Resolution

Introduction

Resolution in fluorescence microscopy is defined as the shortest distance between two points on a specimen that can still be distinguished. This is primarily determined by two factors; microscope resolution, which is the smallest object the microscope can resolve, and camera resolution, which is the ability of the camera to detect what the microscope can resolve.
The maximum resolution of the microscope is a function of the numerical aperture of the objective lens and the emission wavelength of the sample, whereas camera resolution is determined entirely by pixel size.

However, the resolving power of a fluorescence microscope is ultimately restricted by the diffraction limit of light which, when using green light (510 nm) for example, would be around 220 nm. This sets a lower limit on what can be resolved. It is therefore common practice in standard fluorescence microscopy to use a microscope setup capable of reaching this lower limit to detect the smallest resolvable object. Attempting to resolve lower than this isn't possible using conventional microscopy, it's only possible to break the diffraction limit of light using super-resolution techniques.

Microscope resolution

The diffraction limited spot

Light travels as a wave so when it is focused to a small spot with a lens, no matter how good the objective lens is, the focal spot will have a larger size than the actual fluorophore.

This happens because the wavefront of the fluorescence emission becomes diffracted at the edges of the objective aperture. This effectively spreads the wavefront out, widening the fluorescence emission into a diffraction pattern which has a central spot larger than the original fluorophore (Figure 1).

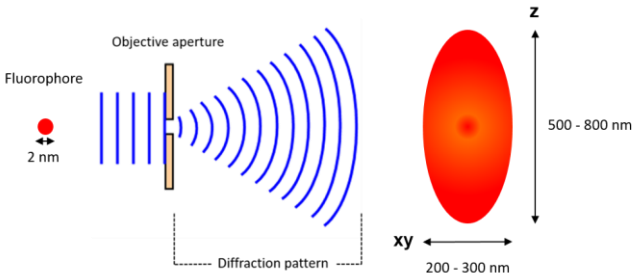


Figure 1: Diffraction pattern of a fluorescence emitter becoming diffracted at the edges of the objective aperture. The final diffraction limited spot may be 200–300 nm in x and y and 500–800 nm in z.

The size of the diffraction limited spot is approximately half the size of the wavelength of light emitted but the full equation, determined by Ernst Abbe in 1873, is:

$$d = \frac{\lambda}{2NA}$$

Where d is the size of the diffraction limited spot, λ is the wavelength of light used and 2NA is 2 times the numerical aperture of the objective.

In the case of GFP, which emits at ~510 nm, and a high (NA 1.4) numerical aperture objective, the size of the fluorophore as resolved by the microscope would be 182 nm. This is much larger than the actual fluorophore which may be just 2 nm.

Airy disks

The diffraction limited spot takes the shape of an Airy disk (Figure 2), named after George Biddell Airy. It consists of a bright central spot with a series of diffraction rings surrounding it. The size of the central spot is determined by the wavelength of light being emitted and the numerical aperture of the objective.

Figure 2 highlights how increasing the numerical aperture of the objective lens reduces the size of the Airy disk and therefore increases the amount of resolvable detail.

Figure 2: Airy disks produced by low, higher and high numerical aperture objectives.

The Rayleigh criterion

The issue with resolving adjacent fluorophores is that as Airy disks move closer together they merge with one another and become unresolvable (Figure 3). Thus, in 1896, Lord Rayleigh refined the Abbe equation to take into account how far apart two fluorophores need to be to differentiate them:

$$d = \frac{1.22\lambda}{2NA}$$

With this refinement, the distance required to differentiate two GFP fluorophores with a high (NA 1.4) objective would be 222 nm.

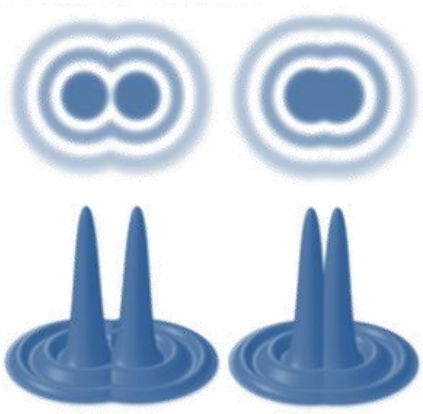


Figure 3: Two Airy disks merge until the two central spots can no longer be differentiated

Resolution limit of objectives

The information in Table 1 highlights the resolution limit possible with a variety of different magnification objectives and numerical apertures using GFP (510 nm) emission.

It's important to note that objective magnification has no impact on resolution, numerical aperture is the only important value. A 100x, 1.40 NA objective has the same resolving power as a 60x, 1.40 NA objective. Equally, a 100x, 1.30 NA objective has the same resolving power as a 40x, 1.30 NA objective.

This leads to the obvious question, why use 100x or 60x magnification if 40x magnification achieves the same resolution? When using higher magnification objectives, field of view is being restricted for no reason.

By using lower magnification objectives, changing from a 100x, 1.30 NA objective to a 40x, 1.30 NA objective, field of view is increased by 450%. That represents a 450% increase in sample area with no loss in resolution.
Equally, by changing from a 60x, 1.30 NA objective to a 40x, 1.30 NA objective, sample area is increased by 200%.

So why use higher magnification objectives at all? This is where camera resolution comes in. Magnification plays a big part in the resolving power of a scientific camera.

Objective (Numerical Aperture)	Resolution Limit (Micrometres)
1x (0.04)	7.8
2x (0.06)	5.2
2x (0.10)	3.1
4x (0.10)	3.1
4x (0.12)	2.6
4x (0.20)	1.55
10x (0.25)	1.2
10x (0.30)	1.04
10x (0.45)	0.69
20x (0.40)	0.78
20x (0.50)	0.62
20x (0.75)	0.41
40x (0.65)	0.48
40x (0.95)	0.33
40x (1.00)	0.31
40x (1.30)	0.24
60x (0.80)	0.39
60x (0.95)	0.33
60x (1.40)	0.22
100x (0.90)	0.35
100x (1.25)	0.25
100x (1.30)	0.24
100x (1.40)	0.22

Table 1: Microscope resolution using different power objectives with GFP (510 nm) emission. All numbers assume a perfectly aligned and optimized optical system.

Camera resolution

Camera resolution is defined as the ability of the camera sensor to sample the image and the resolving power of a scientific camera is entirely dependent on the size of the pixel and by how much it is magnified. Figure 4 highlights how scientific camera pixel size is changed by objective magnification.

