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Article

Adding an Extra Dimension to What Students See through the Light Microscope: A Lab Exercise Demonstrating Critical Analysis for Microscopy Students

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This article describes an undergraduate lab exercise that demonstrates the importance of students thinking critically about what they see through a microscope. The students are given growth data from tip-growing organisms that suggest the cells grow in a pulsatile manner. The students then critique this data in several exercises that incorporate aspects of a problem-based learning approach, envisaging growth not just in two dimensions, but in three dimensions. For some cells, what appears to be pulsatile growth could also be explained by growth at a constant rate up and down in the *z*-axis. Depending on the diffraction pattern generated by the tip of the cell, this movement in the *z*-axis could go undetected. This raises the possibility that pulsatile growth seen in some species may be an artifact generated by the limitations of the light microscope. Students were subsequently asked to rate their awareness of the need to think critically about what they see through a microscope, using a scale of 1 (unaware) to 5 (very much aware). Prior to doing the lab exercise, the mean rating was 2.7; this increased to 4.4 after the lab. The students also indicated a likelihood of being more critical in their thinking in other aspects of their biology curriculum.

INTRODUCTION

A key skill for scientists is the ability to look at experimental data critically. Indeed, critical thought is crucial in all facets of our professional activities. For cell biologists, this need is highlighted by the British Society for Cell Biology statement that "Good scientists working in cell biology are multi-skilled astute observers ... they can think critically, creatively and laterally and use their imagination" (www.bscb.com). Thus, when training the next generation(s) of cell biologists, instructors need to increase student awareness of the need for critical thought.

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This article describes a lab exercise that can be used either as part of a lab course or as a tutorial exercise in which students are given growth rate data for three cell types that extend by the process of tip growth. The cell types are a fungal hypha, an oomycete hypha, and a pollen tube. The students are asked to critically evaluate the data using aspects of a problem-based learning (PBL) approach (White, 1996). Active discussion among students is encouraged, with the lab instructor/lecturer essentially acting as a facilitator of that debate. During this analysis, students should become aware of the possibility that what a microscopist observes through a microscope may not truly reflect reality.

The lab exercise is part of a second-year undergraduate cell biology course that comprises 24 lectures and six 3-h labs. It is the second of two labs specifically concerned with microscopy. The first lab gives the students hands-on experience with Olympus BH2 microscopes and demonstrates Kohler illumination, the limit of resolution, refractive index, polarization, and fluorescence. Previous to this, in their first year, students at the University of Canterbury used microscopes to observe cells and were told about focal planes and depth of field and how the image that is seen can be affected by where the microscope is focused in relation to the cell being viewed.

METHODOLOGY

Introduction to Tip Growth

Students are initially given a lecture that introduces them to the process of tip growth and the cell types that extend by this process. These include the pollen tubes and root hairs of plants, hyphae of fungi and oomycetes, algal rhizoids, and growth cones of nerves in animals. All of these cells are tubular and polarized, with growth occurring at the very tip of the cell. Such localized growth involves wall-yielding at the site of growth and the delivery of vesicles that contain new membrane and wall material (for walled cells) to this site. Concomitant with this is the movement of cytoplasm and organelles to retain the polarized nature of the cell. The process can be demonstrated to the students using videos of tip-growing cells (e.g., see Robinson [2005] or Fungal Cell Biology Group [2011]).

The students are told how tip-growth rates are measured in a research lab. This is done by first focusing the objective lens of a light microscope on the median focal plane of the cell. Because of the shape of the cells (cylinders with hemispherical or semiellipsoid tips), the tip is approximately in focus. The distance this tip travels over a particular period of time along the *x*-axis is measured, and the growth rate is calculated. Through the use of video-enhanced, phase-contrast microscopy, it is possible to detect increments of growth as small as 0.03 μm over 1–5 s in the research lab (Lopez Franco *et al*., 1994), which has enabled the detection of pulsatile growth (Tang *et al*., 1992; Lopez Franco *et al*., 1994; Pierson *et al*., 1995). However, Jackson (2001) argued that some instances of pulsatile growth could be artifacts generated by the cells growing up and down in the *z*-axis of a microscope's field of view. The lab exercise described here is based on this critique.

