MULTIFOCUS IMAGER THEORY AND PRACTICE

Martin Thomas, Cairn Research Ltd, October 2017

SUMMARY

This document describes both the theory and practice of simultaneously acquiring multiple images focussed at different depths into a sample that is being viewed by a microscope. It can be achieved either by use of separate optical pathways going to multiple cameras, or by an image splitter, in which the individual optical pathways are then recombined and focussed onto different regions of the same camera. In both cases this is achieved by use of corrector lenses in one or more of the optical pathways, and the relation between corrector lens strength and focus depth change in the object for a particular microscope configuration is derived.

The advantage of obtaining images at several different depths simultaneously is particularly obvious for moving objects, but in any case, it can increase the overall rate of data acquisition, which is always potentially useful.

However, there is a little more to the subject than this, because changing the focus may also change the magnification of the individual images. While the images can in principle be rescaled to correct for this effect, it is clearly preferable to avoid the need to do so. Therefore, this document also describes how the optical relay system from the microscope to the camera(s) can be configured so as to maintain a constant magnification for all the images. This requires the corrector lenses to be at a location within the infinity space of the relay system that is conjugate with the back focal plane of the objective. Since that location depends on the microscope optics, it needs to be adjustable. The underlying principles are illustrated by diagrams in order to make them clearer.

When simultaneously focussing at multiple depths, the creation of the individual optical pathways is generally going to be done in a wavelength-independent manner using beamsplitters, but the use of depth-correction optics may also be required when selecting on the basis of wavelength instead. This is because of possibly imperfect chromatic correction in the objective, which can be troublesome especially in the deep red and near infrared. The effect of such imperfect correction is that the objective focusses at different depths into the object at different wavelengths, so in this case the corrector lenses are used to compensate for it.

This document also describes the location and characteristics of the image of the objective pupil that is generated within the optical relay system. Depending on the detailed characteristics of the microscope objective, the position of this image is at or close to the optimum location for the corrector lenses. It can be useful to know this location in any case, as the formation of the pupil image here tends to define a known minimum beam diameter at this point.

The conclusions drawn here have all been verified by optical design software. A spreadsheet has been generated to allow the optimum position (no magnification shift) for the corrector lenses to be calculated, and it also calculates the relationship between a given focal length of corrector lens and a given degree of object focus shift.

THE OPTICAL PATHWAY

In order to evaluate the necessary corrections for focussing at multiple depths into an object, we have to consider the entire optical pathway from the sample to the camera(s). To do this, we need to make some simplifications, in particular to model each component by an equivalent “thin lens”, where all the refraction takes place at a single surface. In practice the refraction by each component is going to take place over two or more surfaces that are separated by some distance, and the effect of this simplification is to make the equivalent optical distance through the component somewhat shorter than the actual physical one, as the thin lens approximation sets it to zero.

The effects of this simplification are often overlooked, as they are usually too small to need to be taken into account during the initial design of an overall optical system, but they will be fully taken into account by later design optimisation by software, as this will correctly compute the refraction at each individual surface. We therefore tend to forget about it.

But with microscope objectives it's a different story! They may contain perhaps ten or more elements, so to model such a component as a single refractive surface marks a very significant departure from the actual physical situation. The approximation is still fully valid as far as the subsequent optics are concerned, but it plays havoc with the real distances, as the following will make clear.

The most important parameter for a lens is of course its focal length, and this is manifested as two physical distances from it, namely the front and rear principal focal planes. These are symmetrical and hence interchangeable, being defined by the direction of light transmission through the system. Light from a point somewhere on the front focal plane is refracted by the lens to form a collimated beam, whereas a collimated beam passing through the front focal plane is refracted towards a focussed point somewhere on the rear focal plane. The locations of these points on their respective focal planes is encoded by the angles of the corresponding collimated beams with respect to the optical axis. In mathematical terms the relationship between them is defined by a Fourier transform, but we don't need to involve ourselves with that here, as we can just stick to straightforward ray tracing.