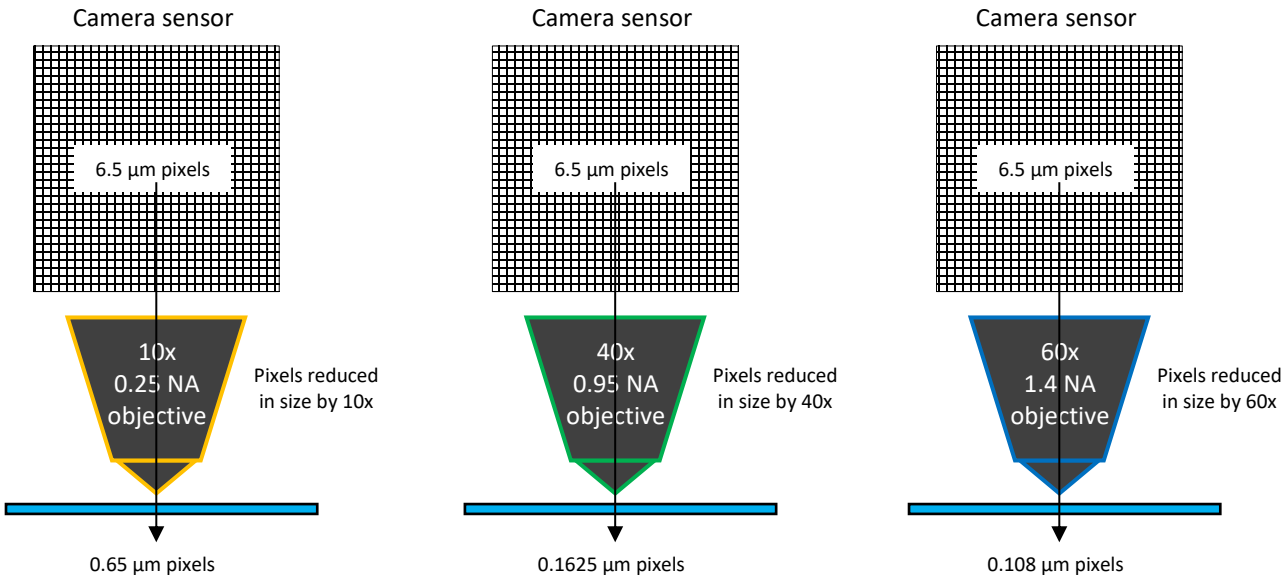


Figure 4: The resolving power of a scientific camera is dependent on pixel size.
6.5 µm pixels reduced in size by a 10x objective result in an effective pixel size of 0.65 µm, a 40x objective results in an effective pixel size of 0.1625 µm and a 60x objective results in an effective pixel size of 0.108 µm

The most obvious way to match camera resolution to microscope resolution would appear to be simply matching the diffraction-limited resolution given in Table 1 to the size of a single pixel. However, this is not the case. The goal isn't just to match the microscope resolution but to distinguish adjacent objects.

Nyquist sampling

As Figure 5 illustrates, if microscope resolution is matched to the size of a single pixel, then it is possible that two adjacent objects could be imaged onto adjacent pixels. In this case, there would be no way of discerning them as two separate objects in the resulting image.

Separating adjacent features requires the presence of at least one intervening pixel with a different intensity value. For this reason, the best spatial resolution that can be achieved requires matching the diffraction-limited resolution of the microscope to two pixels on the sensor. This is called Nyquist sampling and it can be calculated using the equation:

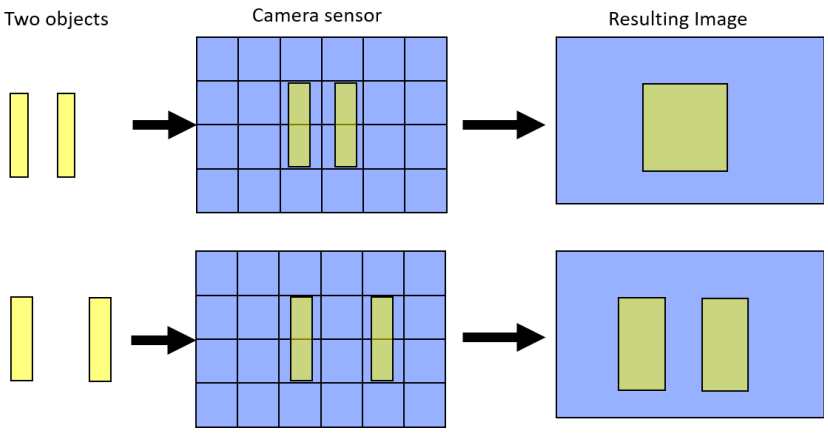


Figure 5: Separating two objects on the camera sensor requires the presence of pixels with disparate intensity between the two objects

$$Camera\ resolution\ = \frac{Pixel\ size}{Objective\ power} * 2.3$$

Matching camera resolution to microscope resolution

The pixel size required to achieve Nyquist sampling at different magnifications is illustrated in Tables 2 and 3. These tables provide the answer to the question posed before: Why use 100x or 60x magnification if 40x magnification achieves the same resolution?

As discussed previously, to achieve Nyquist sampling and match the microscope resolution using GFP (510 nm), the camera resolution should reach 0.22 µm. Table 2 shows that for a 40x objective, a 6.5 µm pixel is too large as the resulting camera resolution is 0.37 µm. A 6.5 µm pixel is considered sufficient for 60x magnification, however, as the resulting camera resolution reaches 0.25 µm.
A smaller pixel is therefore required to achieve Nyquist sampling at 40x. Table 3 shows that a smaller pixel size of 4.25 µm reaches a camera resolution of 0.24 µm with 40x magnification which satisfies Nyquist.

Objective Magnification	Camera pixel size (Micrometres)	Effective pixel size (Micrometres)	Camera resolution (Micrometres)	Nyquist Sampling (510 nm)
10x	6.5	0.65	1.50	X
20x	6.5	0.33	0.75	X
40x	6.5	0.16	0.37	X
60x	6.5	0.11	0.25	✓
100x	6.5	0.07	0.15	✓

Table 2: The impact of objective magnification on camera resolution possible with 6.5 µm pixels and whether Nyquist sampling is achieved

Objective Magnification	Camera pixel size (Micrometres)	Effective pixel size (Micrometres)	Camera resolution (Micrometres)	Nyquist Sampling (510 nm)
10x	4.25	0.43	0.98	X
20x	4.25	0.21	0.49	X
40x	4.25	0.11	0.24	✓
60x	4.25	0.07	0.16	✓
100x	4.25	0.04	0.10	✓

Table 3: The impact of objective magnification on camera resolution possible with 4.25 µm pixels and whether Nyquist sampling is achieved

The data presented in this section illustrates that the lowest magnification that can be used is limited by the pixel size of the camera, which needs to be small enough to match the microscope resolution.