Exercise 1. Growth Data

In the lab exercise, students are first given growth data from three types of tip-growing cell: 1) a hypha of the oomycete *Saprolegnia ferax,* 2) a hypha of the fungus *Trichoderma viride*, and 3) a pollen tube from *Lilium longiflorum.* They are asked to graph growth rate against time for each cell type and, after discussion among themselves, to describe what the graph tells them. An example of the plots they obtain are shown in Figure 1. Students should recognize that growth rates clearly oscillate between faster and slower rates and that growth can be described as pulsatile. The pulses vary in their frequency and amplitude among the three species, but the faster a species grows, the greater the amplitude of the pulses. The students then start a series of exercises that enable them to critique the growth data.

Exercise 2. Critiquing the Growth Data

Part A. Thinking about Growth in Three Dimensions. When a microscopist looks through a microscope, he/she sees a two-dimensional image of the cell and the space through which the cell is growing. Growth would typically be measured along the *x*-axis (i.e., across the field of view) and the *y*-axis (i.e., from the bottom to the top of the field of view). In reality, however, cells and the space through which they grow are not two-dimensional; there is a third dimension, or *z*-axis.

To begin the critique, the students are asked to think of additional directions in which a cell could be growing and how this might affect what is seen through the microscope.

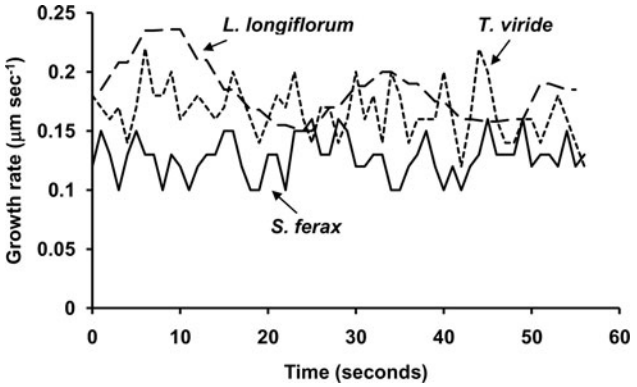

Figure 1. Growth rate data for hyphae of the oomycete *S. ferax* and the fungus *T. viride* and pollen tubes of *L. longiflorum*. Growth appears to occur in a pulsatile manner. Data have been sourced from Figure 2 of Lopez Franco *et al*. (1994; hyphae) and from Figure 1 of Holdaway-Clarke *et al*. (1997; pollen). The plots represent ideal results.

What they often overlook is the fact that a cell could also grow in the *z*-axis (i.e., up and/or down in the field of view). This is important because it can affect the observed growth rate, as demonstrated in Exercise 2B. If a cell is growing up or down in the *z*-axis, this can be observed, as the tip will move out of focus. However, the shape of the cell affects the tip, causing it to move a certain distance in the *z*-axis without any change in focus. The first stage in critiquing the growth data is to calculate this distance. To do this, the students need to consider the limit of resolution and the cells as three-dimensional objects.

When a microscope is focused on the tip of a cell, the observer does not see the tip itself, but the diffraction pattern generated by the tip, as light is scattered by the edge of the cell. Abbe's equation derives the width of this pattern ($d_{\min}$) in terms of the wavelength of the light (λ) and the numerical aperture (NA) of the objective lens, itself the product of the index of refraction (η) and the sine of the half-angle between the axis of illumination and the edge of the objective lens, that is:

$$d_{\min} = \lambda/2\mathrm{NA} \quad (1)$$

The students have been taught this equation and what it means in a previous lab and so are just reminded of it in this lab exercise. In Exercise 1, a lens of 1.25 NA illuminated with light of 550 nm, gives a diffraction pattern with a width of 0.22 μm.

The students are then given published images of the tips of the three species, with the cells being viewed from above (Figure 2A). The next part of the critique requires the students to envisage what the cells look like from the side (in doing this they are starting to think in three dimensions, rather than two). Given that the cells approximate to cylinders with hemispherical or semiellipsoid tips, an assumption is made that a side-on view of these cells (i.e., from the perspective of the *x*- and *z*-axes) is the same as the view from above (i.e., from the perspective of the *x*- and *y*-axes). The students are asked to draw the tips from a side-on view, with the diffraction pattern (drawn to scale) around the tip.

