorescence wide-field microscope. This has an impact on the contrast and theoretical resolution values.

Criterion	Approach	Associated contrast	Theoretical "resolution"	Variation with regards to the Houston criterion
Schuster	Two-point	98.2%	$1.226 \times \frac{\lambda_{exc}}{NA}$	+138%
Rayleigh	Two-point	26.5%	$0.613 \times \frac{\lambda_{exc}}{NA}$	+19%
Houston (FWHM of the PSF)	Single-point	5.7%	$0.515 \times \frac{\lambda_{exc}}{NA}$	0%
Abbe	Contrast transfer function	3.4%	$0.500 \times \frac{\lambda_{exc}}{NA}$	–3%
Sparrow	Two-point	~0%	$0.456 \times \frac{\lambda_{exc}}{NA}$	–11%

Table 3: The criteria within different approaches for a perfect fluorescence confocal microscope, ranked by decreasing contrast magnitude. The contrast is assumed to arise from two fluorescent point-like objects. The theoretical "resolution" is compared with respect to the one defined within the Houston criterion.

Criterion	Approach	Associated contrast	Theoretical "resolution"	Variation with regards to the Houston criterion
Schuster	Two-line	97%	$1.190 \times \frac{\lambda_{exc}}{NA}$	+138%
Rayleigh	Two-line	26.5%	$0.595 \times \frac{\lambda_{exc}}{NA}$	+19%
Houston (FWHM of the LSF)	Single-line	6.2%	$0.501 \times \frac{\lambda_{exc}}{NA}$	0%
Abbe	Contrast transfer function	6.1%	$0.500 \times \frac{\lambda_{exc}}{NA}$	0%
Sparrow	Two-line	~0%	$0.440 \times \frac{\lambda_{exc}}{NA}$	–12%

Table 4: The criteria within different approaches for a perfect fluorescence confocal microscope, ranked by decreasing contrast magnitude. The contrast is assumed to arise from two fluorescent point-like objects. The theoretical "resolution" is compared with respect to the one defined within the Houston criterion.

Note: It is important to remember that all these criteria and theoretical formulae assume an ideal (i.e., perfect) fluorescence microscope, which is a microscope without any optical aberrations capturing noise-free images with infinite sampling rate and dynamic range. In practice, the theoretical resolution is never achieved.

8.3. Discussion

The pertinence of using the different resolution criteria in practice is discussed below.

Single-point/single-line approach

The ***Houston criterion*** is currently the most used criterion to measure resolution because the concept of measuring the PSF FWHM is simple to understand (although many pitfalls make this measurement not that straightforward). However, this criterion does not correspond necessarily to the need of microscope users, which is, in most cases, the ability to discriminate objects that are close to one another. By definition, this criterion does not take into account the concept of contrast, and therefore the influence of noise and background on it. A contrast of 3–6%, corresponding to the spacing between two objects separated by the PSF FWHM, is very weak in practice; it is usually not sufficient in the presence of noise and background to resolve objects close to one another. It is therefore a criterion that provides an advantageous value of resolution, while in practice, for most of the biological objects, the achievable resolution is much less.

Contrast transfer function approach

The ***Abbe criterion*** takes into account the concept of contrast, and therefore the influence of noise and background on it. The cut-off distance of the CTF, determined from the intercept of the CTF with the 0% contrast horizontal line, is therefore subject to measurement variability, which makes this criterion not practical to use.

Two-point/two-line approach

Even for a well-resolvable spacing, unless post-acquisition image processing such as deconvolution is applied, a contrast of 100% is never obtained in practice, mainly because of background and noise. For this reason, the ***Schuster criterion*** is not practical to use.

Similarly, a contrast of almost 0% is hardly useable in practice because the resolvable spacing around 0% of contrast is highly influenced by the acquisition parameters (mainly noise, sampling rate, and dynamic range). Therefore, the measurement variability of a barely resolvable spacing around a contrast of 0% makes the ***Sparrow criterion*** not practical to use.

The ***Rayleigh criterion***, although somehow arbitrarily chosen, seems a good compromise to use for resolution measurements. As such, a 26.5% contrast value to determine an associated resolvable distance, whatever the used approach (two-point, two-line, or CTF), is practically achievable, which makes the measurement of resolvable spacings more reproducible.

9. Parameters influencing resolution

At the first order, the acquisition modality (e.g., wide-field or confocal), the wavelength (excitation and/or emission), and the objective numerical aperture are the parameters influencing the most resolution. These three parameters appear in the following well-known equation:

$$Lateral\ resolution\ = K \times \frac{\lambda_{em}\ or\ \lambda_{exc}}{NA}$$

where λ_{em} is the emission wavelength, λ_{exc} is the excitation wavelength, NA is the numerical aperture of the objective, and K is a factor that contains the specificity of the imaging modality and of the resolution measurement approach.

At the second order, the optical aberrations (mainly spherical aberration, which can be due to refractive index mismatch from the immersion medium or the sample, or a poorly adjusted correction collar) also influence resolution. The optical aberrations can mathematically be described, yet in a more complex way than the previous equation, using the wavefront aberration function of Zernike polynomials formalisms, for instance.

These two sets of parameters are present for any type of fluorescence microscope. However, other parameters such as polarization or pinhole size, specific to some acquisition modalities, also influence resolution. Besides, additional parameters due to digitized modern detectors, such as sampling or noise, affect the image quality, which in turn influences the achievable practical resolution. This section aims to describe these additional parameters.

9.1. Polarization

Polarization influences the resolution in microscopes equipped with lasers because they are polarized. This is usually the case in confocal microscopes.

In confocal microscopes, the PSF is the product of the illumination PSF and the detection PSF:

$$PSF_{confocal}(x;y;z) = PSF_{illumination}(x;y;z) \times PSF_{detection}(x;y;z)$$

While the illumination PSF is influenced by the beam's original polarization, the detection PSF is not, because the fluorescence itself is usually not polarized.

Some confocal laser scanning microscopes illuminate the sample with linearly polarized light, some with circularly polarized light. In the first case, the linear polarization gives rise to an elongation of the illumination PSF along the direction of the incident linear polarization, about 29% [Li2015]. This elongation of the illumination PSF along one direction leads to an elongation of the confocal PSF of about 17% in theory (Figure 13) [Jon2003]. In practice, it

can be more than 17%. In the second case, the circular polarization gives rise to an isotropic illumination PSF [Li2015], and therefore to an isotropic confocal PSF.