In the case of an infinity objective, the sample is therefore at the front focal plane of the equivalent thin lens, in order to form the collimated beam that is refocussed by the tube lens. In the analyses here these lenses will be referred to as L1 and L2 respectively. The situation with a “traditional” noninfinity objective is only slightly different, as in this case the sample is just slightly further away from the front focal plane of the equivalent single thin lens, so that an image is formed 160mm beyond it. However, to allow direct comparison with infinity objectives, we will here model such an objective as having the same two lenses L1 and L2, but with no separation between them.

This all seems straightforward enough, until we ask where the back focal plane of L1 is, which it turns out is something we really need to know. The “usual” answer is that it's at the back *aperture* of the objective (generally known as the *pupil plane*), which itself is usually at or close to the objective's *seating plane*, and which is the surface that mates with the microscope turret when the objective is attached to it. However, that isn't necessarily so, and here is why. Consider for example a Nikon 40x objective, with a parfocal distance (the distance between the sample and the objective seating plane) of 60mm. To get a 40x magnification with a 200mm tube lens, the objective must have a focal length of 200/40 = 5mm, so the equivalent thin lens is going to be that far away from the sample, or 55mm from the seating plane. That would put its back focal plane 50mm from the seating plane, and hence deep into the objective. In practice the multilens nature of the objective means that it won't be that far in (because the equivalent optical length of the objective is shorter than its physical one), but on the other hand it would be quite a coincidence if it were to exactly

coincide with the seating plane.

Similarly, the pupil plane may not be exactly at the seating plane either, although in practice the correspondence between these two locations is likely to be much closer, and its location at the rear of the objective can often be directly observed.

There is potential confusion here, in that some of the basic descriptions of a microscope tend to treat the back focal plane, the pupil plane and the seating plane as being synonymous, whereas in practice they may well differ somewhat. Instead this identity is the “best guess” that one makes in the absence of any further information, as at least the seating plane location is known, so this is what we have done in the accompanying calculations spreadsheet. However, a more accurate position can enter by using an amended infinity distance within the microscope, as we'll see. Unfortunately, the back focal plane location doesn't seem to be specified by the manufacturers (why?), although it can be measured by a variety of methods that are described in the general literature. Generally though, it tends to be deeper into the objective for the higher magnification ones, and might even be beyond the seating plane for some lower magnification ones.

We now turn to the sample focussing and magnification issues, for which there are two potential requirements to deal with. The first is to obtain two or more simultaneous images at slightly different depths into a sample, to form a “mini z stack”. This type of approach is particularly useful, if not essential, if the sample is still sufficiently alive to be actually moving (it does happen occasionally). Although microscope objectives are designed to work at just one distance into the sample, they can be refocussed to slightly different distances by changing the image distance appropriately. The extent to which this can be done before their point spread function significantly deteriorates depends on the type of the objective, with water immersion types being considerably better than oil ones on account of the better refractive index matching through to the sample, but even with oil it should be possible to achieve the few microns that may be all that is needed.

The second is the apparently more prosaic one of simultaneously imaging two or more colours at the same depth into the sample. If microscope objectives were perfect, this wouldn't be a problem, but for reasons we're just about to explain, the problem of chromatic aberration becomes relatively more important at high magnifications. Lenses suffer from chromatic aberration because the refractive index of all optical glasses, and hence their focal length, is to some extent dependent on wavelength. By using multiple lenses of different glasses (of which the “achromatic doublet” is the simplest example), these effects can be reasonably well corrected, but there is a trade-off between the degree of correction and the wavelength range over which it is effective. An inevitable part of the trade-off is that the chromatic performance rapidly deteriorates outside the corrected range, which for microscopes has of course been optimised for visible wavelengths, whereas cameras can operate significantly into the near infrared. Any such variation in the focal length of the objective means that it is focussed at a different depth into the sample, so here one would want to correct wavelength-dependent images in order to bring them all back *into* the same focus.

In order to appreciate the problem that underlies both these potential requirements, we have to appreciate the difference between lateral and longitudinal magnification. The lateral magnification is what it says on the objective, whereas the longitudinal magnification is the *square* of that. This means, that to take the case of a x40 noninfinity objective, which we can model as a single lens, the focus position at the image, for a point on the object that is just one micron deeper than the current in-focus position, we have to move the image sensor by not 40 microns, but by 1.6mm, to bring that point into focus! And for 100x the required shift is a full 10mm....

