To bin or not to bin:

Balancing the trade-off between signal intensity, spatial and time resolution in biological imaging

Sam Swift and Laura Trinkle-Mulcahy

Three of the most important things we ask of the light microscope in cell biology are:

1. **appropriate magnification**
2. **high contrast**
3. **high (spatial or temporal) resolution**

This short paper focuses on issues relating to *digital resolution* and attempts to demonstrate how all three of these attributes of the light microscope influence the decisions we make when using digital technology to record biological events. As such, no single one can be considered in isolation.

Magnification is the simplest term to understand: it is our ability to make small objects appear larger. We generally do this so that we can observe more details. A simple example is presenting a slideshow to a large audience. We can set our laptop on a desk at the front of the room and play the slideshow on the laptop screen, but it would be understandably difficult for the entire audience to clearly see what is going on. What we normally do instead is output our laptop signal to a projector so that the slideshow can be magnified onto the wall and everyone in the room can clearly see it. It is important to remember, however, that our viewing pleasure will depend on the resolution of the images/movies in the slideshow and the levels of contrast that the projector is able to generate; magnification alone isn't always terribly useful.

Contrast refers to how well we can distinguish the object that we are interested in from the background or the noise that is also present. For example, if we put a piece of clear tape on a window, it would be difficult to see the tape because it absorbs very little light. If we instead put a piece of black electrical tape on the window, it absorbs more of the light being transmitted through the window and thus is much easier to see. In other words, the signal to background ratio is now significantly higher. If we take a photo of the window with our digital camera, the noise can be considered to be any random signal imposed on the image by the camera. In the case of digital recording devices, the most significant noise is usually a consequence of thermal noise across the detector. This is the reason that the higher resolution cameras used for microscopy applications are cooled.

Resolution is our ability to distinguish two points in an image as being separate from each other (see Figure 1). A good example of this is to have two people stand next to each other with their arms outstretched. If we stand progressively further away from them, at some distance it is difficult to tell if their hands are touching or not, i.e. at this distance we are unable to determine whether they are one object (touching) or 2 distinct objects (not touching). This article discusses resolution, but it is important to consider that resolution, magnification and contrast do not happen in isolation. A low contrast image taken with high resolution optics is just as useless as a low contrast image taken with low resolution optics.

Figure 2 illustrates how these parameters interact. Digital resolution is determined by the total number of units that define a particular image, illustrated here in panels A-B. The same arrow, drawn in Photoshop and described by either 300 dpi (dots per inch, a standard measure of digital resolution) or 72 dpi, looks identical at a small size because we cannot distinguish individual pixels. When it is enlarged, a resolution of 300 dpi is still sufficient to describe the arrow in detail and it remains sharp and well-resolved. As the 72 dpi arrow is