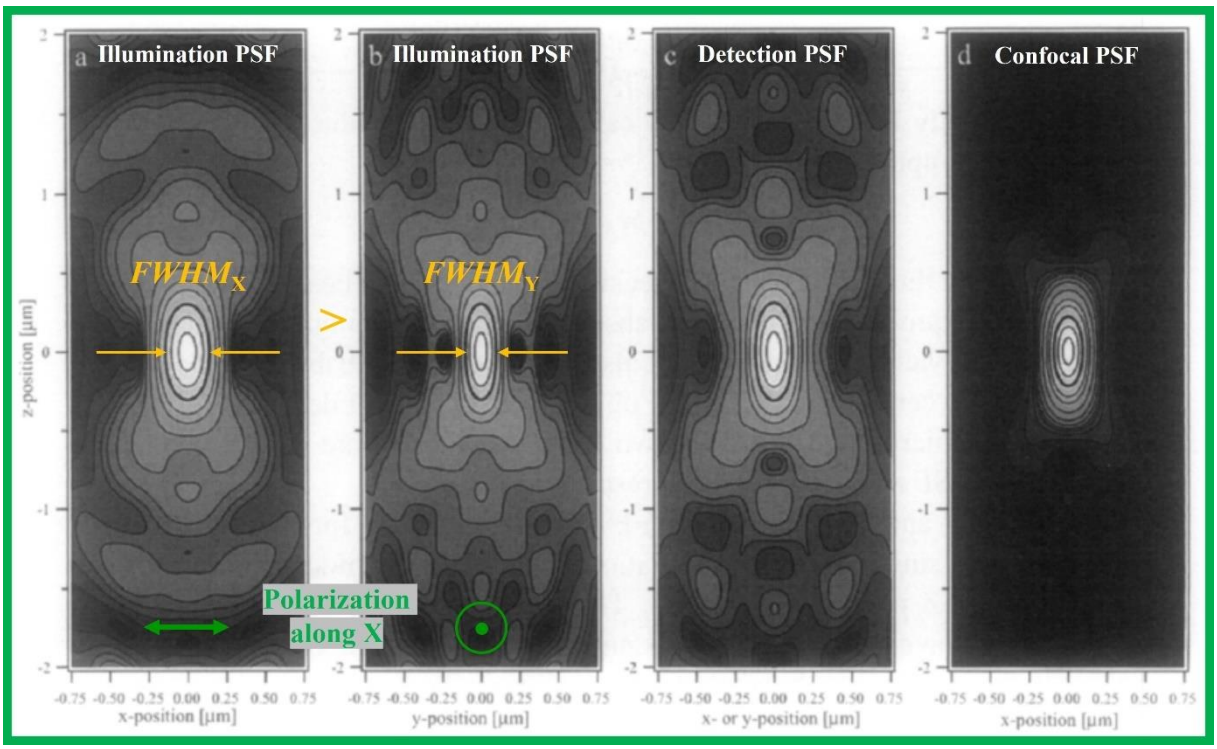

Figure 13: Simulated illumination PSFs along X (a) and Y (b), assuming a laser linearly polarized along X. The FWHM of the PSF along X (i.e., along the polarization direction) is broader than the one along Y. Simulated detection PSF (c), whether along X or Y, not influenced by the polarization. Simulated confocal PSF along X (d), resulting from the product of the illumination PSF (a) and the detection PSF (c).

Does this mean that microscopes in which the illumination is circularly polarized provide a better resolution than those in which the illumination is linearly polarized? Not necessarily, because there is no such thing as a free lunch. Indeed, dichroic mirrors in the microscopes are optimal for linear polarization, which results in better SNR in the images. Since the resolution is limited by the SNR, as will be emphasized later, the “bad thing” (the elongation of the PSF in one direction for linear polarization) can be compensated by a “good thing” (a better SNR in the images).

9.2. Pinhole diameter

The pinhole diameter influences the resolution in microscopes equipped with pinholes whose diameter can be adjusted. This is usually the case in confocal laser scanning microscopes.

In theory, the resolution increases (i.e., degrades) as the pinhole diameter increases, following a sigmoidal-like evolution (Figure 14a) [Huf2015].

Increasing the pinhole diameter above 1 AU (Airy Unit) has no influence on the resolution (Figure 14a), although more signal reaches the detector (Figure 14b), leading to an increase of SNR and signal-to-background ratio (SBR) in the images.

Decreasing the pinhole diameter below 1 AU improves the resolution (Figure 14a), but less signal reaches the detector (Figure 14b), leading to a decrease of SNR and SBR in the images.

An optimum in terms of resolution and signal (i.e., the resolution can be improved without a significant loss of signal) can be reached at about 0.63 AU.

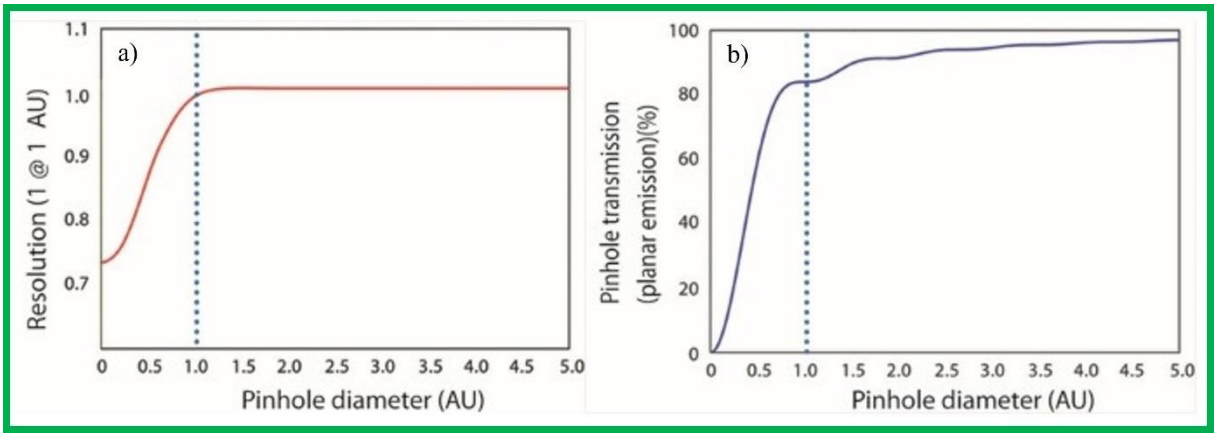

Figure 14: Evolution of the normalized resolution (a) and the normalized pinhole transmission (b) versus the pinhole diameter.

9.3. Noise

Noise can be defined as an undesired statistical fluctuation superimposing on the signal of interest. It can be described by the SNR, used in imaging to characterize image quality. Traditionally, the SNR is defined to be the ratio of the average signal value to the standard deviation of the signal.

Noise induces uncertainty in the intensity measurements and therefore uncertainty in the contrast measurement. In general, this leads to an underestimation of the contrast. Every reduction of the contrast causes an increase in the minimum resolvable distance, and hence a degradation of the resolution [Ste1998]. Thus, the noise influences the contrast, which in turn influences the resolvable distance [Fer2019].

Figures 15a and 15b show an ideal representation of two point-like objects separated by the Rayleigh distance, without any noise and with 20% of noise. Figures 15c and 15d show the same representation, but in a pixelated way. Because of the noise and the uncertainty that it induces on the intensity measurements, both representations, although different in appearance, are analogous from a metrological point of view.

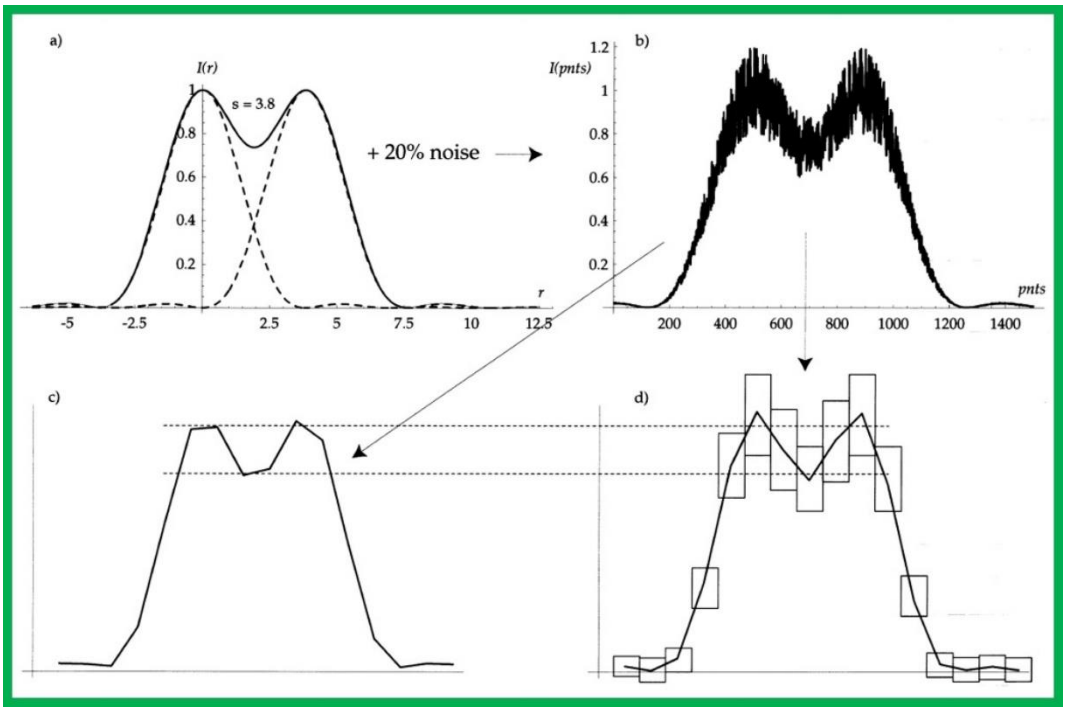

Figure 15: Influence of noise on contrast: (a) ideal image representation of two point-like objects at the Rayleigh distance; (b) the same conditions but with a 20% noise level included; and (c, d) twelve points are used to represent the sum. Both presentations are valid. The boxes indicate the uncertainties associated with each intensity value due to noise.