Although this may sound counterintuitive, it can be derived directly from the standard optical equation for focussing by a single lens

$1/u + 1/v = 1/f$

where u is the object distance, v is the image distance and f is the focal length of the lens.

We also have the simple further relation for the (lateral) magnification m

$m = v/u$

Now let us assume that the object distance changes slightly, from u to u'. Then there must be some consequent change in the image distance, from v to v', such that

$1/u + 1/v = 1/u' + 1/v'$

Hence

$1/v' - 1/v = 1/u - 1/u'$

Rearranging this gives

$(v\text{-}v')/vv' = (u'\text{-}u)/uu'$

Since the differences between the original and revised distances are relatively small, then to a very good approximation we can write

$(v\text{-}v')/v^2 = (u'\text{-}u)/u^2$.

Hence

$(v\text{-}v') = v^2/u^2(u'\text{-}u) = m^2(u'\text{-}u)$

TELECENTRICITY

Such large shifts in v when v/u is high mean that moving the imaging sensor in order to maintain focus is going to significantly change the magnification, which in turn introduces the subject of telecentricity. If the optical system consists of just a single lens, the lateral magnification is the ratio of image distance to object distance as given above, but the longitudinal magnification effect means that at high magnifications, the change in image distance for a given change in object distance is also relatively high. So, in the case of a traditional noninfinity microscope with a 100x objective, and where the image is viewed directly by a camera, then that 10mm image position shift for a 1um object shift in the example mentioned above would change the magnification from 100x to
(160+10)/(1.6-0.001) = 106.32x to two figures, which is clearly quite significant! (Note though that the longitudinal magnification approximates to a square law only at the limit, although in practice the approximation is pretty good – according to the application of the standard optical equation, the new magnification for a 1um object shift is actually 106.74x)