This leads to a new question: If you have a camera with small enough pixels, why use 100x or 60x magnification if 40x magnification achieves the same resolution?

Utilizing smaller pixels for larger fields of view

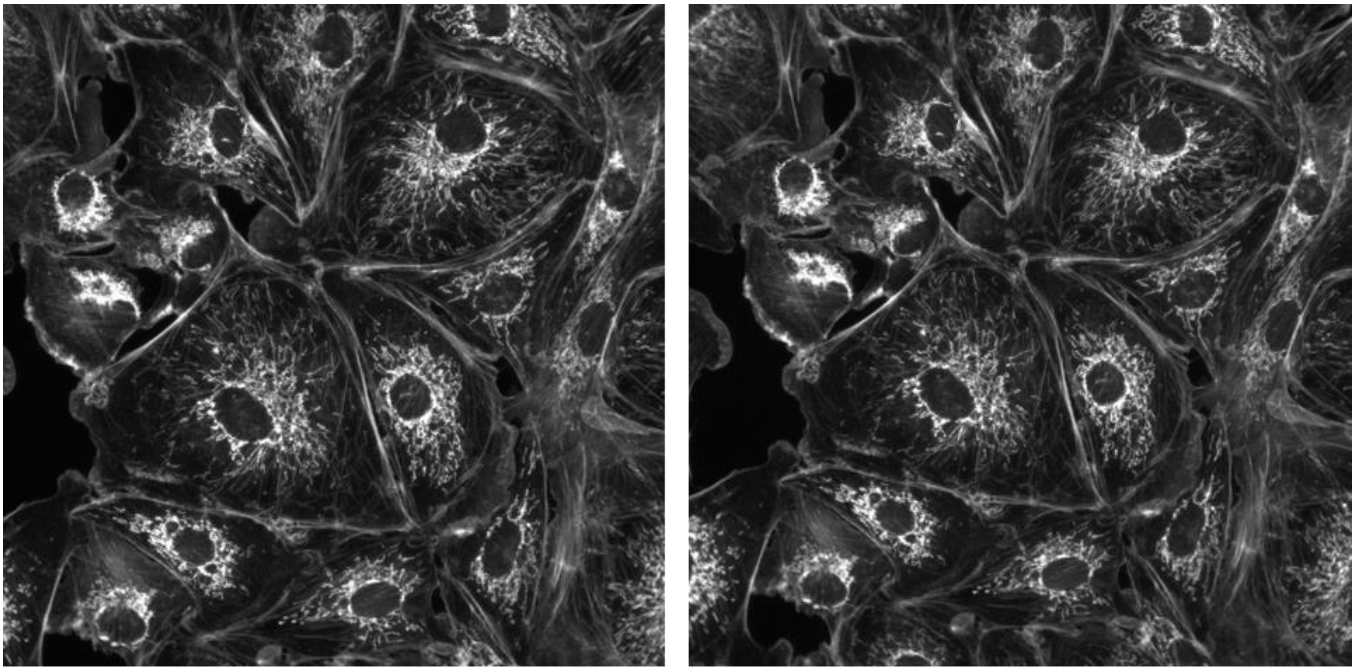
Typical sCMOS cameras, as well as the back-illuminated Prime BSI, have a pixel size of 6.5 µm which makes them perfect for 60x objectives but less suitable for 40x objectives. Likewise, the Prime 95B has been designed with large, 11 µm pixels to fit perfectly with 100x objectives.

The Iris 9 and 15 scientific CMOS cameras, however, have been designed for high-resolution imaging with lower magnification objectives by taking advantage of smaller, 4.25 µm pixels. This comes with multiple advantages.

Field of view

The primary advantage of the Iris cameras is being able to move to lower magnification and thereby increase field of view without sacrificing resolution.

Figure 6 shows that at 60x magnification, the Iris 9 and sCMOS cameras achieve comparable image quality and field of view. The microscope resolution using this sample is 0.23 µm which the sCMOS camera matches with a camera resolution of 0.25 µm and the Iris 9 easily matches with a camera resolution of 0.16 µm.