9.4. Background

Background can be defined as an undesired signal superimposing on the signal of interest. It can be described by the SBR, used in imaging to characterize image quality. There are different sources of background as follows:

- Constant background coming from ambient light, filter bleed-through, or auto-fluorescence of the sample's mounting medium. This type of background can usually be removed with a simple subtractive mathematical operation.
- Out-of-focus background coming from the out-of-focus light due to the sample axial length. This type of background requires complex mathematical operation, such as background correction, deblurring, or deconvolution, to be removed.

For most of the microscopes and imaging conditions, the uniform background can be removed by reducing the impact of the sources it originates from. For microscopes having optical sectioning capabilities, such as confocal fluorescence microscopes, the out-of-focus light is greatly reduced. The way that these two sources of background impact the measurement of the CTF is explained below.

Constant background

Figure 16 shows two simulated images of vertical "gradually spaced lines" of an Argo-HM slide without (Figure 16a) and with (Figure 16b) constant background. The CTFs obtained from these two images superimpose almost perfectly (Figure 16c), evidencing that constant background does not influence the contrast and, therefore, the resolvable distances.

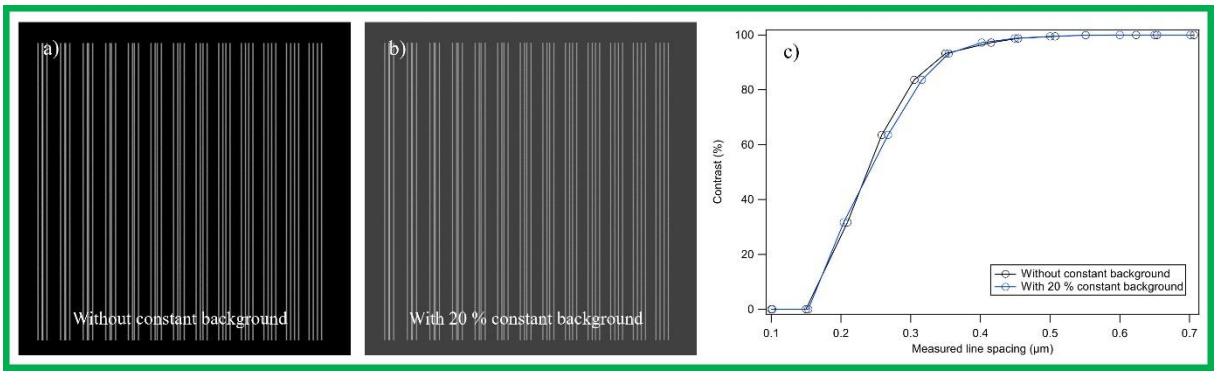

Figure 16: Simulated images of vertical "gradually spaced lines" of an Argo-HM slide (a) without and (b) with 20% of constant background. (c) CTFs obtained from these two images using the analysis "lateral resolution" in Daybook.

Out-of-focus background

Figure 17 shows four simulated images of vertical "gradually spaced lines" of an Argo-HM slide without (Figure 17a) and with (Figures 17b, 17c, and 17d) several levels of local background, the latter emulating out-of-focus light emitted from the lines in the planes above and below the focal plane. The CTFs obtained from these four images are different. As the level of local background increases, the contrast decreases, evidencing that local background such as out-of-focus light has an impact on the contrast, and therefore on the resolvable distances.

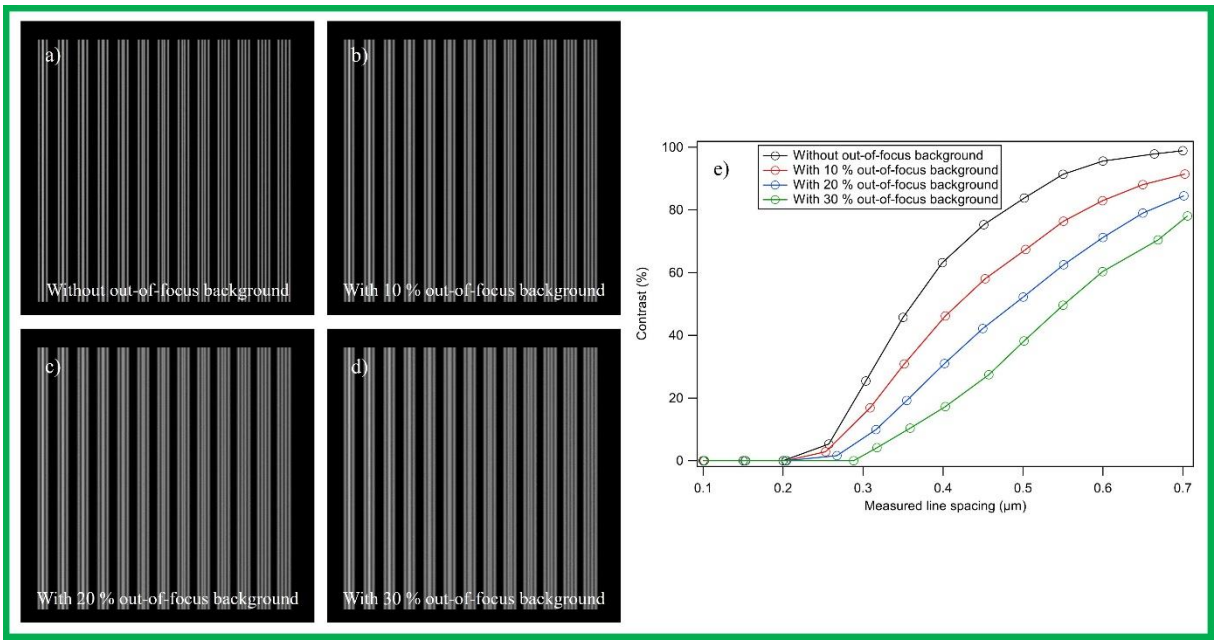

Figure 17: Simulated images of vertical "gradually spaced lines" of an Argo-HM slide (a) without out-of-focus background and with (b) 10%, (c) 20%, and (d) 30% of out-of-focus background. (e) CTFs obtained from these four images using the analysis "lateral resolution" in Daybook.

Recap

While the constant background does not affect the contrast, the out-of-focus background decreases the contrast. Therefore, the out-of-focus light decreases the amount of information that the imaging system can transmit. Distances are resolved with less contrast, affecting the resolution measurement of the microscope.

9.5. Pixelation

Fluorescence microscope images are pixelated, meaning that each pixel in the images corresponds to a certain pixel size at the sample plane. The aforementioned theoretical resolution formulae assume unlimited spatial sampling rate (i.e., infinitely small pixel size). If the spatial sampling rate is insufficient, the boundary of two spots close to one another could be fuzzy. The lateral pixel size is therefore an important parameter to keep in mind for resolution measurement.

Figure 18a shows simulated data from two spots, fitted by two Gaussian functions (with an FWHM of ~260 nm), separated by the Rayleigh distance (~300 nm), providing a contrast of 30%, for an extremely small pixel size (1 nm). As the pixel size is artificially increased, the contrast decreases (Figures 18b and 18c), down to a null value that prevents from discriminating the two spots (Figure 18e) [Li2020]. The contrast starts to decrease significantly for pixel sizes ranging from 50 to 100 nm. From these simulations, using a pixel size that is half the PSF FWHM (i.e., about 130 nm here) is not sufficient to preserve the contrast.