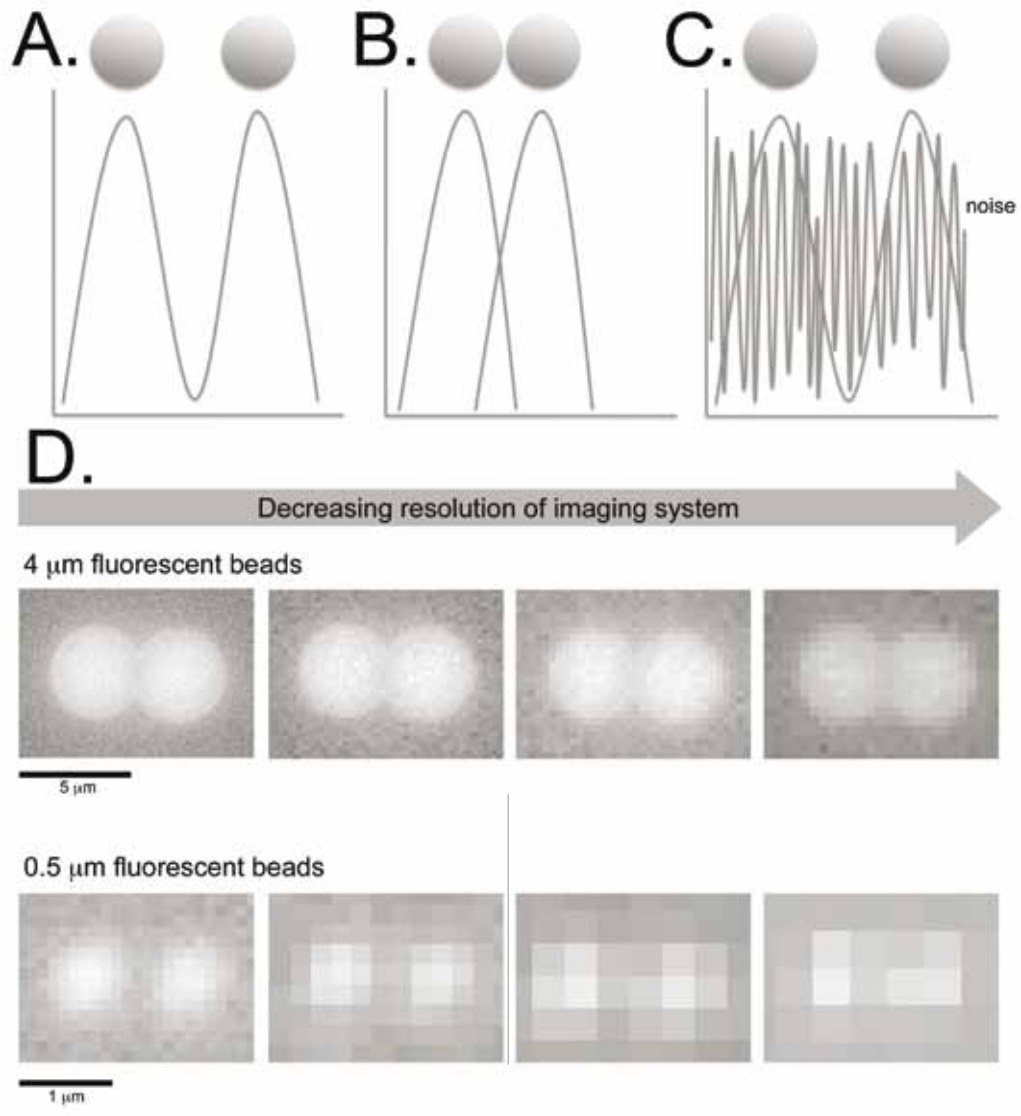

Figure 1. Spatial resolution. The term spatial resolution defines how close two objects can be to each other and still be distinguishable by a particular imaging system based on the signal they generate. In Panel A the two signals are "resolved" as spatially distinct, whereas in Panel B, although the objects do not overlap, the signals generated by the objects do overlap, and we are thus unable to resolve these 2 objects with our imaging system. A low signal:noise ratio compounds the difficulty of resolving two distinct signals (Panel C). Panel D demonstrates how decreasing resolution of the system in combination with a low signal:noise ratio has a more significant impact on the ability to resolve small structures (0.5 μm beads, bottom) compared to larger structures (4 μm fluorescent beads, top).

enlarged, however, the fewer individual pixels that describe it now become evident (image becomes "pixellated"). We extend this same concept to a more complicated digital image in Panel C, which shows a photograph taken with a 1 megapixel (Mpx) mobile phone camera. When a region of the image is digitally enlarged using Photoshop, it looks bigger (so it is magnified), but details cannot be clearly distinguished because the comparatively large pixels in the camera provide poor spatial resolution. Panel D shows a photo taken of the same scene using a digital single-lens reflex (SLR) camera with a 10 Mpx chip. Now, when the same region of the image is digitally enlarged, fine details are more readily discerned because a significantly larger number of pixels describe the same image. In other words, we now have magnification and resolution. Panel E shows the same original image and expanded region as in Panel B, however the contrast has been dramatically reduced. We know that the magnification and resolution are identical, but it is now difficult to make use of them because of the lack of sufficient contrast.

Until the recent introduction of super resolution microscopy techniques such as PALM (Photo-Activated Light Microscopy), STED (Stimulated Emission Depletion Microscopy) and structured illumination, the design of light microscopes had improved to the point where resolution in high quality instruments was generally limited by the wavelength of light (with resolution limited to about half the wavelength of light used) rather than the optical components. Abbe's equation is used to determine the theoretical resolution of an optical instrument and states that for fluorescence microscopy:

$$\text{Resolution} = \lambda\ 0.61 / \text{NA}$$

λ = wavelength of light
NA = numerical aperture of the lens

Numerical Aperture is in turn dependent on the angle of acceptance of the lens (Figure 3A) and on the optical index of the media (Figure 3B).