They do this by tracing around a tip (as shown in Figure 2B) and then reproducing their tip shape on a plot of the *x*- and *z*-axes (as shown in Figure 2C). The diffraction pattern is drawn with additional lines that are 0.11 μm from

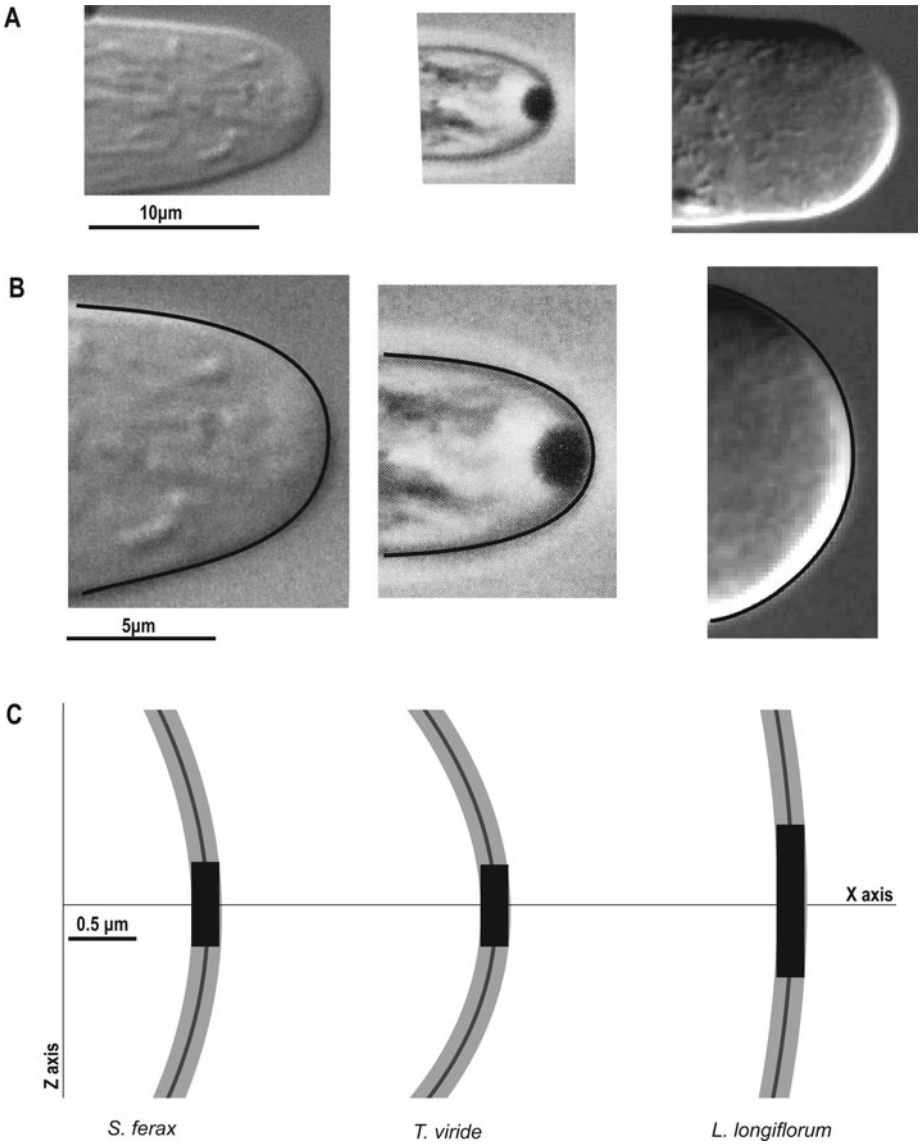

Figure 2. Micrographs (from left to right) of the tips of *S. ferax*, *T. viride*, and *L. longiflorum* as observed through a light microscope using differential interference–contrast or phase-contrast optics (A). In Exercise 2A, students draw a line around the tip as shown in (B) and then enlarge and reproduce the drawing to show the shape of the tip, as viewed from side-on (i.e., the tip is plotted with respect to the *x*- and *z*-axes) (C). The line represents the actual tip; the gray shading shows the diffraction pattern around the tip. The heights of the rectangles at the tip represent the distance the tip could move in the *z*-axis without affecting the diffraction pattern. The plots in (C) represent ideal results. (C) is based on Figure 4 from Jackson (2001). *S. ferax* micrograph courtesy of Prof. R. Lew (York University, Toronto, Canada), *L. longiflorum* micrograph courtesy of Prof. P. Hepler (University of Massachusetts, Amherst, MA) and Dr. A. Lovy-Wheeler (Tufts University, Boston, MA). The image of *T. viride* is reproduced with permission from Lopez-Franco *et al*. (1994); © 1994 National Academy of Sciences.

the inside and outside of the tip (the diffraction pattern is shown by the shading in Figure 2C). From their drawings, it is possible to approximate how far a tip would need to move in the *z*-axis before the diffraction pattern would change (and hence before that movement could be detected through the microscope). This can be done by drawing a box at the tip that has a width slightly less than the width of the diffraction pattern (as indicated in Figure 2C). This box then needs to be extended both up and down in the *z*-axis until the corners at the tip-most edge reach the limit of the diffraction pattern (as shown in Figure 2C, the tips of the boxes reach the edges of the shading). The height of this box at this stage represents the distance the tip could move without any change in the diffraction pattern. Using their scale bar, the students can measure this distance and should obtain distances of approximately 0.6 μm for *S. ferax*, 0.62 μm for *T. viride*, and 1.1 μm for *L. longiflorum*.