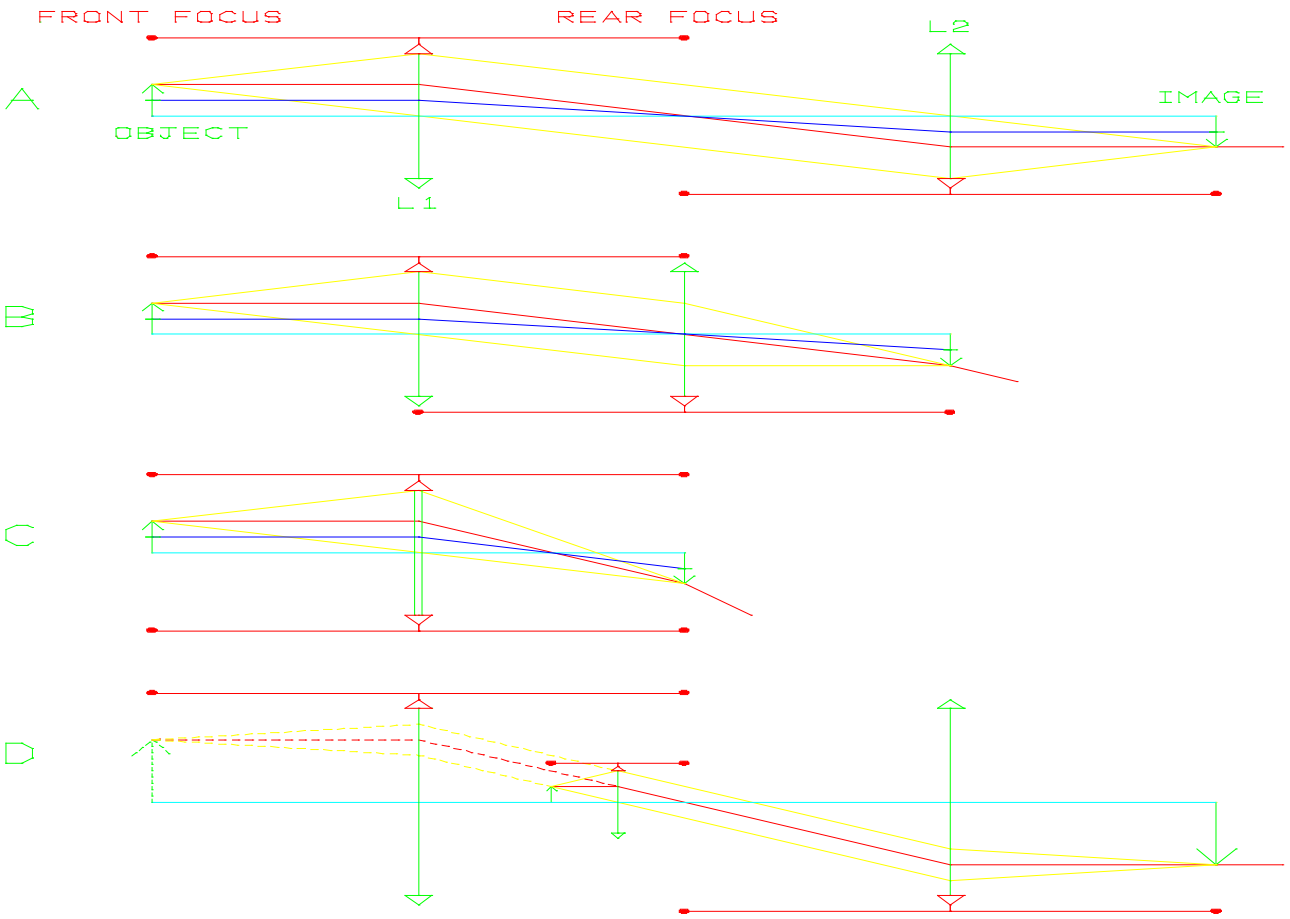

FIGURE 1

We can avoid the magnification change that a single lens would yield, by making the system telecentric, which we do by introducing a second lens as shown in Figure 1A. We can relate such a system directly to an infinity microscope, where L1 is the objective and L2 is the tube lens. In all the Figures the front and rear focus positions of each lens have been shown by red blobs attached to one edge of it. The marked edge alternates between successive lenses in order to prevent any potentially confusing ovelaps, and has no other significance.

For the sake of easy illustration, we initially show the situation for a unity magnification system, where the lenses L1 and L2 have equal focal length. The object is placed at the front principal focus of L1. Each point on the object generates a fan of rays that are collimated by L1, at an angle to the optical axis that corresponds to their position on the object. To understand what is going on, we consider the specific case of the *chief ray*, which is the one that leaves the extreme edge of the object in a direction parallel to the optical axis, and is shown in red in the Figures. This ray will pass through the rear focus of L1. If we place a second lens L2 such that its front focus coincides with the rear focus of L1, then L2 will refract it so that the ray is once again parallel to the optical axis. Since the extreme edge of the image must fall somewhere along this line, the magnification must be independent of the object and image positions, being given by the ratio of the focal lengths of the two lenses, which is unity in this case.

Note though that the distance between the two lenses is a true infinity space only for the case where the object is at the front focus of L1, which places the image at the rear focus of L2. Application of the standard lens equation to L1 and L2 in turn shows that as the object moves further towards L1, the image moves further away from L2 in proportion to the square of the L2/L1 magnification ratio, just as for the single-lens case, except that here the relation is an exact one rather than an

approximation. So in practice we still have the same image shift as for a single lens, but in this case without any change in the magnification.

However, as the two lenses are moved closer together, the telecentricity is lost. This is shown both for an intermediate case as in Figure 1B, where L2 itself rather than its front focus is at the rear focus of L1, and for the "extreme" case of Figure 1C where L1 and L2 are coincident, corresponding to the familiar case of a single lens, which will have a focal length of half that of L1 and L2 to achieve equivalent object and image distances. Figure 1B is reasonably typical for an infinity microscope, whereas Figure 1C represents the noninfinity case. In both cases the chief ray is no longer parallel to the optical axis, so the magnification will change if the object distance changes, with the effect in Figure 1C being that previously given for the single-lens case, and that in Figure 1B being somewhat intermediate.