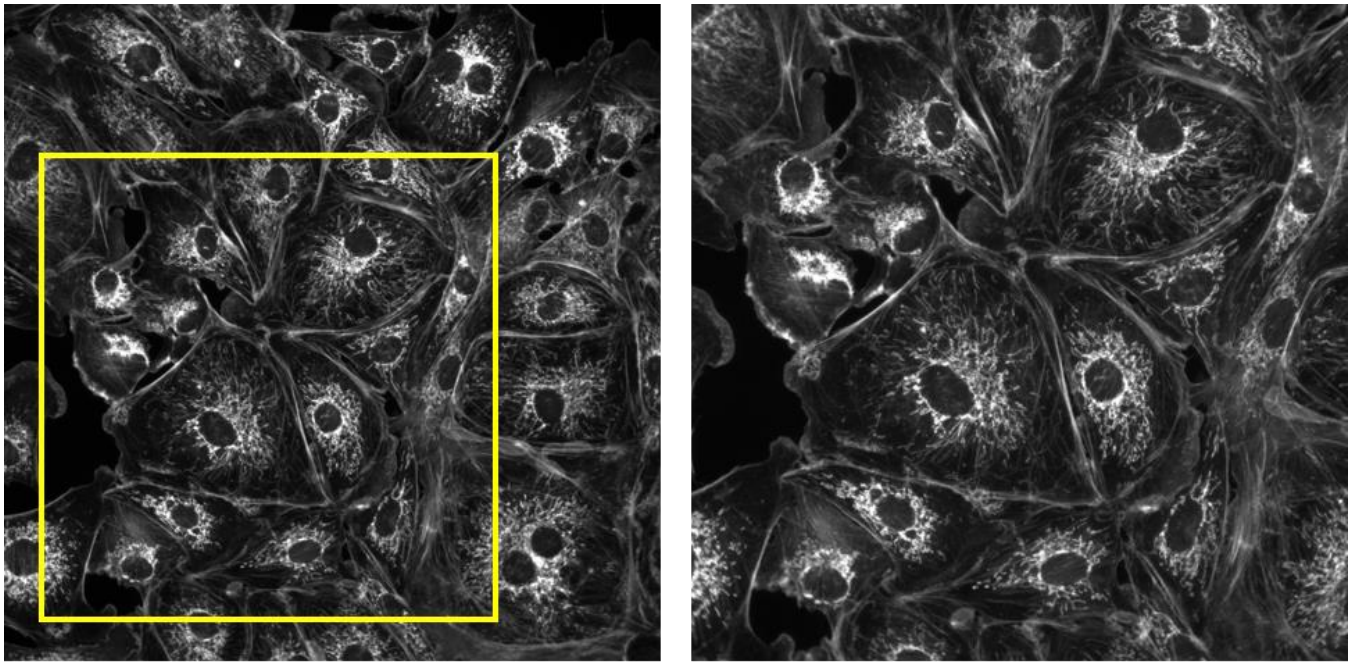


Iris 9 at 60x, 0.07 µm pixel size **82% sCMOS at 60x, 0.11 µm pixel size**

Figure 6: Field of view comparison between the Iris 9 and 82% sCMOS at 60x magnification. Microscope resolution = 0.23 µm, Iris 9 resolution = 0.16 µm, sCMOS resolution = 0.25 µm

The smaller pixels of the Iris 9 however, allow the magnification to be reduced to 40x and still match Nyquist. Figure 7 shows that by reducing magnification to 40x, the microscope resolution (0.24 µm) is matched perfectly by the Iris 9 camera resolution (0.24 µm) whereas the sCMOS camera resolution at 40x is 0.37 µm, 1.5x too large for Nyquist. This

means that for equivalent resolution with these two cameras, the Iris 9 can use 40x magnification but the sCMOS has to use 60x magnification.

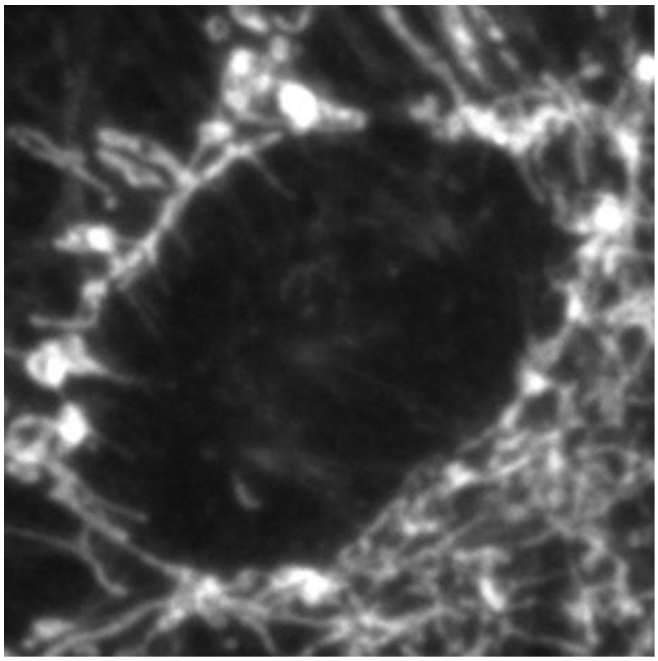


Iris 9 at 40x, 0.11 µm pixel size **82% sCMOS at 60x, 0.11 µm pixel size**

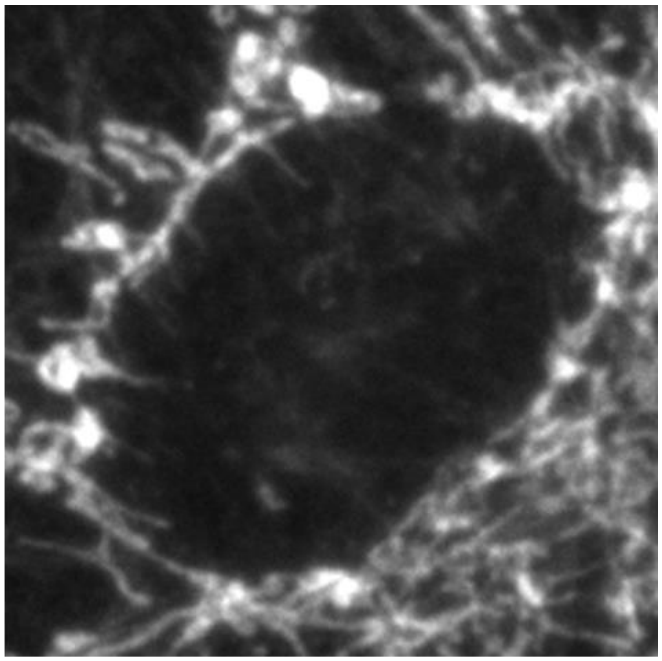
Figure 7: Field of view comparison between the Iris 9 at 40x magnification and 82% sCMOS at 60x magnification. The yellow box on the Iris 9 image highlights the field of view captured with the 82% sCMOS at 60x magnification.
Microscope resolution at 60x = 0.23 µm, Iris 9 resolution at 60x = 0.16 µm, sCMOS resolution at 60x = 0.25 µm
Microscope resolution at 40x = 0.24 µm, Iris 9 resolution at 40x = 0.24 µm, sCMOS resolution at 40x = 0.37 µm

Figure 8 goes on to show that there is truly no loss in resolution between the Iris 9 at 40x and the sCMOS at 60x. Both cameras have the same effective pixel size of 0.11 µm and therefore both achieve Nyquist. The only difference is that the Iris 9 uses lower magnification.

By utilizing smaller pixels, larger fields of view can be achieved by moving to lower magnification objectives. The field of view increase moving from 60x to 40x magnification is 150% which translates directly into 150% more data throughput – a tremendous advantage of moving to a smaller pixel camera.



Iris 9 at 40x, 0.11 µm pixel size



82% sCMOS at 60x, 0.11 µm pixel size

Figure 8: Resolution comparison of the Iris 9 at 40x and 82% sCMOS at 60x magnification. images from Figure 7 zoomed 300%.

Experimental analysis of resolution improvement

Experimental design

To demonstrate the resolving power of a smaller pixel camera at lower magnification, experimental analysis was performed. The nature of biological samples often renders them unreliable for accurate measures of resolution, for this reason, a known standard was preferred. To this end, experiments were performed using the Argo-HM slide from Argolight (http://argolight.com/argo-hm/). This slide is designed for calibrating and monitoring fluorescence systems through the use of stable fluorescence patterns of known size and fluorescence intensity.

One of the patterns on the Argo-HM slide is specifically designed for quantifying resolution (Figure 9). This pattern consists of thirteen pairs of lines with varying central gap distances. These 'gap pairs' are arranged top-to-bottom, where the top gap pair is separated by a distance of 100 nm and the thirteenth gap pair is separated by a distance of 700 nm. The gap pairs increase in separation distance by 50 nm as they go down.