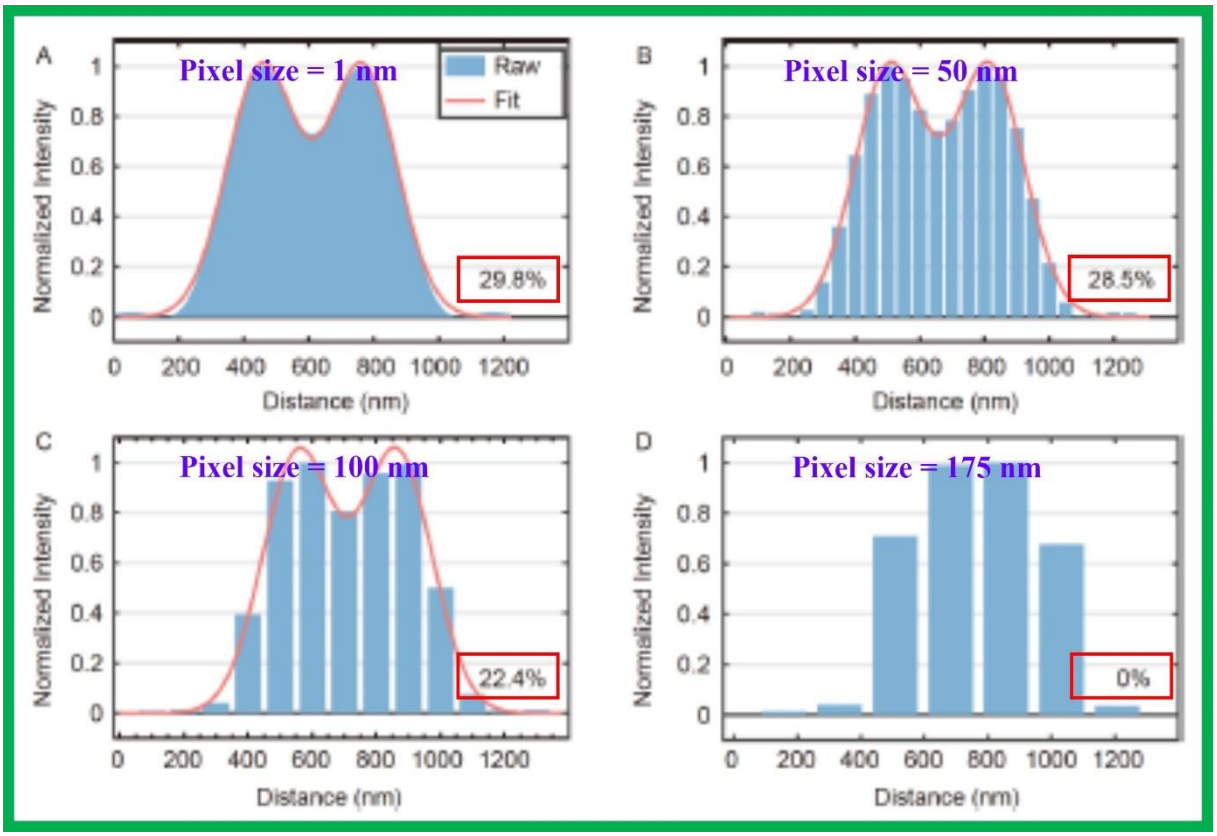

Figure 18: Simulated images of two spots separated by about 300 nm, providing a contrast of about 30%, for several pixels sizes: (a) 1 nm; (b) 50 nm; (c) 100 nm; and (d) 175 nm. The contrast starts to significantly decrease for pixel sizes ranging from 50 to 100 nm.

Instead, to preserve the contrast, a spatial sampling rate of eight points per Airy Unit was suggested [Ste1998]. However, such a high sampling rate is extremely difficult to achieve in practice: for camera-based imaging systems, it would require cameras with very small pixels, and/or relay lenses increasing the overall microscope magnification; for laser-scanning systems, it would require extremely long frame acquisition times. A sampling period of one-third of the theoretical resolution (as defined within the single-point approach) appears to be a good compromise.

9.6. Detector bit depth

The detector bit depth is the range on which the information is coded by the detector. It most commonly takes 8- or 16-bit values, but can also take 10-, 12-, or 14-bit values. For example, an 8-bit depth detector can digitize intensity values from 0 to 255, while a 16-bit depth detector can digitize intensity values from 0 to 65,535. Because of this significantly larger number of gray values accessible with the 16-bit depth detector, more accurate intensity measurements are possible than for an 8-bit depth detector.

A low detector bit depth induces a larger uncertainty in the intensity measurements than a higher detector bit depth, and hence a larger uncertainty in the contrast measurement. In general, this leads to an underestimation of the contrast. Every reduction of the contrast causes an increase in the minimum resolvable distance, and hence a degradation of the resolution. Thus, the detector bit depth influences the contrast, which in turn influences the resolvable distance [Fer2019].

9.7. Recap: resolution is a multiparameter feature

Here are the different elements previously discussed throughout this document:

- Contrast and resolvable distance are related terms.
- Pixelation reduces the contrast.
- Noise reduces the contrast.
- Out-of-focus background reduces the contrast.
- Detector bit depth influences the contrast.

To summarize, noise, background, pixilation, and detector bit depth influence the contrast and the accuracy of fitting, which in turn influence the minimum resolvable distance of the imaging system (Figure 19). All these parameters should therefore be considered when measuring resolution.

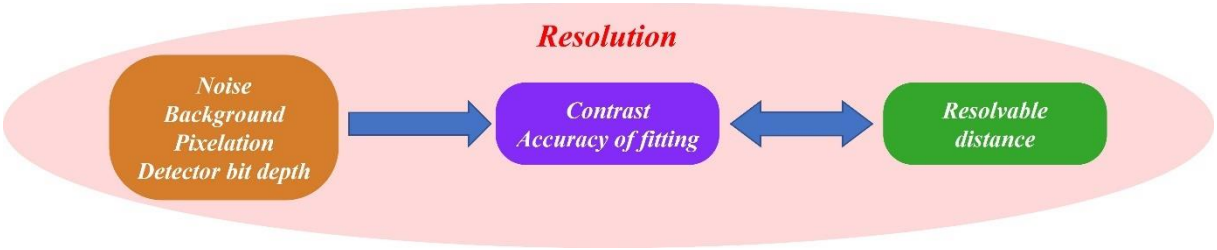

Figure 19: Schematics representing the closely related parameters influencing the resolution.

10. Argolight's approach to measure resolution

10.1. Concept

The concept of Argolight's approach relies on a fluorescent pattern featuring "gradually spaced lines," associated with a dedicated image analysis algorithm available in Daybook. The "gradually spaced lines" pattern consists of pairs of lines whose spacing gradually increases (Figure 20). The image analysis algorithm allows the measurement of the CTF from this pattern (i.e., a graph plotting the contrast versus the distance between the two inner lines) associated with the SNR and SBR of the image. Part of this algorithm was published by Korobchevskaya and colleagues [Kor2017].

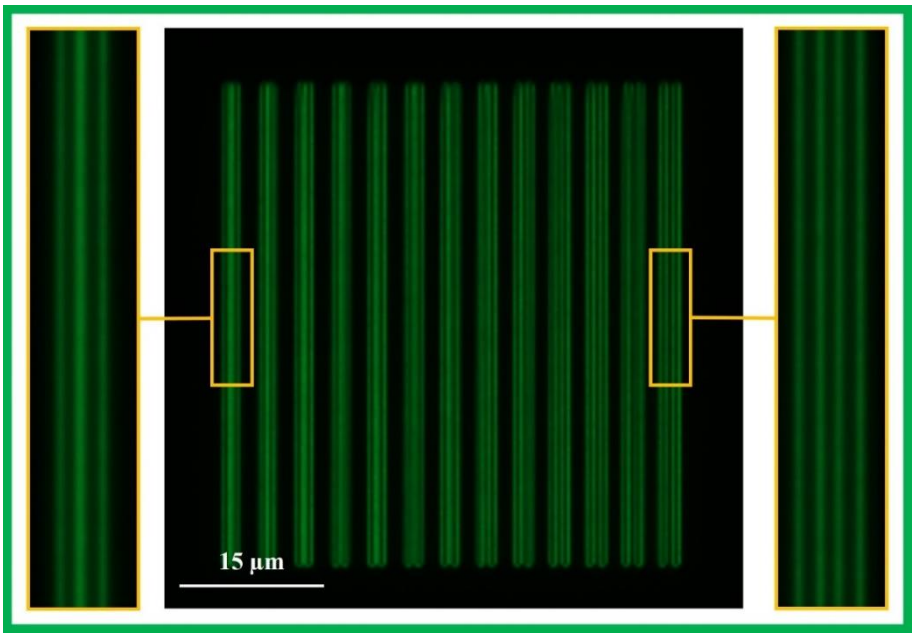

Figure 20: Wide-field fluorescence image of vertical "gradually spaced lines" of an Argo-HM v1.0 slide. The inserts on the left and on the right show the group of lines in which the two inner lines are the closest and the furthest apart, respectively.

10.2. Measurement

The detailed description of the image analysis algorithm can be found in the documentation of the "lateral resolution" analysis in Daybook. A lighter description is given below.

1) Contrast measurement

A mean intensity line profile is drawn perpendicular to the lines, within a region of interest (ROI) with a width of about one-fourth the length of the lines. The peaks and the valleys between the inner lines are detected and labeled in red and blue on the graph, respectively (Figure 21).