The reason why we can use the convenient unity-magnification case to illustrate this effect is shown in Figure 1D. Here L1 has only one-quarter the focal length of L2, giving a magnification of four. However, as far as the image is concerned, we might just as well have been viewing an object at unity magnification, with an L1 of correspondingly longer focal length, placed correspondingly further away. The important point here is that if the rear foci of the two L1 choices coincide as shown, the ray pathways through the subsequent optics are identical. Therefore we can consider the telecentricity of a system of any magnification with reference to its unity magnification equivalent. This in turn shows that for an optical system to be telecentric, L1 and L2 must be separated by the sum of their focal lengths. It also means that, at least from the point of view of telecentricity, we can take the magnification of the objective out of our analysis, and just deal with the much more convenient unity magnification case from here on. The objective magnification will of course be important for calculating the focus shifts, but that comes later.

Although these Figures are primarily for illustration, they do also have a solid theoretical basis. They represent the standard *paraxial* approximation, where the true angles with respect to the optical axis are much smaller than they have been shown here for illustration. This approximation holds for as long as the sine of the angle (which determines the refraction according to Snell's Law) approximates to the angle itself when measured in radians. This is also true with respect to the tangent, which collectively means that the angular deviation of a ray by a lens is proportional to its distance from the optical axis. So, for the paraxial case (which means we have an *aplanatic* system, i.e. free from spherical aberration and coma), these Figures actually constitute geometric proofs.

These Figures also show some other rays in addition to the chief one, as their intersections with it define the image location. For systems that aren't fully telecentric, a ray that we can call the "imaging chief ray" is shown in purple. This is a ray that arrives at the microscope image parallel to the optical axis, and that has been extrapolated back to the object. Some other rays are shown in yellow, and are generally drawn to exploit simple particular cases, for example the one where a ray crosses the optical axis at the lens itself, and hence isn't deviated at all. Finally, it turns out to be very instructive to to show one or more rays that leave from other points on the object, and are also initially parallel to the optical axis. These are shown in blue.

So why aren't infinity microscopes made fully telecentric? There are basically two reasons for this. First, the infinity region adds an extra physical distance between the sample and its primary image, and hence generally to the eyepieces as well. That's not always going to be so convenient. For introducing epi-illumination or other optics into the infinity space, this region usually needs to be no more than 50-100mm, although optionally in some microscopes it can be made somewhat longer to allow a "double deck" configuration with stacked epi-illumination ports. However, that is less ideal for another reason.

If one compares Figure 1A with Figure 1B, one can see that the tube lens L2 needs to be of larger diameter as the infinity space is made longer. The beam diameter through the infinity space therefore needs to be correspondingly larger for a given field of view, which introduces practical problems for the introduction of other optics within this space, and which can perhaps only be accommodated by reducing that field.

RESTORING TELECENTRICITY

The question therefore arises as to whether we can add further optics to convert these practical cases into fully telecentric ones, and the answer is yes. One can of course connect a camera directly to a port that accesses the rear focus of the tube lens, but in the image-splitting or multicamera cases that we're considering here, we're going to need an optical relay system in any case. This brings us to Figure 2, which introduces the further lenses L3 and L4, where L3 recollimates the microscope image and L4 refocusses it. In order to keep this Figure a manageable size, it has been rescaled so that L1 and L2 have half the focal lengths that were shown in Figure 1. Initially we'll consider a unity magnification relay of the same focal length as the tube lens, so L2, L3 and L4 will be identical. We'll then extend the analysis to cover other relay magnifications and focal lengths.