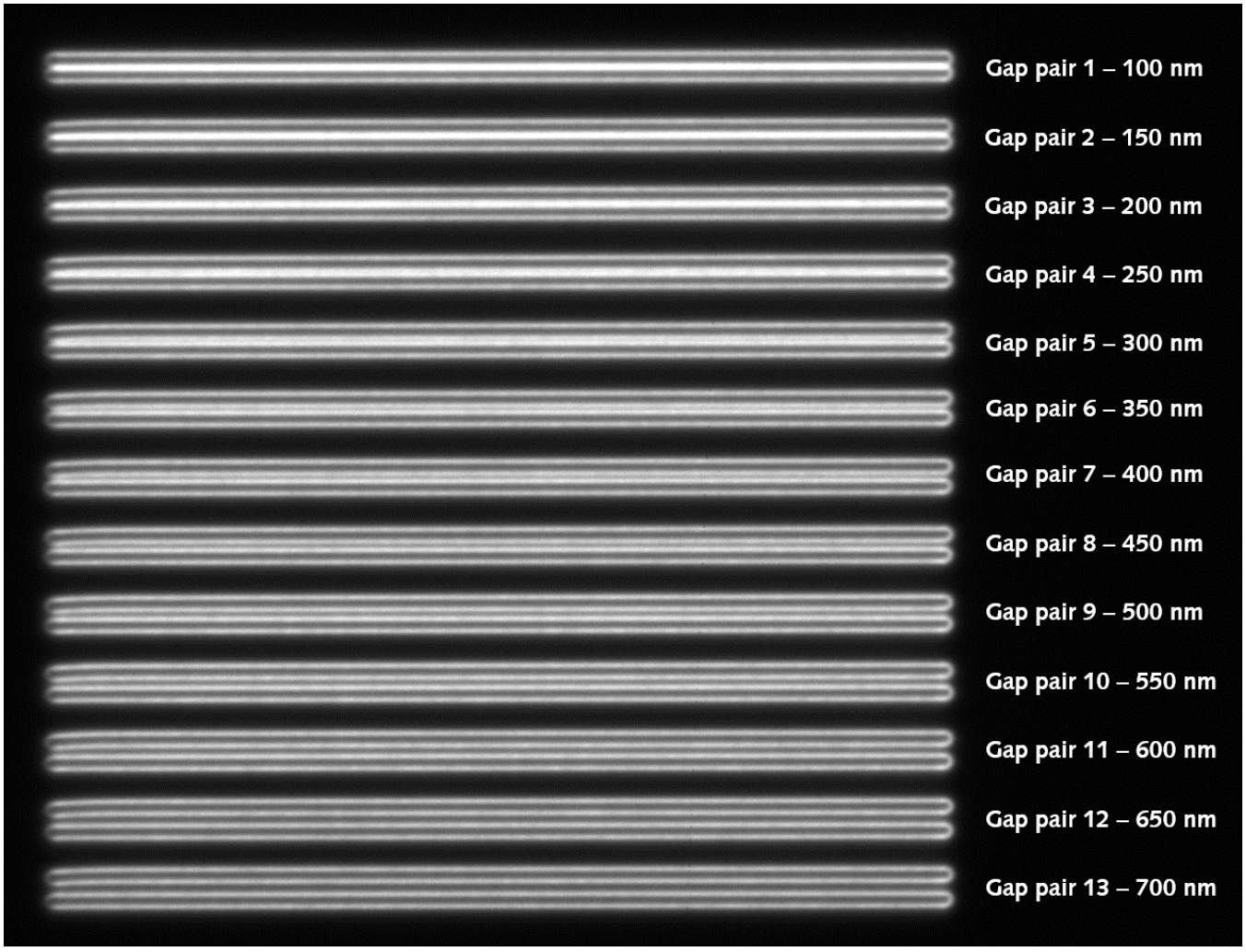
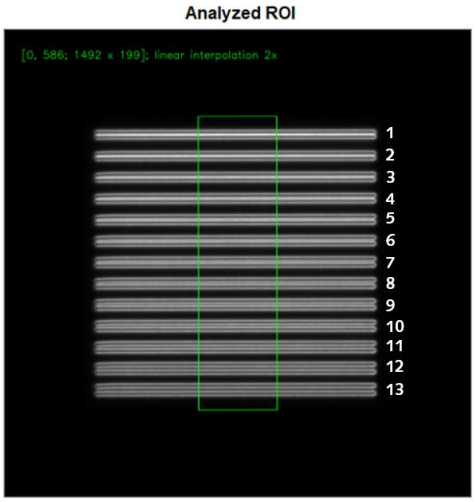
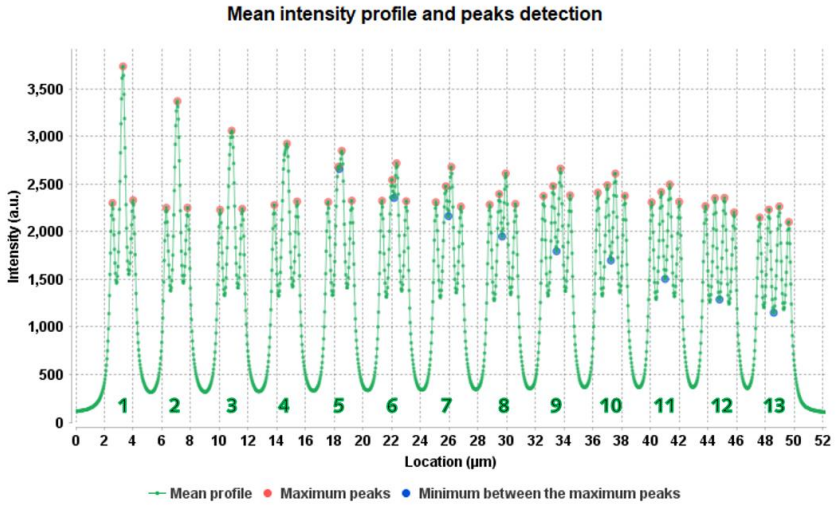


Figure 9: Gradually spaced gap pairs on the Argolight Argo-HM slide using the Iris 9 at 60x magnification.
The pattern consists of 13 pairs of 50 µm-long lines with a variable central gap distance. The gradually increasing gap distance runs from 100 nm (gap pair 1) to 700 nm (gap pair 13).

Using this slide, it's possible to calculate which gap pair the camera can accurately resolve as two separate lines.