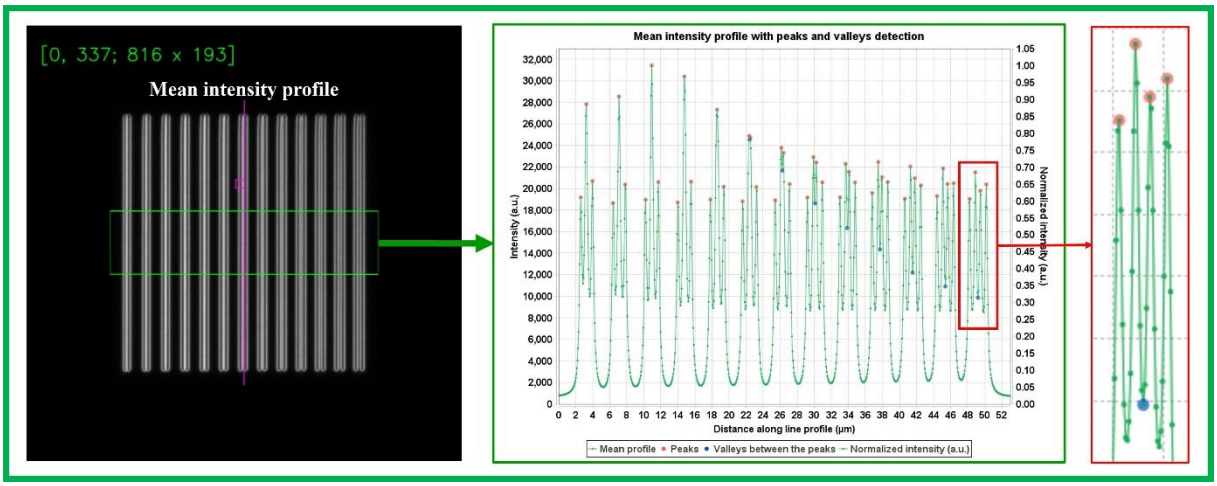

Figure 21: Mean intensity line profiles drawn perpendicular to the lines. Peaks and valleys are labeled in red and blue, respectively.

For each group of lines in which four lines are resolved, the mean contrast is calculated, based on the intensities of the peaks and valleys, according to the following equation:

$$Contrast = 100 \times \frac{\overline{I_{max}} - I_{min}}{\overline{I_{max}}}$$

where $\overline{I_{max}}$ is the average intensity of the two inner lines' magnitude and I_{min} is the local minimum intensity between the two inner lines.

2) Line spacing measurement

For each group of lines, the mean intensity profile is fitted with Gaussian or Lorentzian functions. The spacing between the two inner lines of each group is determined from the position of their respective fitting functions (Figure 22). Empirically, Lorentzian fitting functions are best suited for wide-field microscopes, while Gaussians fitting functions are best suited for confocal microscopes, SIMs, and deconvolution-based microscopes.

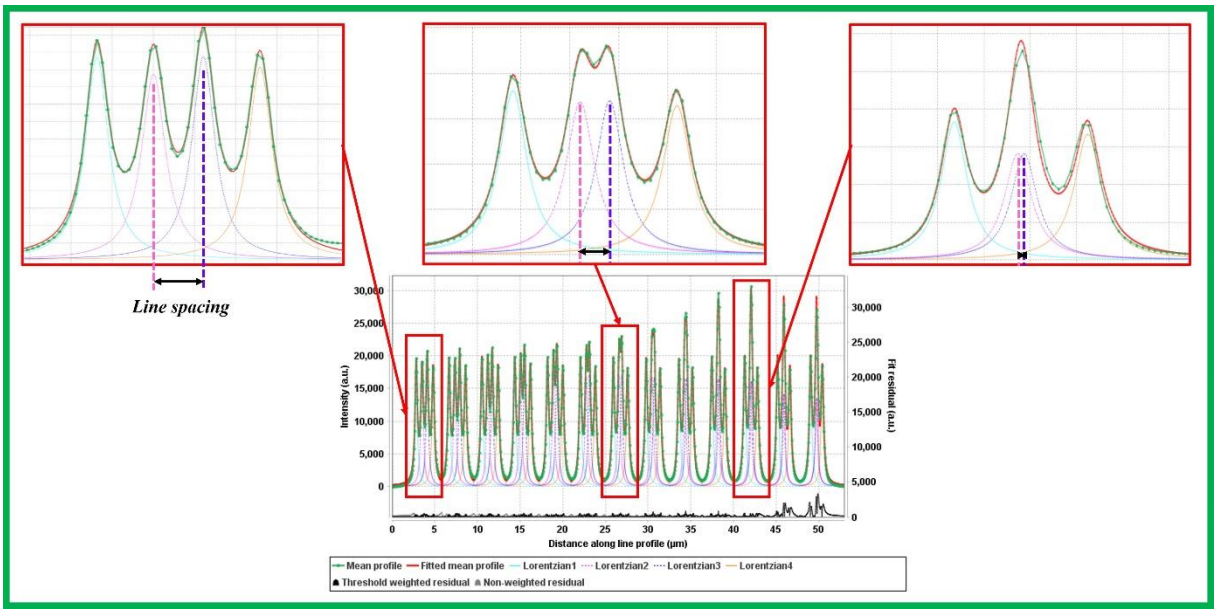

Figure 22: Each group of lines is fitted with Gaussian or Lorentzian function. The position of the fitting functions of the two inner lines of each group allows to determine the spacing between them.

3) Contrast transfer function measurement

The two previous steps allow the measurement of the contrast and the line spacing for each group of lines. Plotting the contrast versus the line spacing gives rise to the CTF (Figure 23). For a given contrast, one obtains the associated resolvable spacing. In Figure 23, the imaging system can resolve spacings of about 0.38 µm with a contrast of 26.5% (Rayleigh criterion).

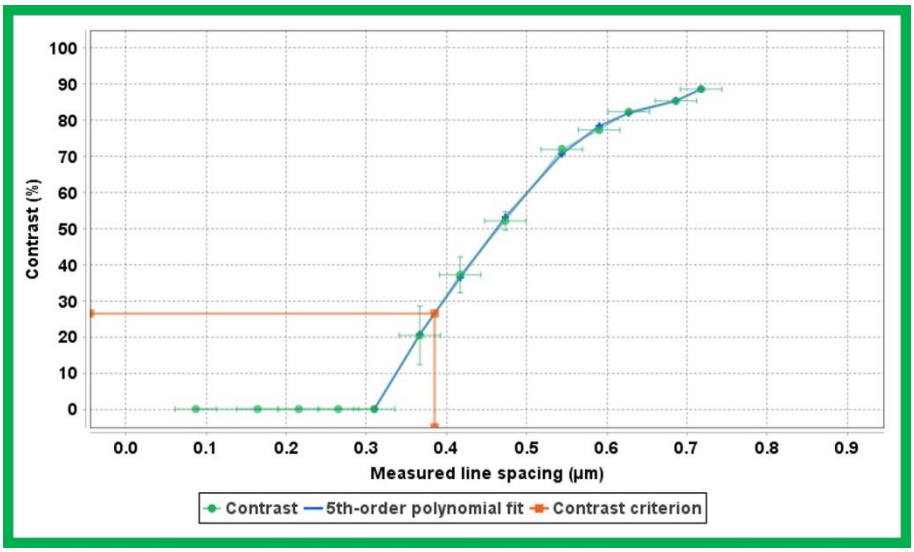

Figure 23: Contrast transfer function (i.e., the measured contrast versus the measured spacing between the two inner lines of each group).

4) SNR measurement

The SNR is measured from the outer lines of each group, within the same ROI used for the contrast and line spacing measurements. Intensity line profiles are drawn along the outer lines; they contain both the signal and noise contributions (Figure 24). For each line, the mean value and the standard deviation are calculated. All the obtained mean and standard deviation values are then averaged over the number of line profiles. Finally, the SNR is calculated according to its original definition (i.e., the ratio of the mean signal value to the standard deviation of the signal).