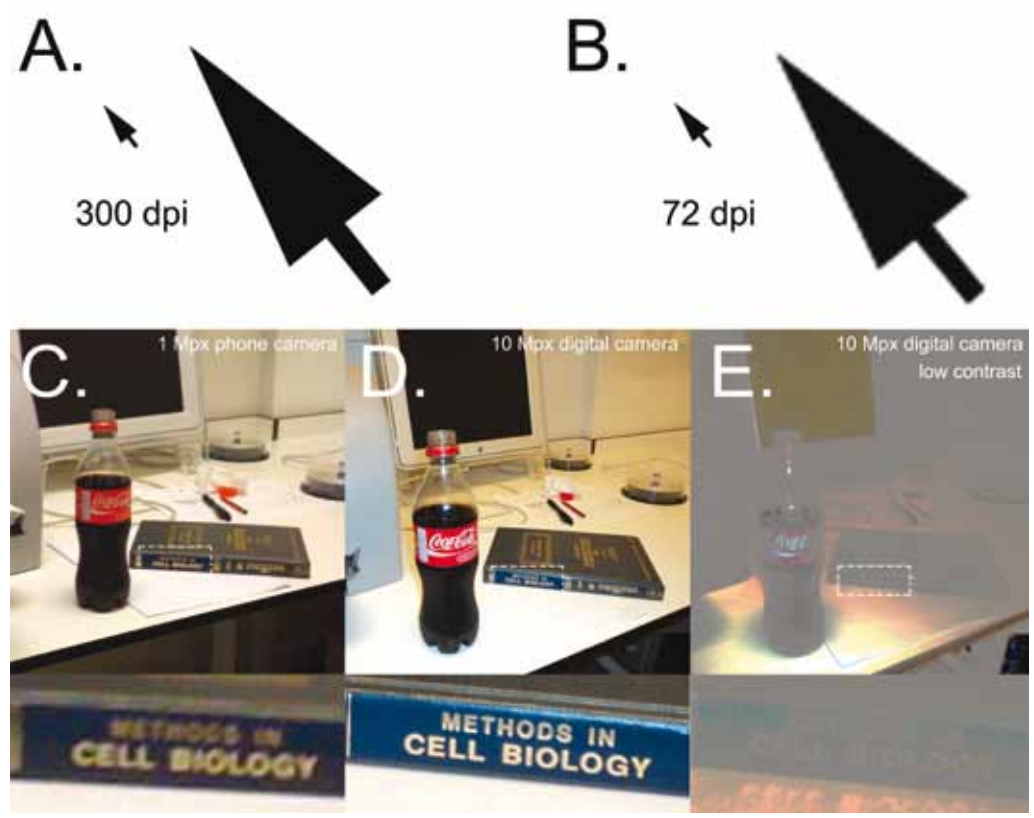

Figure 2. Magnification, resolution and contrast are overlapping properties. Digital resolution, normally expressed as "dots per inch" (dpi), determines how many independent points describe an image. For example, the small arrows shown in Panels A and B look identical, but are described by 300 dpi and 72 dpi, respectively. This difference in resolution becomes apparent when the arrows are stretched to a larger size, as the 300 dpi arrow remains crisp and well-resolved whereas the 72 dpi arrow becomes fuzzy and "pixelated". We can extend this concept to a digital camera capturing a particular field of view as an image. Panel C shows a photograph taken using a 1 megapixel (Mpx) mobile phone camera. A region of the image (hashed rectangle) was digitally enlarged in Photoshop. Although magnified, the resolution of this enlarged region is poor because the pixel size of the camera is relatively large. Panel D shows a photograph of the same scene taken using a digital SLR with a 10 Mpx chip. Now when the same region is digitally enlarged, fine details are retained, because this region is described by 10-fold more pixels. This demonstrates that we now have both magnification and resolution. Panel E shows the effect of decreasing the contrast of the image shown in Panel D: magnification and resolution are the same, but they are useless without contrast.

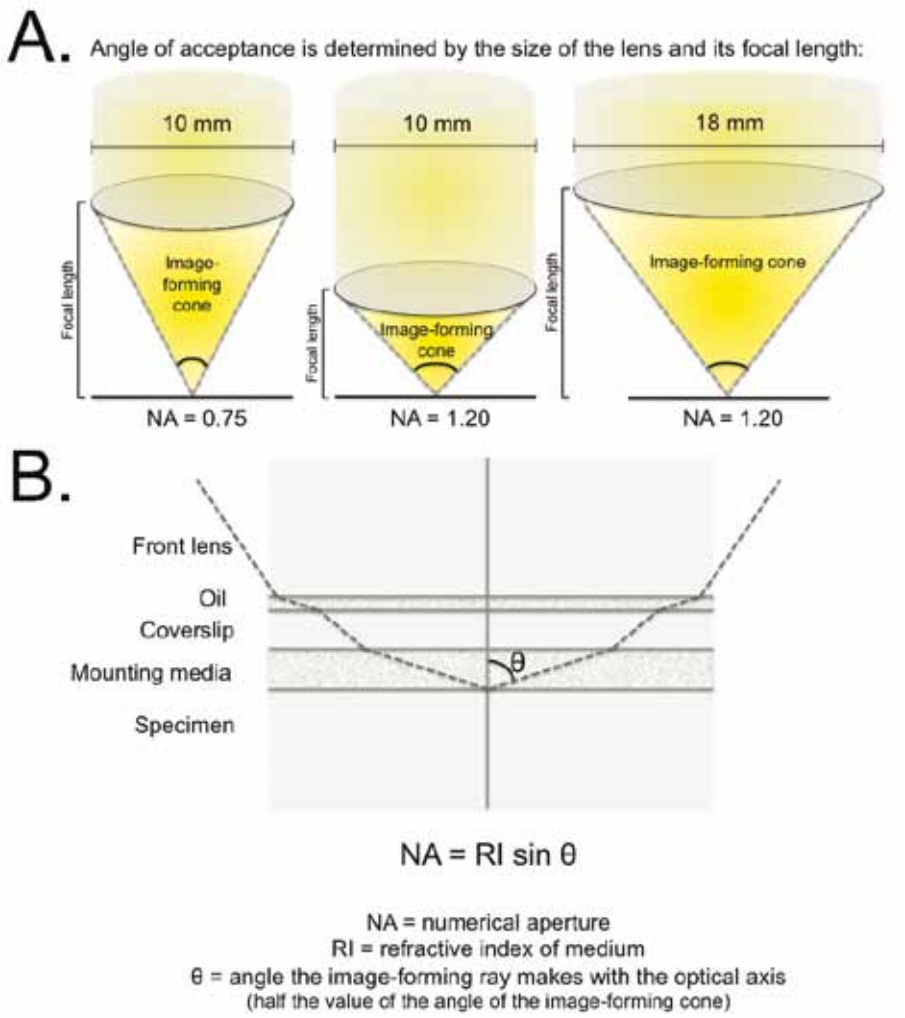

Figure 3. Numerical aperture (NA) is a measure of an objective's ability to gather light and resolve detail at a fixed distance. As defined by Abbe's Equation, resolution is a function of both the wavelength of light and the NA of the lens. The NA is in turn a function of the angle of acceptance of the lens (i.e. angle of the image-forming cone). As shown in Panel A, this value is determined by the size of the lens and by its focal length. Mapping a ray path from a covered specimen in mounting media to an oil immersion objective illustrates the dependence of NA on both the angle the image-forming ray makes with the optical axis (θ) and the refractive index of the medium (B). As θ can clearly never be larger than 90°, and the sine of 90° is 1, no objective can have an NA numerically larger than the RI of the medium. And in practice, as the largest angle of acceptance of a glass lens in any medium is ~140° (rather than the predicted 180°); the NA of an objective is always less than the theoretical maximum.