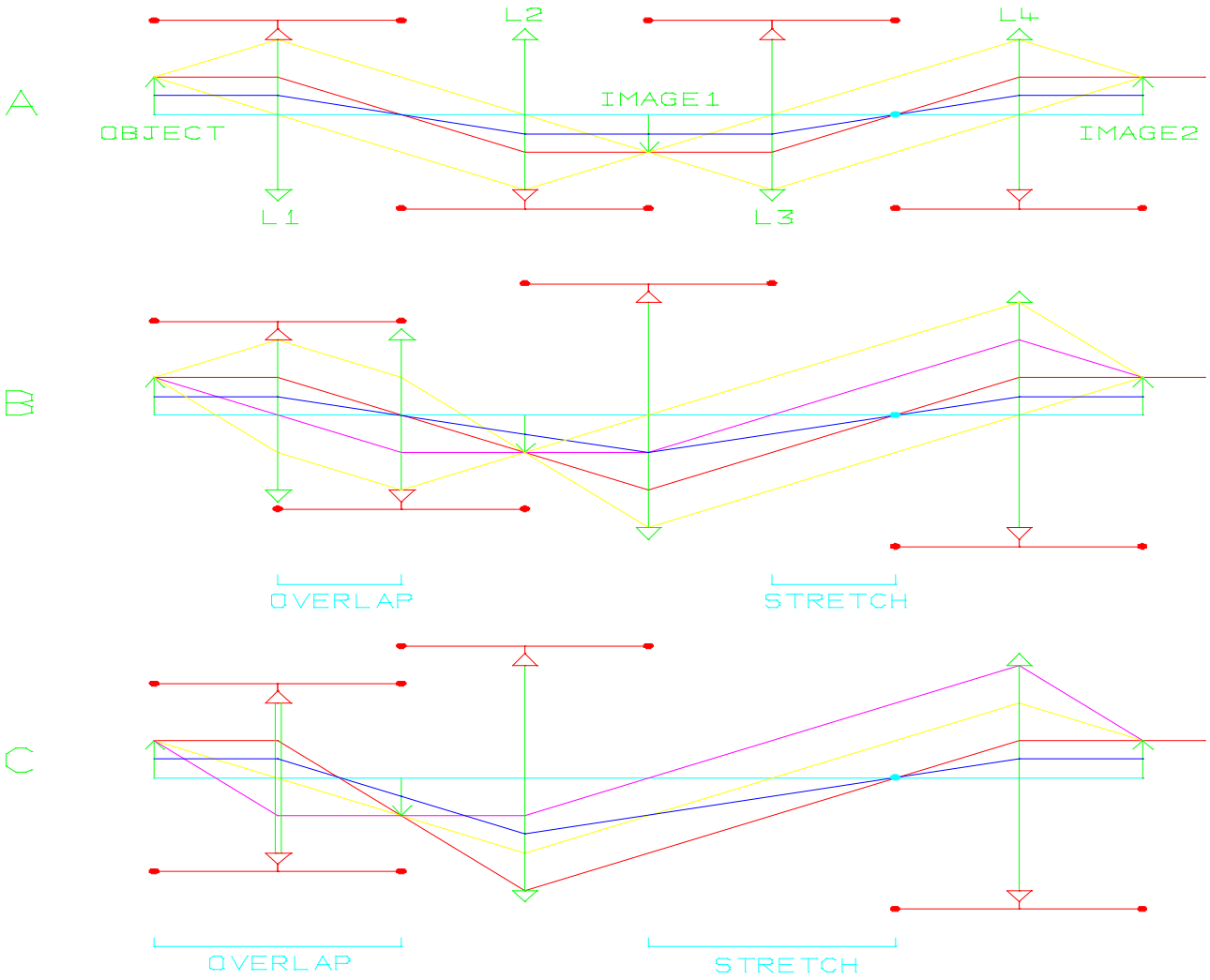

FIGURE 2

In Figure 2A we follow a telecentric configuration with a telecentric relay, so the system is telecentric throughout. There is nothing remarkable here. However, in Figures 2B and 2C we have

“stretched” the infinity space within the relay so that it is now greater than the sum of the focal lengths of L3 and L4. The easiest way to analyse this is with reference to the purple “imaging chief ray”, as this is the chief ray for the relay. It is therefore refracted through the rear focus of L3, which defines the angle in infinity space for all rays coming from the edge of the object. We can therefore extrapolate the direction of the original (red) chief ray to where it meets L3. Here we know that it will be refracted at an angle to make it parallel to the “new” (purple) chief ray, so now we can make that construction. Note that the relative “penalty” for the nontelecentric cases is that is that the beam diameter at L3 is going to be greater than for the telecentric case. However, this is the existing situation that we're stuck with, so we can (and must) take it into account in the design of the relay optics.