The data is analysed in Daybook, a software designed by Argolight for image analysis. The raw data is uploaded and the type of analysis (lateral resolving power) is selected. The analysis calculates the separation distance between the gap pairs, showing which gap pairs can be resolved and which ones can't. The output table displays the calculated separation distance as well as the actual separation distance for comparison. The contrast mean can be used as a confidence interval to determine whether the gap can be resolved. The Dawes criterion (5%) or the Rayleigh criterion (26.3%) are considered desired contrast values for this purpose. Some of this data is shown in Figure 10.





Graph values

Peaks set index	Computed distance	Specified distance	Contrast mean
1	NA	0.1000 ± 0.1100	not detected
2	NA	0.1500 ± 0.1100	not detected
3	NA	0.2000 ± 0.1100	not detected
4	NA	0.2500 ± 0.1100	not detected
5	0.2450 ± 0.0700	0.3000 ± 0.1100	1.9854 ± 1.5247
6	0.3150 ± 0.0700	0.3500 ± 0.1100	5.5374 ± 1.6596
7	0.3850 ± 0.0700	0.4000 ± 0.1100	8.7521 ± 1.9588
8	0.4550 ± 0.0700	0.4500 ± 0.1100	12.4634 ± 2.1213
9	0.5250 ± 0.0700	0.5000 ± 0.1100	17.7671 ± 1.7421
10	0.5600 ± 0.0700	0.5500 ± 0.1100	20.0399 ± 1.1462
11	0.5950 ± 0.0700	0.6000 ± 0.1100	24.0824 ± 0.7518
12	0.6650 ± 0.0700	0.6500 ± 0.1100	29.2849 ± 0.0442
13	0.7350 ± 0.0700	0.7000 ± 0.1100	32.3646 ± 0.3283

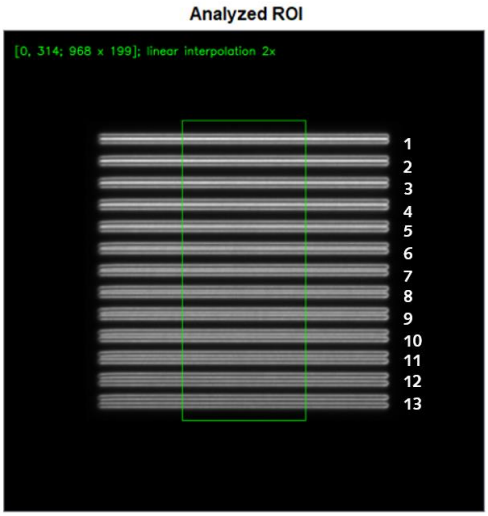
Figure 10: Selected graphs and tables from Daybook analysis of gradually spaced gap pairs using the Iris 9 (4.25 µm pixels) at 60x magnification.
Top left Single frame, raw data of gradually spaced gap pairs with an automatically drawn ROI for analysis.
Top right Graph generated from the resolution information in the ROI. Red circles show intensity peaks whereas blue circles show whether a minimum intensity was detected between the peaks. Blue circles therefore represent a detected separation of the gap pairs. The first detected separation is in gap pair 5.
Bottom right Table of graph values. Peaks set index corresponds to gap pair number. Computed distance (µm) is the separation distance detected by the camera, specified distance (µm) is the expected separation distance. Separation was first detected in gap pair 5 which corresponds to a separation distance of 0.3 µm.

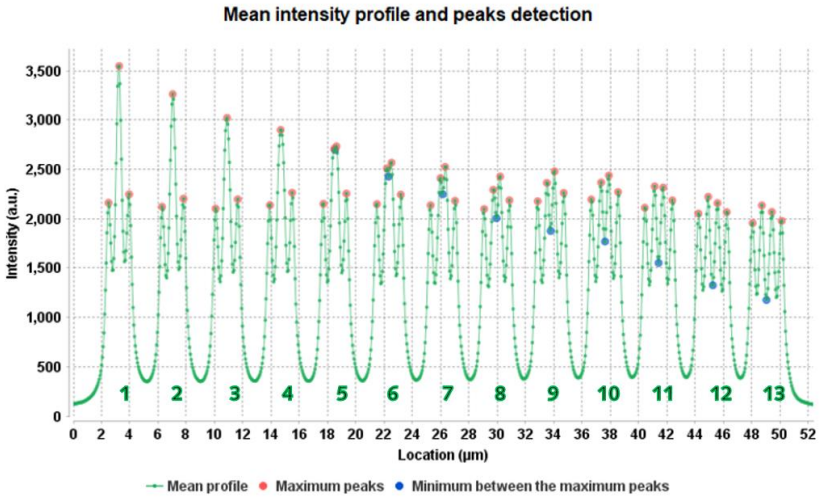
Daybook analysis of Iris 9 and sCMOS resolution

The Iris 9 (4.25 µm pixels) and 82% sCMOS (6.5 µm pixels) were connected to a microscope with a Cairn TwinCam 50/50 image splitter (https://www.cairn-research.co.uk/product/twincam/) to acquire images simultaneously on both cameras. The Argo-HM slide was brought into focus and parfocality was checked and corrected. Images were then acquired on both cameras with a 60x, 1.35 NA oil objective and a 40x, 0.95 NA air objective for comparison.
The comparison was made with a lower NA 40x objective to replicate the most likely 40x objective already present in the laboratory. Data using a 40x 1.30 NA objective is also available on request.

60x Analysis

The Iris 9 data was shown previously in Figure 10 and the sCMOS data is shown in Figure 11.





Graph values

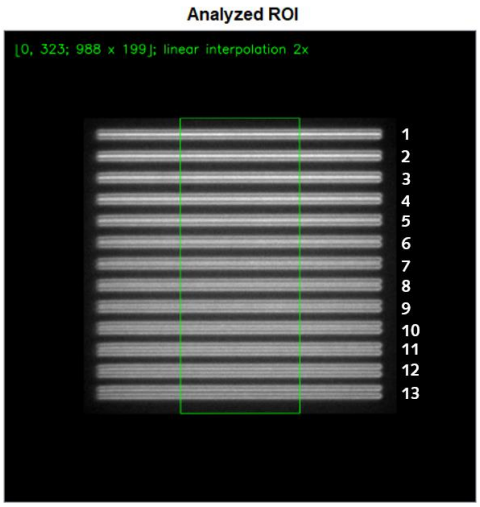
Peaks set index	Computed distance	Specified distance	Contrast mean
1	NA	0.1000 ± 0.1100	not detected
2	NA	0.1500 ± 0.1100	not detected
3	NA	0.2000 ± 0.1100	not detected
4	NA	0.2500 ± 0.1100	not detected
5	0.1624 ± 0.1083	0.3000 ± 0.1100	0.4398 ± 0.2217
6	0.3249 ± 0.1083	0.3500 ± 0.1100	2.2531 ± 0.5842
7	0.3249 ± 0.1083	0.4000 ± 0.1100	4.6748 ± 1.1529
8	0.4873 ± 0.1083	0.4500 ± 0.1100	8.1729 ± 1.4038
9	0.5415 ± 0.1083	0.5000 ± 0.1100	12.6796 ± 1.1681
10	0.5415 ± 0.1083	0.5500 ± 0.1100	15.2098 ± 0.7309
11	0.5957 ± 0.1083	0.6000 ± 0.1100	19.9098 ± 0.1341
12	0.6498 ± 0.1083	0.6500 ± 0.1100	24.6078 ± 0.6901
13	0.7039 ± 0.1083	0.7000 ± 0.1100	28.2465 ± 0.7309