These two factors, combined with the wavelength of light, determine the theoretical resolution of a microscope.

To give an example, if we have a good quality lens with an NA of 1.2 and we image blue fluorescence with a wavelength of 500 nm, the best lateral resolution we can expect from the optics of the microscope is about 250 nm. If we image red light at 600 nm, our lateral resolution is ~300 nm. The take home message is that the resolving power of the microscope has nothing to do with magnification and everything to do with the wavelength of light and the numerical aperture of the lens.

In practice, a good way to measure resolution with the light microscope is to use fluorescent spheres or "beads" of a known size and to measure at what point you can distinguish individual beads. Figure 1 illustrates this point and, furthermore, demonstrates the deleterious effect that poor contrast (i.e. signal:noise) can have on resolution.

Having thought about the optical resolution of our light microscope, we can now consider digital resolution. Often, microscope users are primarily concerned with the NA of the lens (i.e the resolution of the optics), but we also need to think about the pixel size of the digital capture device (typically a CCD [charge-coupled device] camera) that will be used to record images. This is exactly the same principle as discussed in Figure 2, where we compared a 1 Mpx phone camera image to a 12 Mpx SLR image. Although a lens may have high numerical aperture and the potential to resolve structures to as little as 250 nm, if the pixels on your CCD camera are large (for argument's sake, 1 cm each), it doesn't matter that the lens can provide a resolution of 250 nm because the camera will only provide 2-3 cm resolution. It is also important to recognise that the pixel size on the camera needs to be several times smaller than the maximum resolution of the microscope. The reasons for this are explored in Figure 4.

Chip use and file size

As a consequence of this, most digital capture devices contain a large chip composed of numerous small pixels in order to take advantage of the maximum optical resolution and provide a useful field of view. Provided that the pixel size of the chip is sufficiently small relative to the lens that you're using, however, it is not always necessary to use the full area of the chip (see Figure 5). For example, collecting a 1024 x 1024 image on a 1024 x 1024 chip (a common size) utilises the full area of the chip (i.e. every available pixel; Figure 5A). If you're only interested in a single cell in the middle of the field of view, however, you don't need to capture the entire field of view. A file collected at 512 x 512 has the same resolution but only uses a quarter of the chip and thus only collects pixels from the centre of the field of view (Figure 5B). The resulting file size for a z-stack of 20 images of this field of view is significantly smaller (e.g. 19 Mb vs. 76 Mb). And if you're only interested in a small region of the single cell at the centre of the field of view, a z-stack file collected at 256 x 256 again has the same resolution, yet only uses 1/16 of the area of the chip, collecting pixels from a smaller region of the field of view and generating a much smaller file size (4.8 Mb; Figure 5C).

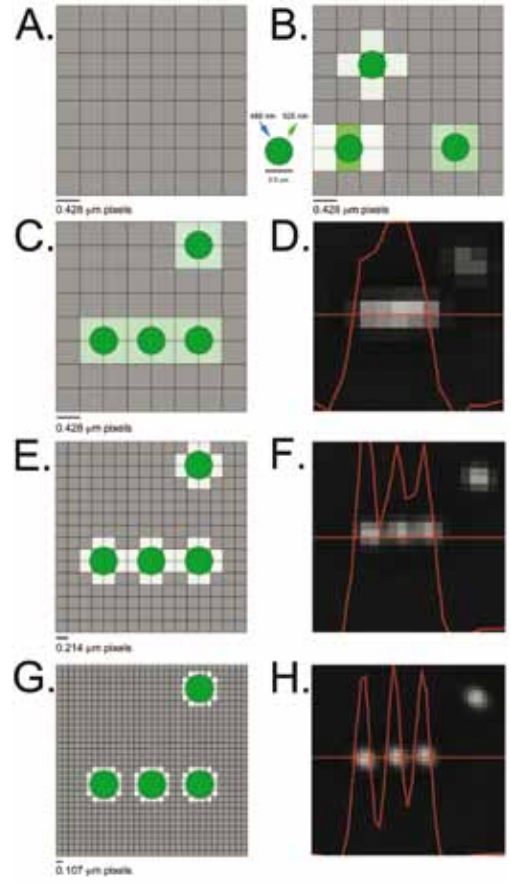

Figure 4. Relationship between pixel size and spatial resolution. A. The 8 x 8 grid shown here represents a CCD chip with a pixel size of 0.428 µm, and in this case we are imaging fluorescent beads (excited with 488 nm light and emitting light at 525 nm) with a diameter of 0.5 µm. Although it seems sensible to have a pixel size similar to that of the objects to be measured, we can see here that this is not the case. Panel B shows three possible fluorescence intensity patterns that might be observed using this combination, depending on how the signal from the beads (i.e. photons emitted from the fluorophore) is projected onto the pixels of the CCD chip (intensity increases from light green to dark green). In this case the beads are sufficiently far apart to be resolved. If we look at the bead layout in Panel C, however, the bead in the top right corner can be resolved from the others, but the three beads in the center are too close to be resolved as three independent objects using this pixel size (see line profile in panel D). Our resolving capability increases if we decrease the pixel size to 0.214 µm in a 16 x 16 grid (E, F). And even better resolution is obtained if we further decrease the pixel size to 0.107 µm in a 32 x 32 grid (G, H).

