

Confocal Application Notes

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On Resolution, Pixels, Zoom and Scan Format

1. Optical resolution of the objective

The theoretical lateral optical resolution in wide field microscopy is given as:

$$r_{xy} = 0.61 * \lambda / N.A. \text{ (Rayleigh criteria)}$$

Due to the narrower point spread function of the confocal microscope, lateral resolution is improved and one can use:

$$r_{xy} = 0.4 * \lambda / N.A.$$

For example, a 40x objective with a N.A. of 1.25 will have a theoretical xy-resolution of 156.2 nm at 488 nm.

$$r_{xy} = 0.4 * 488nm / 1.25$$

$$\underline{r_{xy} = 156.2nm}$$

This 40x/1.25 N.A. objective will be used as an example for this note.

2. Scan Format and Scan Field Size

The scan format determines how many lines are scanned and how many samples are taken along each scan line. The scan format is expressed as:

Number of pixels per line x Lines per frame.

A scan format of 512x512 therefore means that the sample area is scanned at a resolution of 512x512 pixels.

For a given microscope, the size of the scan field (the x-y dimensions of your image) itself is determined by the objective magnification and the zoom factor used (we will talk about zoom later).

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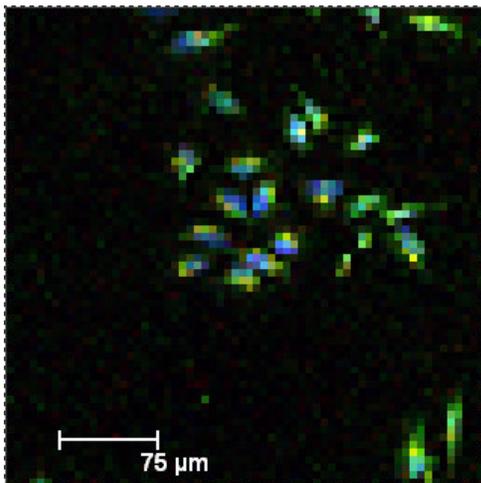
| Objective | Scan field at zoom 1 (μm) |
|-----------|--|
| 10x | 1500x1500 |
| 20x | 750x750 |
| 40x | 375x375 |
| 63x | 238x238 |
| 100x | 150x150 |

Table 1: Scan field size for different objective magnifications.

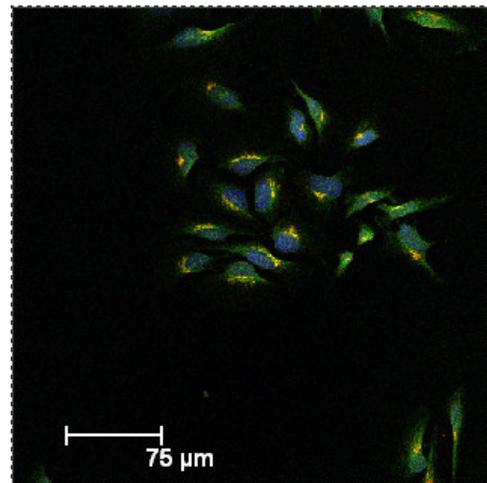
The size of the scan field is independent of the selected scan format. However, the scan format will dictate how many pixels are used to cover the scan field and as a result, the size of the pixels will change with the format selected.

For example: Using a 40x objective and zoom 1, the scan field is 375x375 μm . If the selected scan format is 64x64, then these 375x375 μm are covered by 64 lines with 64 pixels in each line. Every pixel will be 5.86x5.86 μm ($375 \mu\text{m}/64=5.86$). If you choose a scan format of 512x512 the pixel size will be reduced to 0.73 μm ($375 \mu\text{m}/512=0.73$).

To illustrate this here are two images taken under the same conditions except that the scan format for the left image is set to 64x64; while the scan format for the right image is 512x512, resulting in a much smaller pixel size. One can clearly see the difference in resolution.



64x64



512x512

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| Scan format | Pixel size (nm) @ zoom 1 | Pixel size (nm) @ zoom 10 |
|-------------|-----------------------------|------------------------------|
| 64x64 | 5860 | 586 |
| 128x128 | 2930 | 293 |
| 256x256 | 1460 | 146 |
| 512x512 | 732 | 73 |
| 1024x1024 | 366 | 36 |
| 2048x2048 | 183 | 18 |
| 4096x4096 | 91 | 9 |

Table 2: Scan format and pixel size for a 40x/1.25 N.A. objective at zoom 1 and zoom 10.