At some point that original (red) chief ray will cross the optical axis, but since it wasn't parallel to it before L3, this will occur some way beyond the rear focus of that lens. The trick now is to move L4 further away from the Figure 2A position, so that the crossing point coincides with the front focus of L4. This ray will now emerge from L4 parallel to the optical axis, thereby restoring the telecentricity. Comparison between the Figure 2B and Figure 2C situations shows that the greater the departure from telecentricity, the further out we must move L4. In fact, if the focal lengths of L2 and L3 are the same, the distance by which the L1/L2 pair is “short”, or “overlapping” of being telecentric can be seen to define the distance by which we must “stretch” the L3/L4 pair. This is shown by the equal lengths of the two distance markers. Note that it doesn't matter what the focal length of L4 is, just as long as its front focus is at this correct point, so we aren't restricted to a unity magnification relay. We can refer to this point as the “telecenticity position”, and in view of its importance it has been marked by a blob on the optical axis. Its real significance is that it is conjugate with the rear focus of the objective lens L1, which means that anything at that position is refocussed here. In fact we don't actually need the front focus of L4 to be in this conjugate position, for reasons that we'll go into later, but to keep things simple for now we'll assume that it is.

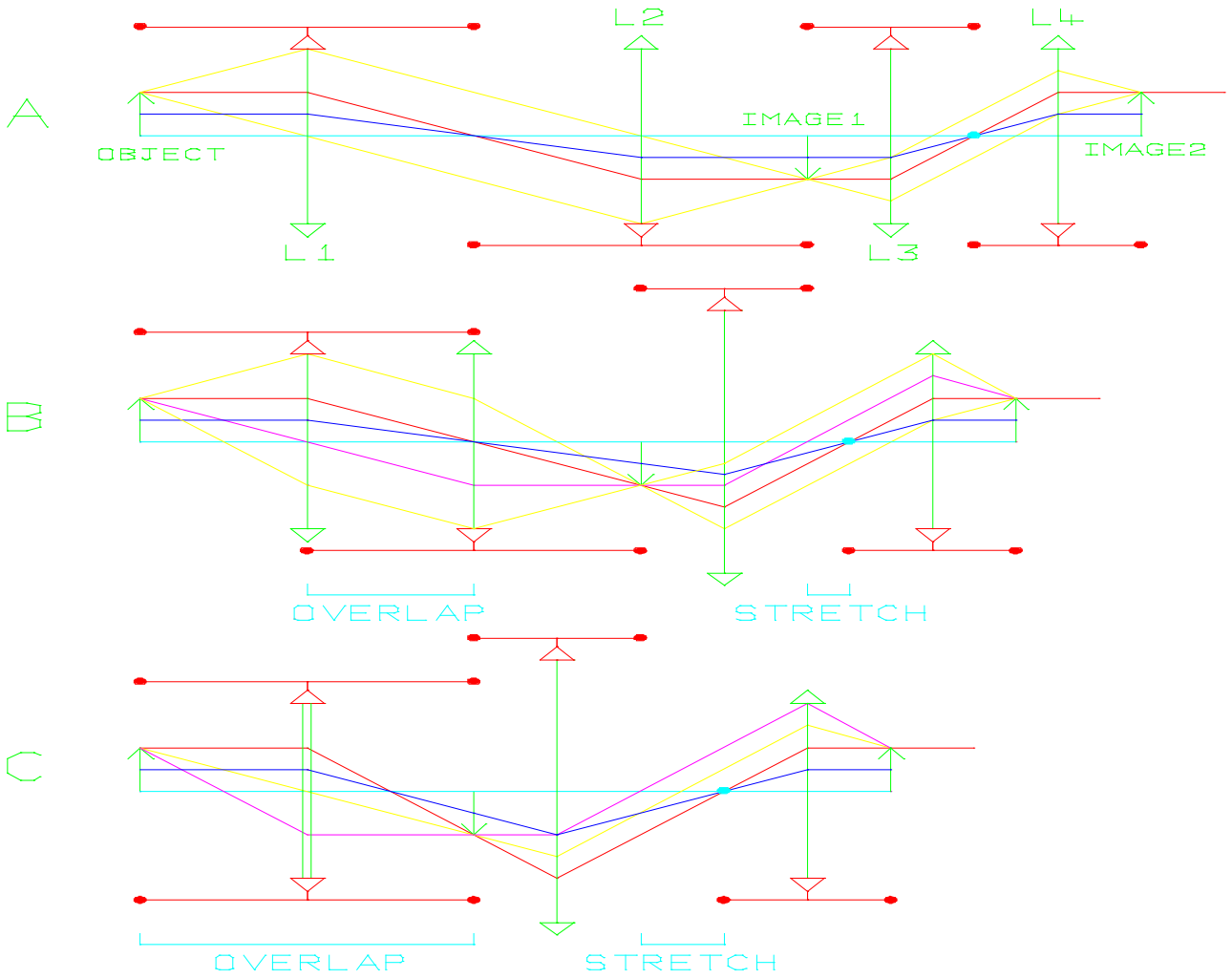

FIGURE 3

But what if the focal lengths of L2 and L3 are different? This situation is shown in Figure 3, where we have halved the focal length of L3, and also that of L4 in order to retain unity magnification through the relay. Here we have two effects. First, in the fully telecentric case of Figure 3A, the length of the relay has halved as expected, and a similar effect also applies to the equivalent nontelecentric case of Figures 3B and 3C, analogous to those of Figures 2B and 2C. However, because of the reduced magnification, the ray angles in the infinity space of the relay, between L3 and L4, are twice those in the equivalent Figure 2 versions for a given position on the object. Therefore, the distance by which we must "stretch" the infinity space for a given shortage in the infinity space of the microscope, is only half as great. What may not be so immediately obvious, although it is clearly shown in the Figures, is that the two effects combine, so that in this case halving the focal length of the relay has reduced the amount of infinity space "stretching" to restore telecentricity to just one quarter of its previous distance. Again, this is shown by the relative lengths of the two distance markers. So more generally, we clearly have a square law effect here.