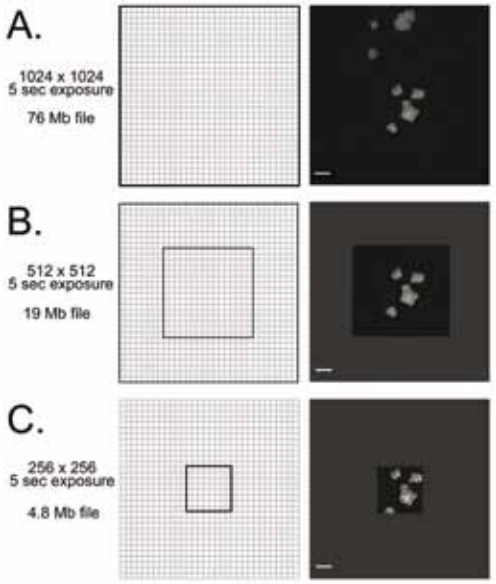

Figure 5. Optimising use of chip size. It is not always necessary to utilise the entire area of the camera chip when collecting images. In this case the region of interest is the centre of the field of view. While this region is included in the image collected in Panel A, there is a large amount of extraneous data and the file size is quite large (76 Mb). The image shown in Panel B uses ¼ of the chip size to collect the same region of interest with the same spatial resolution and signal intensity. The resulting file is significantly smaller (19 Mb). Because the region of interest is so small, it is possible to use even less of the chip (1/16) as shown in Panel C, again retaining the same resolution and signal intensity, with a resulting file size of 4.8 Mb.

The major benefit of smaller file sizes is the ability to store and back up larger numbers of datasets cost effectively, and to reduce the computational time required to process them. A typical live cell imaging experiment might easily involve capturing 20 z-sections every 10 minutes for 24 hours. At a file size of 1024 x 1024, this will generate 10.944 Gb of data. In comparison, at a file size of 256 x 256, only 691.2 Mb of data are generated. Furthermore, if you were using a programmable stage and visiting 20 different fields of view in this time course, the difference is a total of 218.88 Gb of data vs. 13.824 Gb of data. The end result is that we still image the object of interest and at the same resolution, but with a much more manageable data size.

Exposure time and binning

An important factor in imaging is the ability to measure genuine signal above the background signal and noise inherent in the sample and imaging system, respectively. Good contrast is a requisite in exploiting the full resolution of the microscopy system (Figure 6A). Background is non-random signal inherent to the sample (e.g. autofluorescence

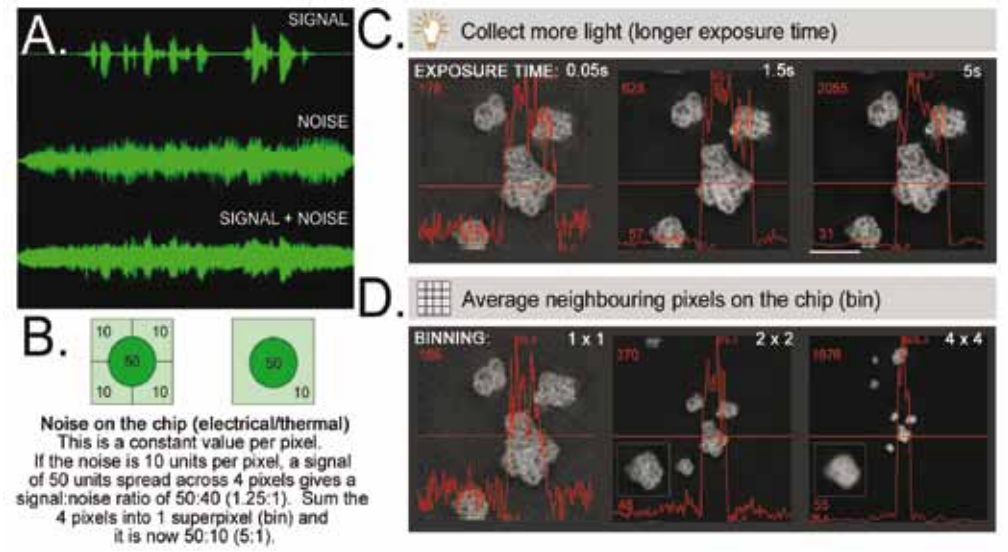

Figure 6. Signal:noise ratio. An important factor in imaging is the ability to sufficiently resolve a signal above the background signal and the noise inherent in the sample and the imaging system respectively (A). Panel B illustrates how the effect of the random electrical/thermal noise on the chip on signal:noise ratio can be minimized by summing neighbouring pixels (binning). Increasing exposure time is one way to increase signal intensity and improve the signal:noise ratio (C), but the increased light exposure is deleterious to the cell. Binning will increase signal intensity (D) without increasing exposure time, however there is a consequential sacrifice in spatial resolution as effective pixel size increases and the same structures are defined by fewer pixels (see inset).