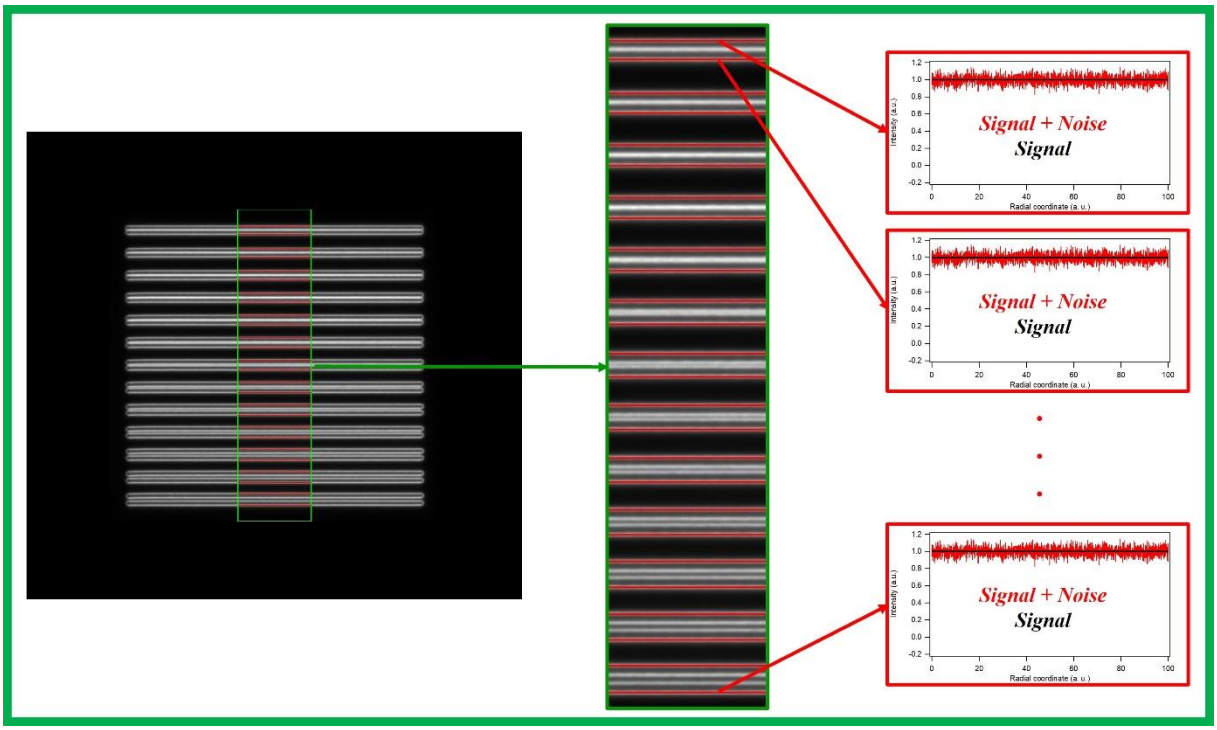

Figure 24: Intensity line profiles drawn along the outer lines of each group. For each line, the mean value and the standard deviation are calculated.

5) SBR measurement

The SBR is simply calculated by dividing the measured mean signal by the measured mean background (Figure 25).

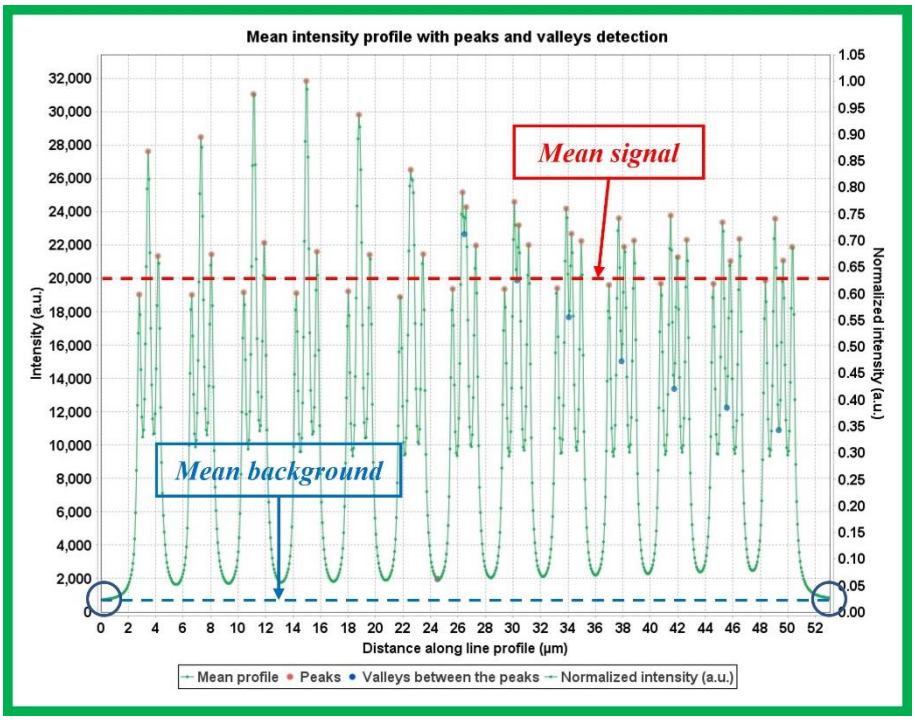

Figure 25: Mean signal (red) and mean background (blue) measured from the mean intensity profile, extracted from the analyzed ROI in the raw image.

10.3. Advantages and limitations

To the best of the author's knowledge, this approach is the only one (commercially available) that provides simultaneous measurements of resolvable distances between lines close to each other, for a given contrast value, with associated SNR and SBR values in the image. As such, it takes into account all the parameters influencing the resolution.

11. Remarkable results obtained with Argolight's approach

Thanks to the stability and durability of the "gradually spaced lines" pattern, and because Argolight's approach allows the simultaneous measurement of the resolvable distance, contrast, SNR, and SBR, it is possible to carry out measurements that would not otherwise be possible. Two examples are provided in this section:

- The evolution of the resolution versus the pinhole diameter of a confocal laser-scanning microscope.
- The improvement in resolution of different imaging modalities and image processing methods.

11.1. Evolution of resolution versus pinhole diameter

Images of horizontal "gradually spaced lines" from an Argo-HM v2.0 have been acquired using a fluorescence confocal laser scanning microscope, for several pinhole diameters from 0.2 to 3.0 Airy Units (Figure 26).

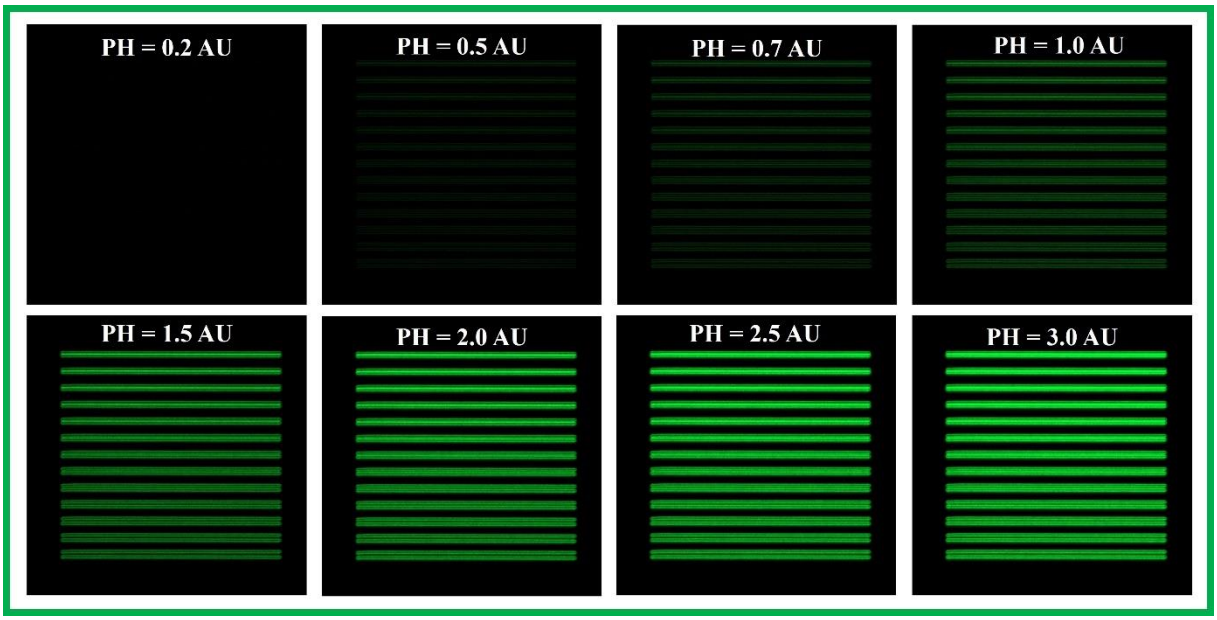

Figure 26: Images of horizontal "gradually spaced lines" from an Argo-HM v2.0 captured using a fluorescence confocal laser scanning microscope, for several pinhole diameters from 0.2 to 3.0 AU (all the other acquisition settings have been kept the same). The experimental conditions were as follows: 63×/1.4 oil Plan Apochromat objective; PMT GaAsP detector; 488 nm illumination wavelength; 490–560 nm detection spectral range, 1024 × 1024 pixels image size; 63 nm lateral pixel size; no averaging; active autofocus (no Z-stacking); 1 AU = 50 µm (at 520 nm).