The ability to detect movement in the *z*-axis is further influenced by the depth of field of the microscope lens. Anything within this section will appear in focus; thus, a cell can grow within this section and still appear in focus (and that growth will therefore go undetected). For the growth data described in Figure 1, the depth of field is 0.41 μm, which should be added to the distances calculated (*S. ferax*: 0.6 μm; *T. viride*: 0.62 μm; *L. longiflorum*: 1.1 μm). The depth of field is calculated as: $\lambda/4\text{n}\sin^2(\theta/2)$, where λ is the wavelength of light used, n is the refractive index of the immersion medium, and θ is the half-angle of acceptance for the objective lens, as described by Inoue (1986). As for Abbe's equation, the students have been taught this in the previous lab and are just given values. The addition of 0.41 μm gives final distances of 1.01 μm for *S. ferax*, 1.03 μm for *T. viride*, and 1.51 μm for *L. longiflorum*. These distances will be used later in Exercise 2C.

Part B. Growth in the x- and z-Axes. The students then consider what effect growth in the *z*-axis might have on the growth rate measured by a microscopist looking through the microscope. They are asked to envisage two cells, one

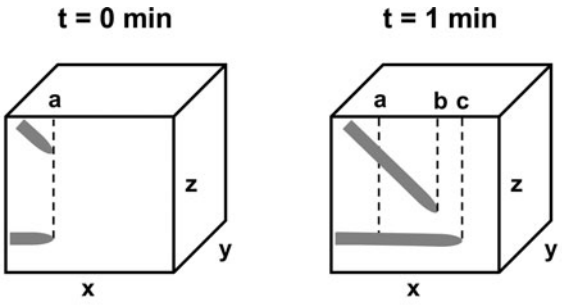

Figure 3. Two tip-growing cells growing at different angles through the field of view. The top cell is growing at an angle of 45° relative to the bottom cell (which is growing horizontally). Both cells are growing at the same rate and after 1 min of growth will have grown the same distance. To the observer looking through the microscope, the top cell appears to have grown a shorter distance (i.e., from a to b) than the cell growing horizontally (which would appear to grow from a to c). The top cell therefore would appear to be growing at a slower rate. The figure represents ideal results.