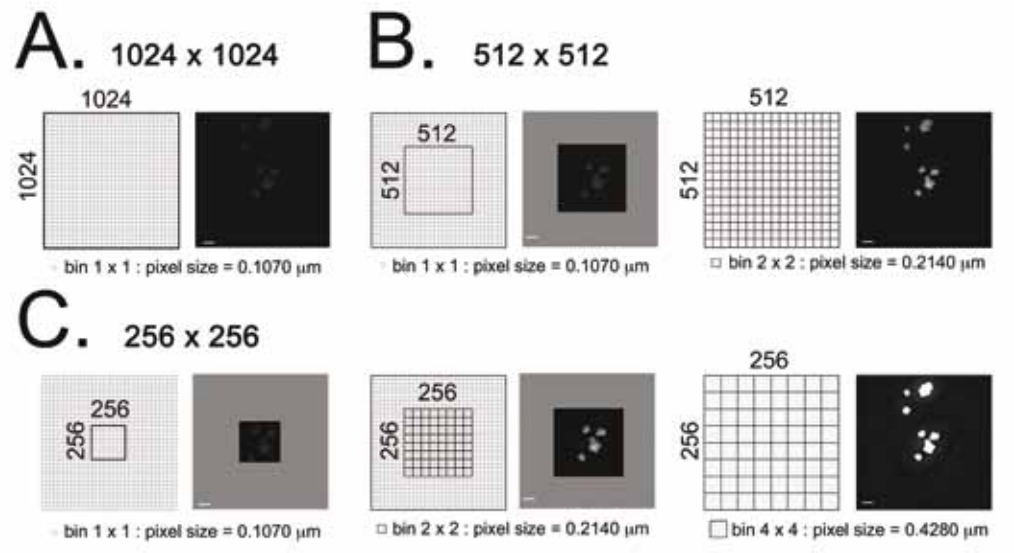

Figure 7. Binning. Signal intensity can be increased without adjusting the exposure time. This is done by combining an area of pixels on the CCD chip in order to create one larger, "superpixel", which now collects light from a larger area of the sample and will thus register a higher signal intensity. Panel A demonstrates the highest resolution and largest field of view that can be collected using this particular CCD chip. Under normal circumstances (i.e. no binning), when we use the entire area of the chip available to us, we can collect light from a total 1024 x 1024 pixels, each of which has an area of 0.107 µm x 0.107 µm. The signal is fairly weak, because each pixel has a small area and is therefore only collecting light from a tiny part of the sample. In Panel B we can see what happens if we use a 512 x 512 area of the chip without binning: we retain the same pixel size and signal intensity, but we are only imaging a smaller region in the centre of the field of view (using ¼ of the area of the CCD chip). If we now bin the chip by combining 2 x 2 areas of pixels to create superpixels, each resulting superpixel is now 4 times the area (0.214 µm x 0.214 µm) of a single non-binned pixel. We can fit 512 x 512 of these superpixels on the chip, and we can again see the entire field of view, albeit at a lower spatial resolution because we are describing the same field of view with fewer pixels. Importantly, we have now increased the signal intensity because each superpixel is collecting light from a larger area of the sample. Similarly, in Panel C we can see the effect of using 256 x 256 pixels with various levels of binning. With no binning (first panel) we're only using 1/16th of the area of the chip and see a limited field of view. When we bin at 2 x 2 (middle panel) we again form superpixels that are each 4x the area of a non-binned pixel, and thus collect more light (note that this uses the same area of the chip as a 512 x 512 resolution image with no binning). We also have the option to bin 4 x 4, i.e. combine 16 pixels into one superpixel with an area of 0.428 µm x 0.428 µm. In this case we're again imaging the entire field of view, however it is only described by 256 x 256 large pixels. Spatial resolution has decrease quite a bit, but signal intensity has increased substantially because each superpixel is collecting light from a much larger area of the sample.

in the media, non-selective labelling, dirt, etc.), it is most easily dealt with experimentally (e.g. change the sample preparation and/or experimental conditions). Noise, however, is due to the collection of randomly generated signal, such as electrical noise across the detector. This is an important difference that we can use to our advantage: if you double the camera exposure time you will collect approximately double the signal from your sample, with only a small increase in noise for any given pixel. Indeed, collecting more light by using a longer exposure time can significantly increase signal:noise ratio, as demonstrated in Figure 6C. In this example, increasing exposure time from 0.05 to 1.5 to 5 seconds steadily increases the signal:noise ratio. If we look at the minimum and maximum values for a horizontal line sampled across the image, the signal:noise ratio increases from 2.7 (0.5 sec exposure) to 11 (1.5 sec exposure) to 66 (5 sec exposure). A drawback of increasing exposure time, however, is the deleterious effect of light on the cells. While this may not always be an issue with fixed cell imaging (assuming photobleaching of the fluorophore is not significant), it is a serious concern with live cell imaging (where phototoxic effects can alter the behaviour of cells or, more often than not, kill them outright). Fortunately, we can exploit another property of the optical system to increase signal intensity without increasing exposure time.