Using the method described earlier, the CTFs have been measured from these images. Some of them are superimposed in Figure 27. Decreasing the pinhole size leads to an increase in the contrast for the same line spacing. Furthermore, as the pinhole size decreases, smaller resolvable distances can be measured.

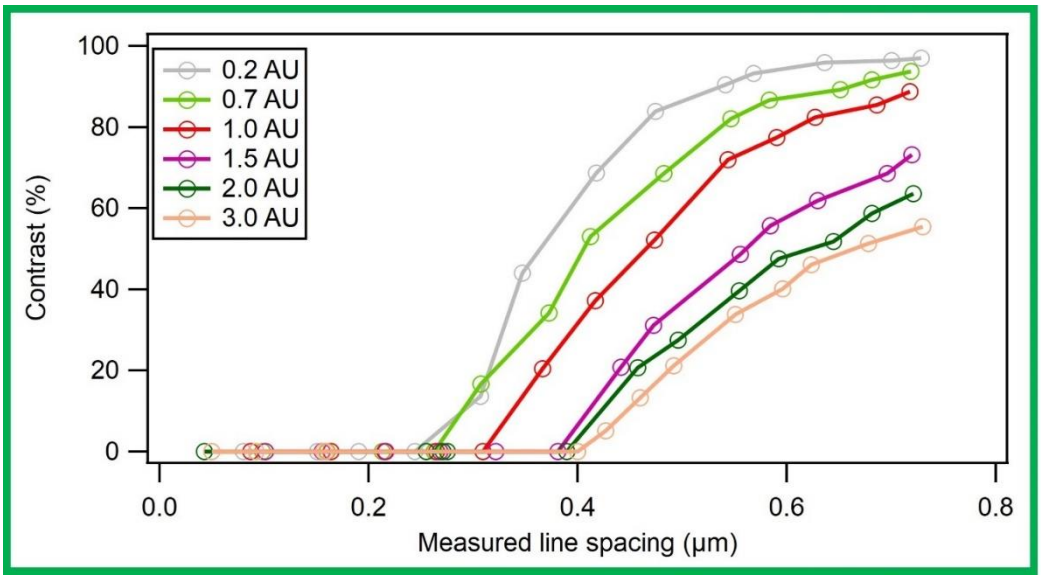

Figure 27: Contrast transfer functions measured from images of horizontal "gradually spaced lines" acquired for different pinhole diameters (0.2, 0.7, 1.0, 1.5, 2.0, and 3.0 AU).

Figure 28 shows the evolution of the resolvable distance at Rayleigh contrast, the minimum resolvable distance (i.e., at 0% contrast), and the SNR and the SBR versus the pinhole size. As expected from the theory, these four parameters increase as the pinhole size increases, following a sigmoidal-like evolution.

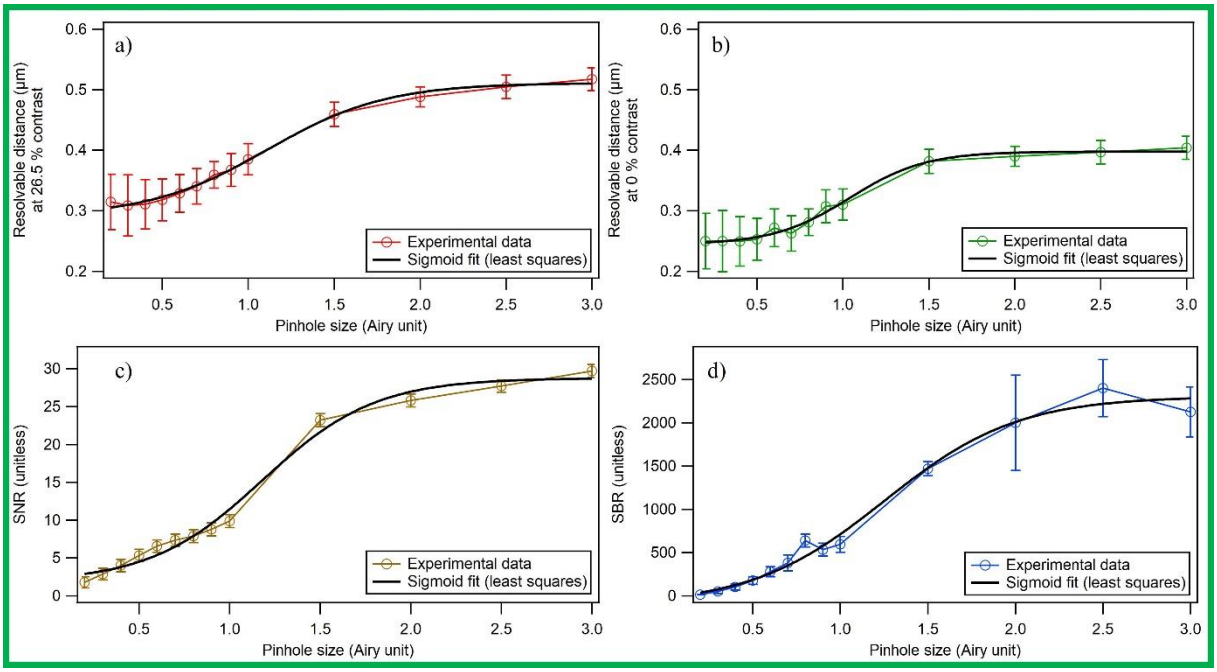

Figure 28: (a) Resolvable distance at Rayleigh contrast versus pinhole size; (b) minimum resolvable distance versus pinhole size; (c) SNR versus pinhole size; and (d) SBR versus pinhole size. All the data were fitted using Sigmoid functions with different fitting parameters.

11.2. Evolution of resolution versus image processing method

Images of vertical “gradually spaced lines” from an Argo-SIM v1.1 have been acquired using different imaging modalities and image processing methods: fluorescence wide-field microscopy, deconvolution, SIM, and “improved” SIM (Figure 26). The resolution is expected to improve in this order.