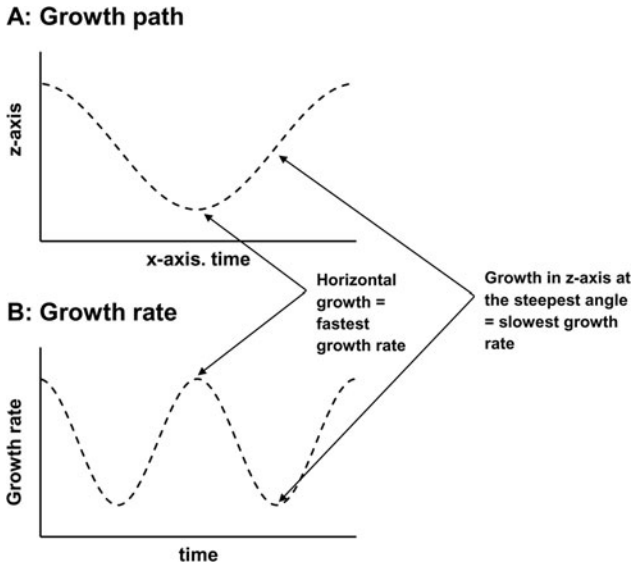

Figure 4. The relationship between a sinusoidal growth path (A) and growth rate (B). As illustrated in Figure 4, horizontal growth (i.e., at the top or bottom of the sine curve) would appear to be faster than growth at an angle through the *z*-axis (i.e., midway between the top and bottom of the sine curve). (B) The figure represents ideal results.

of which is growing horizontally (i.e., along the *x*-axis) and the other of which is growing downward at an angle of 45° relative to the first (i.e., along both the *x*- and *z*-axes; Figure 3). The cells grow for 1 min at a constant rate of 12 μm/min; both of these cells will therefore grow an actual distance of 12 μm. Looking through the microscope, the microscopist would see a two-dimensional image and, as illustrated in Figure 4, would observe that the cell that was growing downward had grown a shorter distance than the cell that was growing horizontally. Rather than the 12 μm that it had actually grown, the cell that was growing downward would appear to have grown only 8.4 μm, and it would appear to be growing at a slower rate (8.4 μm/min). If this growth downward were not sufficient to move the tip out of focus, it would be undetected (and the microscopist would falsely assume the cell was growing horizontally). The apparent growth rate of 8.4 μm/min would appear to be the actual growth rate.

In this exercise, students are asked to come up with a means of working out the apparent growth rates for the two cells. They are given the directions in which the cells are growing and an actual growth rate of 12 μm/min. A simple way of doing this is for them to make drawings, as shown in Figure 3. It is not the values here that are important, but the principle that the direction of growth can affect what the microscopist observes.

This line of thinking is then expanded by considering a cell that has a more complicated growth path, one resembling a sine curve when plotted relative to the *x*- and *z*-axes (Figure 4A). Such patterns of growth are possible, given reports of helical growth by tip-growing cells (Kaminskyj and Heath, 1992). The students are given the plot shown in Figure 4A and are asked to plot the growth rate a microscopist would observe against time. To do this correctly, they need to have understood the principle discussed in the previous two paragraphs. The axes for this exercise are given to the students directly below the plot of the growth path, such that the growth rates they plot relate to a specific part of the growth path (as shown in Figure 4). The growth rates are shown in relative terms (i.e., faster or slower) and not with actual values (Figure 4B). What the students should learn from this is the cell is growing at a constant rate, but the direction of growth up and down in the *z*-axis gives the impression of pulsatile growth (as shown in Figure 4).

Part C. Are the Cells Growing in a Pulsatile Manner? The students then return to the growth data they have plotted in Exercise 1 and critique them, using some of the principles they have learned in Exercise 2B. They are asked to hypothesize that the pulsatile growth is not real and may, in fact, be an artifact of the cells growing in a sinusoidal manner. To test this hypothesis, they first need to calculate the distance the tips would have to move in the *z*-axis to generate the impression of growth pulses.

The students are asked to come up with a way of calculating this distance using Pythagorean geometry (a gentle reminder of Pythagoras's theorem is helpful at this stage). To do this, they need to consider the schematic representation of growth shown by the top cell in Figure 3. They should come up with the scheme shown in Figure 5, and thus need to work out the apparent (i.e., observed) and true (i.e., real) distances grown by a cell growing at an angle in the *z*-axis.