As we have seen, a pixel on a camera chip is a given size, and you can choose the area of the chip that

you use for capturing your image. If you opt to use a smaller area of the chip, you can now take advantage of the excess, unused area on the chip to increase signal intensity. This is done by summing neighbouring pixels into one super pixel in a process called "binning". Importantly, the electrical/thermal noise on the chip is a constant per pixel, and thus will not increase upon binning (Figure 6B). As shown in Figure 6D, binning 2 x 2 (i.e. summing 4 neighbouring pixels) while maintaining the same exposure time significantly increases the signal:noise ratio, and this can be further increased by binning 4 x 4 (i.e. summing 16 neighbouring pixels). The drawback is that although binning can dramatically increase the signal:noise ratio, by definition it also increases the effective pixel size and thereby decreases spatial resolution. Thus, it is more commonly employed to increase the signal:noise ratio under conditions such as live cell imaging, in which it is important to limit the amount of light the cells are exposed to. Some sacrifice in spatial resolution is acceptable in this case, as it is the only way to keep the cells viable.

The concept of binning and the resultant increase in effective pixel size is detailed in Figure 7. When you choose 1 x 1 binning for a digital capture device, you are using the smallest pixel size available on that chip. For example, using our 60X/NA 1.42 objective combined with a decent quality cooled CCD camera, the smallest available pixel size is 0.1070 µm. This pixel size and corresponding signal intensity remain constant no matter what area of the chip you use for imaging: the full size of 1024 x 1024 (Fig. 6A), ¼ of the size - 512 x 512 (Fig. 6B) or 1/16th of the size - 256 x 256 (Fig. 6C). All that changes is the total area of the field of view that is imaged. If we now bin 2 x 2 (i.e. combine the signals collected in 4 adjoining pixels), there is a four-fold increase in the size of each pixel (now 0.2140 µm by 0.2140 µm). We can now only fit 512 x 512 of these superpixels on the chip, as shown in Fig 7B. Importantly, the signal intensity has increased significantly without needing to increase exposure time. Thus, binning can increase signal:noise ratios while minimizing light exposure, with the caveat that pixel size will increase and spatial resolution will necessarily decrease.

Figure 7C demonstrates the maximum binning available on a 1024 x 1024 chip. When an image size of 256 x 256 is chosen, this uses 1/16th of the area of the chip. It is then possible to bin 2 x 2, in which case the resultant pixel size of 0.2140 mm is the same as that for 2 x 2 binning of a 512 x 512 image (Figure 7B). It is also possible to bin 3 x 3 (sum 9 neighbouring pixels with a resulting pixel size of 0.3210mm; not shown), or to use the full area of the chip and bin 4 x 4, summing 16 neighbouring pixels. This maximum binning results in an effective pixel size of 0.4280 mm (Figure 7C). If you compare the resulting image to that taken at 1024 x 1024/ bin with the same exposure time, the field of view that has been imaged is identical; however there is a significant increase in signal intensity. Were you to digitally enlarge this image, however, you would quickly notice the large pixel size and low spatial resolution, much as we did when we compared our 1 Mpx image to our 12 Mpx image in Figure 2.

The trade-off triangle

We have demonstrated that there is a clear trade-off between signal intensity, spatial resolution and temporal resolution in biological imaging. For example, if you are imaging fixed cells under conditions where photobleaching or UV-induced damage is not an issue, your priority may be a strong signal and high spatial resolution, shown at the bottom of the trade-off triangle in Figure 8. HeLa cells stably expressing GFP-tagged PP1γ were imaged at various stages of cell division to map subcellular localisation patterns. In this case we have used the full size of the chip (1024 x 1024) and long exposure times to maximize spatial resolution and signal intensity. Each image is of a different cell, because these conditions lead to mitotic arrest and cell death.