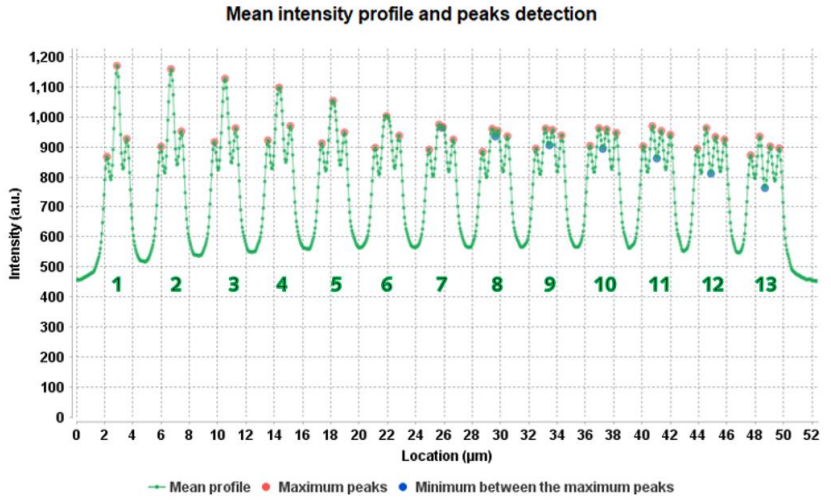
Figure 11: Selected graphs and tables from Daybook analysis of gradually spaced gap pairs using an 82% sCMOS (6.5 µm pixels) at 60x magnification.
Top left Single frame, raw data of gradually spaced gap pairs with an automatically drawn ROI for analysis.
Top right Graph generated from the resolution information in the ROI. First detected separation in gap pair 5.
Bottom right Table of graph values. Separation was first detected in gap pair 5 which corresponds to a separation distance of 0.3 µm.

The data agrees with what was discussed in the previous section, at 60x magnification both cameras reach Nyquist and therefore have similar resolving power. Both cameras are able to detect the 300 nm separation in gap pair 5 which means both cameras match the microscope resolution which is only limited by the diffraction limit of light.

However, the Iris 9 does appear to show an improvement when resolving the separation in gap pair 5. The data in Figure 10 shows that the Iris 9 detects the 300 nm separation with a contrast mean of 1.98 whereas the data in Figure 11 shows that the sCMOS detects the 300 nm separation with a contrast mean of just 0.44. The Dawes criterion (5%) was satisfied by the Iris 9 at 350 nm separation but was only reached with the sCMOS at 400 nm separation (within error).

40x Analysis

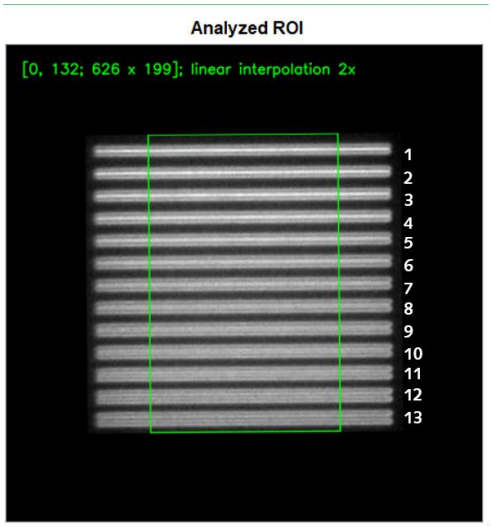


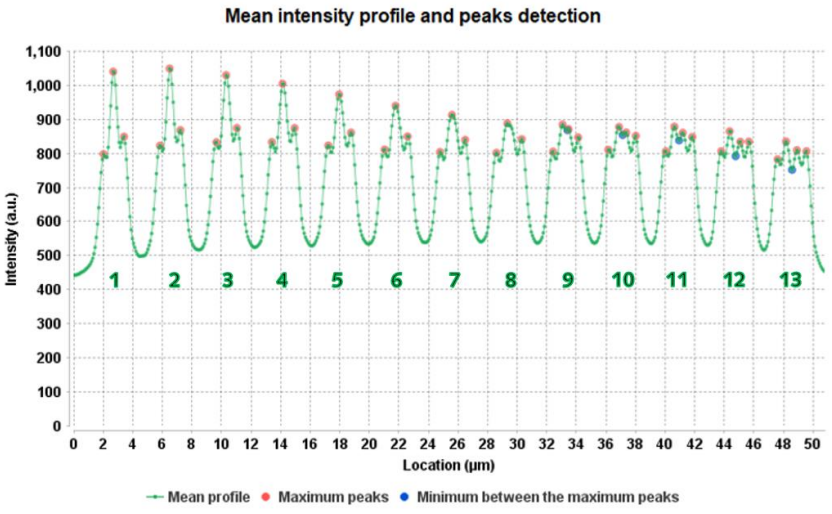


Graph values

Peaks set index	Computed distance	Specified distance	Contrast mean
1	NA	0.1000 ± 0.1100	not detected
2	NA	0.1500 ± 0.1100	not detected
3	NA	0.2000 ± 0.1100	not detected
4	NA	0.2500 ± 0.1100	not detected
5	NA	0.3000 ± 0.1100	not detected
6	NA	0.3500 ± 0.1100	not detected
7	0.2655 ± 0.1062	0.4000 ± 0.1100	0.3487 ± 0.2099
8	0.3717 ± 0.1062	0.4500 ± 0.1100	1.1714 ± 0.1649
9	0.4779 ± 0.1062	0.5000 ± 0.1100	2.8825 ± 0.1205
10	0.5310 ± 0.1062	0.5500 ± 0.1100	3.6177 ± 0.1094
11	0.6372 ± 0.1062	0.6000 ± 0.1100	5.4837 ± 0.3997
12	0.6372 ± 0.1062	0.6500 ± 0.1100	7.7842 ± 0.7780
13	0.7434 ± 0.1062	0.7000 ± 0.1100	9.2309 ± 0.8929

Figure 12: Selected graphs and tables from Daybook analysis of gradually spaced gap pairs using the Iris 9 (4.25 µm pixels) at 40x magnification.
Top left Single frame, raw data of gradually spaced gap pairs with an automatically drawn ROI for analysis.
Top right Graph generated from the resolution information in the ROI. First detected separation in gap pair 7.
Bottom right Table of graph values. Separation was first detected in gap pair 7 which corresponds to a separation distance of 0.4 µm.