The apparent distance grown and the true distance grown can be calculated using the growth data from Exercise 1. The students need to figure out that the true distance grown is equal to the product of the pulse length (average time between peaks of fastest growth from Figure 1) and the maximum speed (the actual value of the fastest growth from

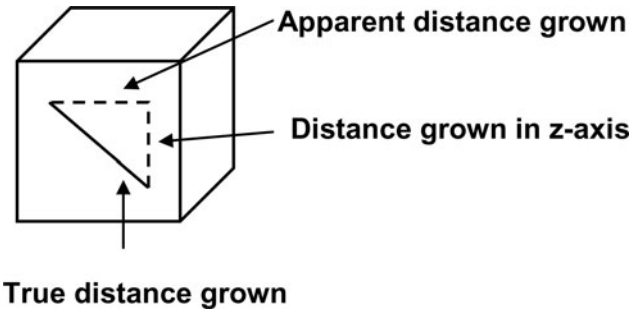

Figure 5. The distance grown in the *z*-axis is estimated by Pythagorean geometry using the apparent distance grown and the true distance grown, as indicated.

Figure 1). From their plots of growth rates, as shown in Figure 1, they should obtain approximate pulse lengths of 4 s for *T. viride*, 7.3 s for *S. ferax*, and 30 s for *L. longiflorum*. Maximum growth speeds are 0.21 μm/s for *T. viride*, 0.16 μm/s for *S. ferax*, and 0.24 μm/s for *L. longiflorum*. These give true distances grown of 0.84 μm for *T. viride*, 1.17 μm for *S. ferax*, and 7.2 μm for *L. longiflorum*.

The students should also figure out that the apparent distance traveled is the product of the pulse length and the average speed (this is a weighted average). The average speeds are 0.16 μm/s for *T. viride*, 0.13 μm/s for *S. ferax*, and 0.19 μm/s for *L. longiflorum*; these give apparent distances grown of 0.64 μm for *T. viride*, 0.95 μm for *S. ferax*, and 5.7 μm for *L. longiflorum*.

The distances the cells would have to grow in the *z*-axis can then be calculated using Pythagoras's theory, as follows:

$$\text{Distance in z} - \text{axis}^2 = \text{true distance grown}^2 - \text{apparent distance grown}^2 \quad (2)$$

Thus, the distances that the cells would need to move vertically in the *z*-axis to generate artifactual growth pulses are 0.59 μm for *T. viride*, 0.68 μm for *S. ferax*, and 4.4 μm for *L. longiflorum*.

Once the students have calculated these distances, they compare them with the values they calculated in Exercise 2A and are asked to come to a conclusion with respect to their hypothesis. If the distance in the *z*-axis is more than the sum of the depth of focus and the shape-dependent depth for no change in the diffraction pattern at the tip, then the hypothesis above can be rejected. In such an instance, the students can be confident that these cells are growing in a pulsatile manner, though perhaps not as extremely as may appear. If the cells were growing at a constant rate with a sinusoidal growth path, the distance the cells would need to grow in the *z*-axis to give the appearance of pulsatile growth is far greater than the distance these cells can move undetected in the *z*-axis. So, if they were growing with a sinusoidal growth path, the tip would move out of focus. If a microscopist were to observe the tip staying in focus when measuring growth rates, he/she could be confident the cells were in fact growing horizontally and in a pulsatile manner. In contrast, if the distance is less than the sum of the depth of focus and the shape-dependent depth for no change in the diffraction pattern at the tip, then the hypothesis cannot be rejected. These cells could generate the appearance of pulsatile growth by growing in a sinusoidal manner and still remain in focus. In this instance, it is not possible to unequivocally state that these cells are growing in a pulsatile manner, as the changing growth rates could simply be the tips growing up and down. From their calculations, the students should conclude that *L. longiflorum* is growing in a pulsatile fashion, but pulsatile growth of *S. ferax* and *T. viride* could be explained by sinusoidal growth at a constant rate due to movement in the *z*-axis.

At the end of this, and in the final summing up of the lab exercise, the lab instructor explains to the students that their critical evaluation of the data provides a stronger argument for pulsatile growth in *L. longiflorum*. Importantly, it is also stressed that their critique does not disprove pulsatile growth in *S. ferax* and *T. viride.* Instead, they are providing another possible explanation for the observation of growth pulses. Whether or not growth pulses occur in all tip-growing species is still an area of debate among researchers (Sampson *et al.*, 2003), although, as discussed by Money (2001), given the complex nature of the growth process, it is difficult to imagine that growth rates never vary.