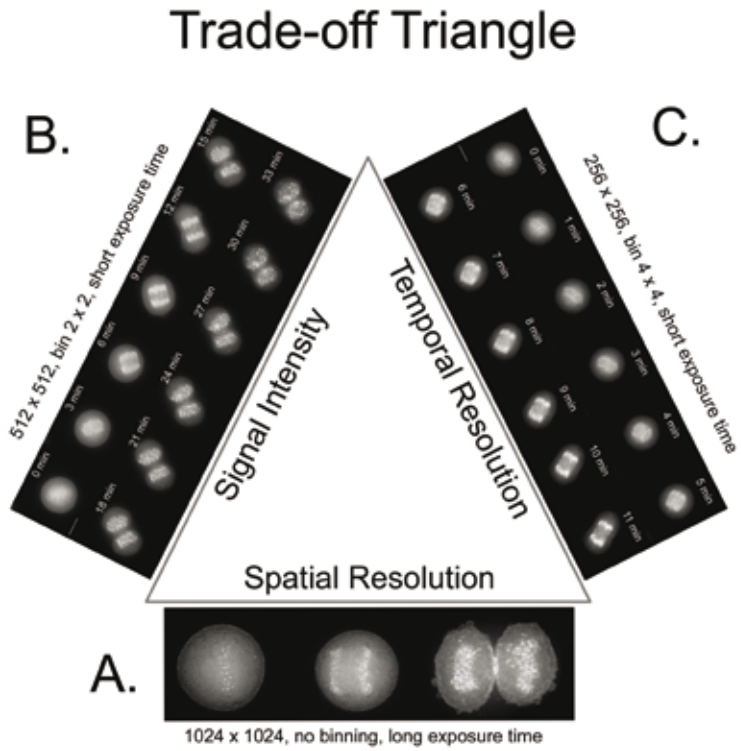

Figure 8. Trade-off triangle. Imaging is a trade-off between spatial resolution, temporal resolution and signal intensity. To demonstrate this, HeLa cells expressing GFP-tagged PP1γ, which exhibits distinct localisation patterns throughout mitosis, were imaged under 3 different conditions: A. 1024 x 1024 with no binning and long exposure times; B. 512 x 512, bin 2 x 2, shorter exposure times and Z-stacks collected every 3 min; C. 256 x 256, bin 4 x 4, even shorter exposure times and z-stacks collected every 1 min. Panel A demonstrates the highest spatial resolution and signal intensity that can be obtained by imaging separate cells, when photobleaching/UV damage from prolonged light exposure is not an issue. The time-lapse series in Panels B and C show how spatial resolution must be sacrificed in favour of both signal intensity and time resolution in order to maintain cell viability (in this case, following the same cell throughout mitosis without activating mitotic checkpoints by UV-induced DNA damage).

When the main priority is that the cell survives, for example when you want to image the same cell over time, there is a necessary compromise of signal intensity and/or spatial resolution in order to acquire images at an appropriate rate and maintain the viability of the cells. Cellular events are dynamic, and therefore it is important to image cells with an appropriate time resolution to fully capture the events in which you are interested. For example, if a CCD camera mounted in a car park takes an image of the car park every 5 min, you might see that a white VW Golf was damaged at some point between 1:05 p.m. and 1:10 p.m., but if the incident happened at 1.07 p.m. you may not know what actually caused this. If our temporal resolution were better, e.g. we acquired an image every 10 seconds, we might see that another car ran into the Golf at 1.07 p.m. and drove off at 1.09 p.m. If we also had sufficient spatial resolution, we might have learned what make and model of car hit the Golf, its number plate and various physical attributes of the driver, which could prove more useful.

With respect to cellular imaging, however, we have demonstrated the trade-off between time and spatial resolution and signal intensity with two examples. On the left-hand side of the triangle we selected a 512 x 512 image (1/4 of the chip) and then binned 2 x 2 (summing 4 neighbouring pixels). We were then able to use a shorter exposure time to successfully image the same HeLa $^{GFP\text{-}PP1\gamma}$ cell through mitosis, from metaphase into late telophase. The signal:noise ratio was sufficiently high and we were able to collect 5 z-stack images every 3 min without damaging the cell (which would have activated the mitotic checkpoint and caused cell division to arrest).

We did lose some spatial resolution, however, as the effective pixel size increased to double the minimum pixel size of the system.

When we required higher time-resolution, i.e. 5 z-stack images every 1 min, it was necessary to increase binning to achieve the required signal intensity with an even shorter exposure time, because the increased number of images taken increased the total light to which the cell was exposed. This is demonstrated on the right-hand side of the triangle, where we again prioritised time resolution by choosing a 256 x 256 image with 4 x 4 binning. This necessarily decreased spatial resolution even further, with an effective pixel size four-fold greater than the minimum pixel size of the system.

In summary, we have demonstrated that a microscope is required to provide magnification (so that we can see small objects), resolution (so that we can distinguish them from each other) and contrast (so that we can distinguish them from background noise). Understanding these parameters and how they interact allows us to systematically design experiments that prioritise the most important results we require. This may involve some degree of compromise. For example, when we don't require temporal resolution (e.g. for fixed cell imaging with a stable fluorophore), we can prioritise spatial resolution and contrast. In live cell imaging experiments, however, we may need to compromise spatial resolution in order to obtain sufficient temporal resolution. Clever use of the signal intensity/spatial resolution/temporal resolution triangle in the design of imaging experiments can therefore help to maximise your success.

Sam Swift, s.swift@dundee.ac.uk

Head of Imaging, College of Life Sciences, University of Dundee, UK

Sam Swift directs the College of Life Sciences Imaging Facility at the University of Dundee, a multi-user facility for College and External research staff hosting Electron Microscopy and Advanced Light Microscopy including Structured Illumination (OMX), MPLSM, CLSM, TIRF, FLIM and Deconvolution. Please see http://microscopy.lifesci.dundee.ac.uk/ and http://microscopy.lifesci.dundee.ac.uk/omx/ for more details.

Laura Trinkle-Mulcahy

Department of Cellular & Molecular Medicine and Ottawa Institute of Systems Biology, University of Ottawa, Ottawa, ON, Canada

Laura is an Assistant Professor at the University of Ottawa in Canada, who combines live cell imaging and quantitative proteomics to map targeted protein phosphatase activity in human cells.

Acknowledgments

We thank our colleagues in the Trinkle-Mulcahy Laboratory and the Light Microscope Facility at the University of Dundee for helpful discussions and suggestions. Work in the Trinkle-Mulcahy lab is funded by a Canadian Cancer Research/Terry Fox Foundation New Investigator Award (Ref: 20148) and an NSERC Discovery Grant (Ref: 372370), and LTM holds a Canadian Institutes of Health Research New Investigator Salary Support Award.