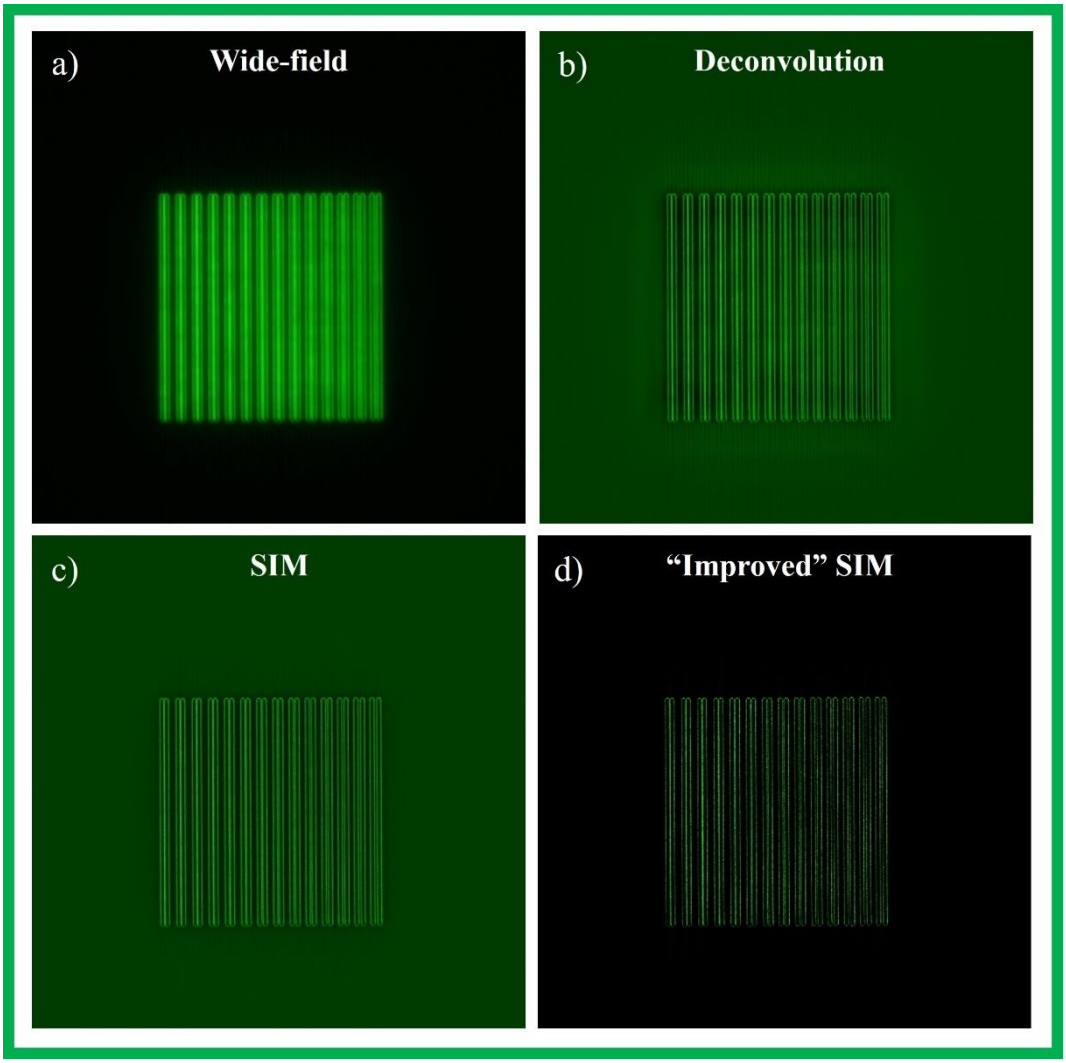

Figure 29: Images of vertical “gradually spaced lines” from an Argo-SIM v1.1 captured using: (a) fluorescence wide-field microscope; (b) deconvolution microscope; (c) SIM; and (d) “improved” SIM. The experimental conditions were as follows: 63×/1.46 oil Plan Apochromat objective; 488 nm illumination wavelength; 520 nm detection wavelength, 5120 × 5120 pixels image size; 20 nm lateral pixel size; Z-stack over 2.92 µm with a Z-step of 100 nm.

Using the method described earlier, the CTFs have been measured from these images. They are superimposed in Figure 30. As expected, the contrast increases with the processing method for the same spacing and the minimum achievable resolvable distance decreases with the processing method.

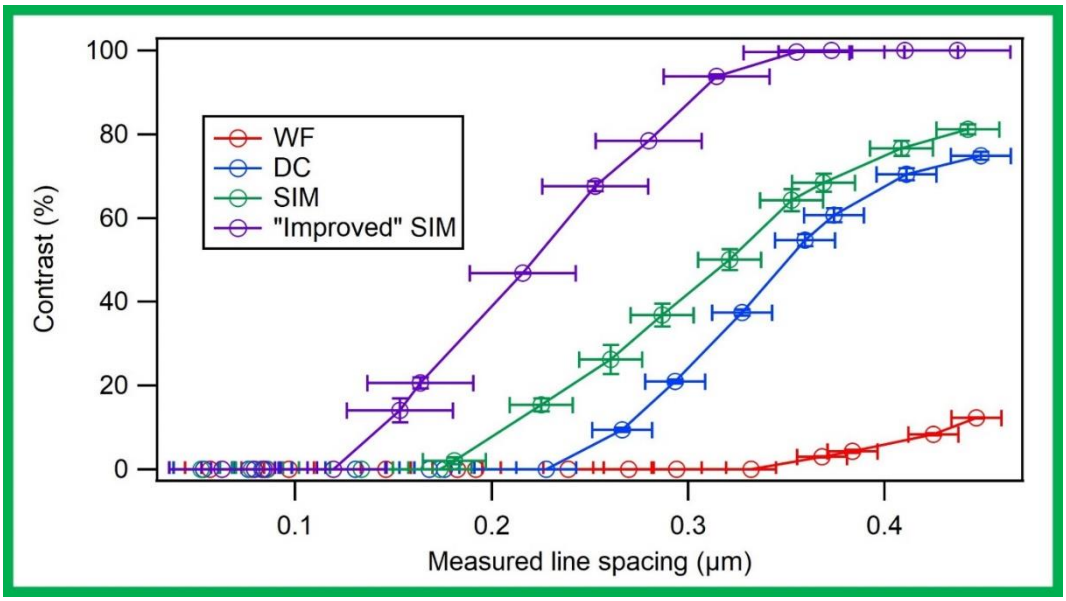

Figure 30: Contrast transfer functions measured from images of vertical “gradually spaced lines” acquired with different imaging modalities and image processing methods: fluorescence wide-field microscopy (WF), deconvolution (DC), structured illumination microscopy (SIM), and “improved” SIM.

12. Conclusion

In this white paper, five different approaches to measure the resolution have been described, both from a conceptual and practical perspective: the single- and double-point approaches, the single- and double-line approaches, and the CTF approach. The presented approaches have their own advantages and drawbacks, samples availability, community habit, and acceptance.

All these approaches provide values that can be transposable from one to another, only in the ideal case of a perfect fluorescence microscope. In real life, these approaches provide different results that cannot be strictly compared. The choice of the suited approach will depend on the application: biological imaging, material imaging, system performance monitoring, and so on.

A review of the parameters influencing resolution measurements has been conducted, which provides insight into extra-parameters (generally related to image quality, such as SNR and SBR) that are usually not considered.

Finally, remarkable results obtained from Argolight's approach have been presented, evidencing the potential of this approach, notably in modern computationally enhanced resolution microscopy.

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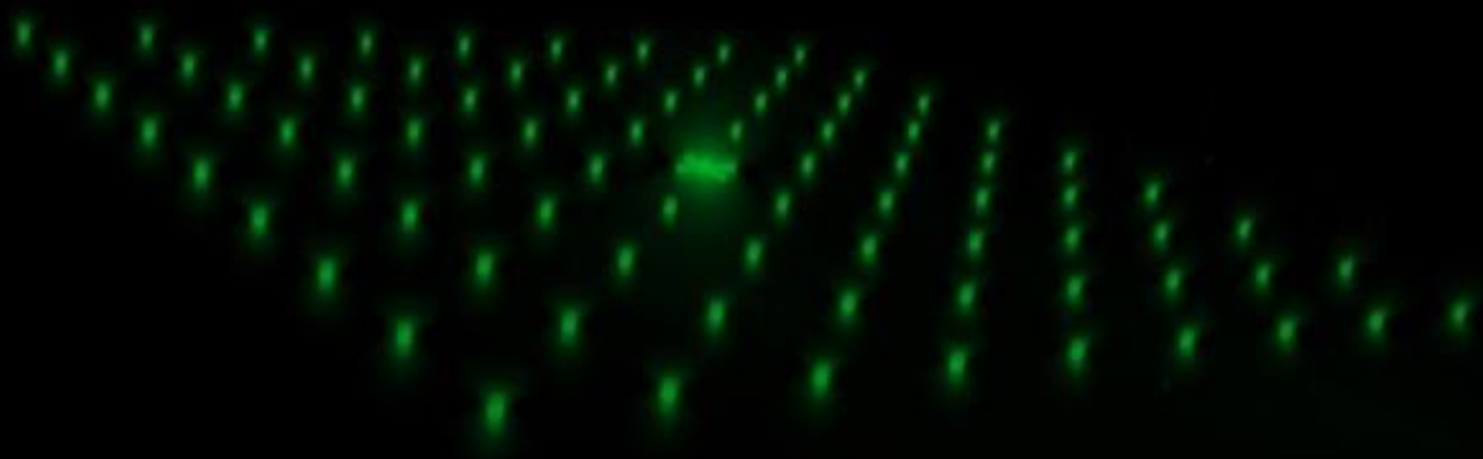

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