RESULTS AND DISCUSSION

As detailed earlier, the aim of this lab exercise was to increase students' awareness of the need to think critically about a microscope image. To assess whether this aim was achieved, I emailed a short questionnaire to three separate lab sections after the exercise was run in 2010. Students were asked to respond anonymously, via a drop-off box, and numerically rate, on a scale of 1 to 5, their awareness before and after completing the lab exercise of the need to think critically about their interpretation of a series of microscope images. They were also asked to rate the likelihood of their thinking more critically in other areas of their biology curriculum. The questions, ranking guide, and the actual responses, expressed as a mean and a range, are given below.

1. How would you describe your awareness as a microscopist of the need to think critically about what you see through the microscope **prior** to doing the lab? Mean: 2.7 (range 1–5; $n = 21$)
2. How would you describe your awareness as a microscopist of the need to think critically about what you see through the microscope **after** completing the lab? Mean: 4.4 (range 4–5; $n = 21$)
3. How would you describe your awareness of possible artifacts that can arise with the use of a light microscope? Mean: 4.2 (range 3–5; $n = 21$)
4. Are you now more likely to think more critically in other aspects of your biology curriculum? Mean: 4.0 (range 2–5; $n = 21$).

Students were also given the opportunity to comment on the lab exercise. The vast majority of these comments, as for the ratings above, suggested that the lab exercise had achieved its goals. A selection of comments is summarized as follows: "Lab was very engaging. I could actually see and understand where a scientist may go wrong"; "Prior to completing this lab I hadn't considered the possibility that what I observe down a microscope may not be accurate, so this

lab was very useful as it opened our eyes to the realities of making accurate observations"; "Despite the tricky calculations, it was actually a very interesting lab"; "The lab completely introduced to me the possibility of artifacts occurring when doing microscope work, especially when observing live cells. The concept made complete sense once explained in the lab; it simply had not occurred to me beforehand, that what I might be seeing in microscopy data may not reflect the situation at the actual cellular level. The lab was rather theory heavy, but I don't see a way around this, as it was all new and relevant material"; and "I liked this lab; it spoke well to my common sense." More negative comments were "I did have trouble taking everything in in this lab"; and "It was a long lab to demonstrate one small point." One note of caution should be added: responses were received from only 21 students out of 90 contacted. Given the low response rate, the conclusions as such are based on a small, self-selected subset of students.

The applicability of PBL for effective teaching of cell biology is becoming increasingly apparent (Allen and Tanner, 2003; Spiegel *et al*., 2008). The nature of this lab exercise means that it is particularly well suited for courses where a PBL format is used. As described, it does not adhere strictly to a PBL approach, in that the students do not define the problem and are at times given, rather than having to identify and find, information, but this is largely due to the time constraint of a 3-h lab. There are aspects of PBL, in that students are asked to work collectively, to share information, and to pose questions to themselves and their peers. The time constraints mean that the lab instructors set goals that need to be reached at the end of each exercise. The instructors can provide guidance if and when necessary, but the onus is on the students to work together to apply their prior knowledge of microscopy and the properties of light and of simple geometry and to start to picture what a microscopist sees down the microscope in three dimensions.

For our second-year cell biology course at the University of Canterbury, the exercise is run as a laboratory class that lasts 3 h, although it would also be suitable for tutorial classes, given that there is no actual practical work. There are no lab equipment needs. Assessment is based on short answers and the students' calculations and final conclusions. In 2010, this was run through three lab classes with approximately 30 students in each class. This required the presence of one lab instructor and two teaching assistants for each class.

In summary, a lab/classroom exercise is described that aims to demonstrate to students the importance of thinking critically about what they see through the microscope and, in principle, to think critically about what any technology seems to show. This is based around a series of exercises that incorporates aspects of PBL. Student self-assessment of their critical thinking suggested that this aim was largely attained. While this has been given as a cell biology course at the University of Canterbury, it is applicable to any course in which microscopes are used. However, the skills learned are not just applicable to microscopists, as critical thinking is a key skill for any scientist.

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