3. Zoom factor

The zoom factor will determine the scan angle of the scan mirrors. Zooming in will reduce this angle, which in turn reduces the size of the scanned area. The scan format is unchanged and you now scan a smaller area of your sample with the same number of pixels that you used before, therefore the pixel size is reduced. The image is magnified to a greater extent and resolution is improved. Please keep in mind that this improvement of resolution is limited by the optical resolution of the objective! One cannot arbitrarily increase the zoom factor and improve resolution indefinitely. Oversampling will not add any more details to the image (see Nyquist theorem below)!

We have to distinguish between this optical zoom and the digital zoom we will find in image processing software. A digital zoom works by mathematically increasing the image size using the original number of pixels and the image will become blurry.

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| Zoom factor | xy-image dimensions (μm) | Pixel size (nm) |
|-------------|--|-----------------|
| 1 | 375 | 732.42 |
| 2 | 187.5 | 366.21 |
| 4 | 93.75 | 183.11 |
| 8 | 46.88 | 91.55 |
| 16 | 23.44 | 45.78 |
| 32 | 11.72 | 22.9 |

Table 3: Zoom factor vs. image and pixel size. Scan format 512x512, 40x/1.25 N.A. objective.

4. Pixel size and the Nyquist theorem

The Nyquist sampling theorem states that the sampling frequency must be greater than twice the bandwidth of the input signal to reconstruct the original input from the sampled data.

What does this mean for scan format and pixel size?

The pixel size should be set to about one-half to one-third of the minimum spacing that one wants to resolve.

Let's go back to the 40x objective. The objective has a theoretical optical resolution of 156 nm. To make full use of this resolution the pixel size should be approximately:

$$\text{pixel size} = \frac{r_{xy}}{2.5} = \frac{156\text{nm}}{2.5} = 62.4\text{nm}$$

At a scan format of 512x512 and zoom 1 the pixel size is 730 nm. This pixel size is therefore too big to make full use of the resolving power of the 40x objective. Using a zoom factor of 12x with the 512x512 format will reduce the pixel size to 61 nm. We

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could also change the scan format to 4096x4096 at zoom 1 to achieve a pixel size of 91 nm.

At this point you have to ask yourself if you really need 60 nm resolution? Are you trying to resolve details in your sample that small? Please keep in mind that scanning an image at 400Hz and 4096x4096 resolution will take 10 seconds. Now photobleaching and movement of your live-cell preparation may create a new problem.

5. Output of your digital image using third-party software, i.e. Adobe Photoshop

When you open a confocal image in Adobe Photoshop and look at the image size, Photoshop may show you an image resolution of 72 dpi and an image size of 7x7 inches. This obviously does not reflect the physical size or resolution of your confocal image. Photoshop is rather displaying the image optimized for the output device; in this case your computer monitor (72 dpi is the standard resolution of most monitors).

Let's say you are asked to submit your image files at a resolution of 300 dpi. At a printer resolution of 300 dpi a 512x512 image will yield a 1.7x1.7 inch printout without introducing pixilation ($512/300=1.7$). A 1024x1024 image will allow for a $1024/300=3.4$ x3.4 inch printout ($1024/300=3.4$).

| Scan format | Printout size (inch) | |
|-------------|----------------------|-----------|
| | @ 300 dpi | @ 600 dpi |
| 64x64 | 0.2 | 0.1 |
| 128x128 | 0.4 | 0.2 |
| 256x256 | 0.8 | 0.4 |
| 512x512 | 1.7 | 0.8 |
| 1024x1024 | 3.4 | 1.7 |
| 2048x2048 | 6.8 | 3.4 |

Table 4: Scan format and recommended size of the printout at 300 and 600 dpi printer resolution.

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If you have to change the dpi resolution of the image in Photoshop to match the output device (printer), please turn off “resample image”. This will allow you to change the output resolution of the image without changing the number of pixels. Resampling the image can introduce artifacts; in order to create new pixels the software has to interpolate.

Summary

1. Try to achieve the highest optical resolution that you need for the job by selecting the right objective, scan format and zoom factor for your sample.
2. Match the scan format to the desired output resolution.