Graph values

Peaks set index	Computed distance	Specified distance	Contrast mean
1	NA	0.1000 ± 0.1100	not detected
2	NA	0.1500 ± 0.1100	not detected
3	NA	0.2000 ± 0.1100	not detected
4	NA	0.2500 ± 0.1100	not detected
5	NA	0.3000 ± 0.1100	not detected
6	NA	0.3500 ± 0.1100	not detected
7	NA	0.4000 ± 0.1100	not detected
8	NA	0.4500 ± 0.1100	not detected
9	0.4062 ± 0.1625	0.5000 ± 0.1100	0.5411 ± 0.3827
10	0.4875 ± 0.1625	0.5500 ± 0.1100	0.8905 ± 0.4604
11	0.5687 ± 0.1625	0.6000 ± 0.1100	1.8469 ± 0.5172
12	0.7313 ± 0.1625	0.6500 ± 0.1100	3.5219 ± 0.9063
13	0.7313 ± 0.1625	0.7000 ± 0.1100	4.4942 ± 0.7716

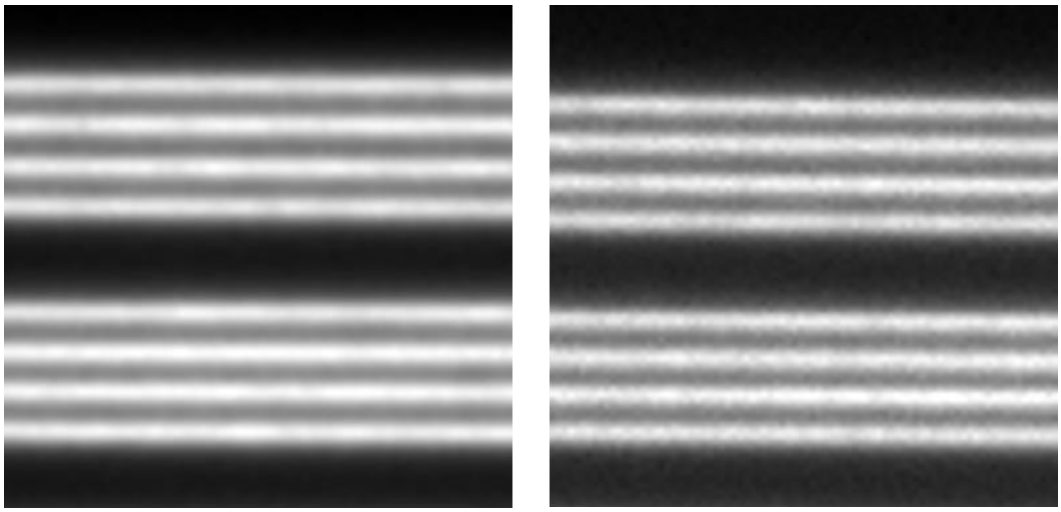
Figure 13: Selected graphs and tables from Daybook analysis of gradually spaced gap pairs using an 82% sCMOS (6.5 µm pixels) at 40x magnification.
Top left Single frame, raw data of gradually spaced gap pairs with an automatically drawn ROI for analysis.
Top right Graph generated from the resolution information in the ROI. First detected separation in gap pair 9.
Bottom right Table of graph values. Separation was first detected in gap pair 9 which corresponds to a separation distance of 0.5 µm.

The results generated in Figures 12 and 13 agree with what was expected. At 40x, the 4.25 µm pixels of the Iris 9 reach Nyquist and were able to detect the 400 nm separation in gap pair 7. The 6.5 µm pixels of the sCMOS, however, don't reach Nyquist and were only able to detect the 500 nm separation in gap pair 9. At 40x magnification, the larger 6.5 µm pixels do not have the resolving power necessary to separate any further gap pairs.
The Iris 9 satisfies the Dawes criterion (5%) at gap pair 11 whereas this was only reached by the sCMOS at gap pair 13 (within error).

Taken together, these results demonstrate that using a smaller pixel camera allows for Nyquist sampling at lower magnification objectives. This means that larger fields of view, and therefore greater data throughput, are possible with a simple change in objective magnification. Furthermore, there was no disadvantage to using a smaller pixel camera with higher magnification objectives, making it a more versatile choice.

Smaller pixels with higher magnification objectives

As mentioned in the previous section, smaller pixel cameras perform equally well when using higher magnification objectives. By oversampling (using a pixel size lower than Nyquist), images appear smoother and with less blockiness (Figure 14) which may be of value when creating higher quality images for publication. Oversampling is also used when performing deconvolution, an important post-processing tool used to reduce the impact of out-of-focus light.



Iris 9 at 60x,
0.07 µm pixel size

82% sCMOS at 60x,
0.11 µm pixel size

Figure 14: Image quality comparison between the Iris 9 and 82% sCMOS using gap pairs 12 and 13 zoomed 300%.

Conclusion

There are two types of resolution; microscope resolution and camera resolution. Microscope resolution is determined by the numerical aperture of the objective and the emission wavelength whereas camera resolution is determined by pixel size.

It's possible to reach diffraction-limited resolution with lower (40x) magnification objectives but pixel size becomes limiting. To remedy this, cameras such as the Iris 9 and 15 have been developed with a smaller pixel to allow researchers to move to lower magnification objectives. This has the advantage of increasing the field of view without sacrificing resolution. Researchers can expect to increase throughput 200% just by switching from 60x to 40x.

With the addition of the Iris cameras, we are now able to offer cameras that achieve Nyquist sampling at 40x, 60x and 100x to meet the varied needs of the most challenging samples.

References

Cairn Research - https://www.cairn-research.co.uk/

Argolight - http://argolight.com/