Hence the rules for constructing a relay to give telecentric behaviour overall are as follows. First, measure the infinity distance in the microscope, and calculate the distance by which it is short of being telecentric. If one assumes (or is forced to assume!) that the rear focus of the objective is at its seating plane, then the distance by which we are short is given by the focal length of the tube lens minus the physical length of the infinity space. If we have a better estimate of the objective's rear focus, then we use the distance between that point and the tube lens instead. Then in the case where the focal length of the input lens (L3) of the relay is equal to that of the tube lens (L2), we increase

the infinity distance in the relay by that amount from its true telecentric one. If these focal lengths are different, we multiply the correction distance by the square of the ratio of the focal length of L3 divided by that of L2.

To make the system telecentric overall for any given microscope, it is therefore going to be necessary to adjust the distance between the relay's collimating lens L3 and the corrector lens location, in order for that location to be conjugate with the rear focus of the objective, and the accompanying spreadsheet can calculate what this distance should be. Or it could be determined experimentally. There is nothing wrong with the experimental approach, especially in view of the potential uncertainty in the location of the rear focus of the objective L1. Either way, our image splitters and camera adapters can be fitted with variable length “trombone” couplers to allow this condition to be achieved. It should be clear from the preceding analysis that the focal length of L3 significantly affects this distance, so in principle that can be changed in order to bring the adjustment to within a convenient range, but at the expense of making a possibly undesirable change to the overall system magnification. However, for *viewing* purposes, the same overall magnification could be achieved by making the same relative change to the focal length of L4 compared with that of L3.

FOCUS SHIFTING

So now we've made the optical system telecentric overall, but how do we shift the focus? The blue rays in the Figures provide the clue. These represent the chief ray from a subregion of the object, specifically a point half way between the edge of the object and the optical axis. This ray crosses the optical axis at the same place as the “full-size” chief ray, namely the front focus of L4. By further plotting, this can be shown to be the case for on-axis rays leaving *any* point on the object. And by further extension of that, although rays leaving the object at *other* angles won't cross the optical axis at the front focus of L4, all the rays leaving from *different* points on the object at any given angle will converge to a *common* point somewhere on the *front focal plane* of L4 or more generally (and importantly), at a conjugate plane to the rear focus of L1. Therefore, the placement of a further lens at this point cannot affect the magnification of the system, although it must have some sort of focussing effect, because it is in what was a pure infinity space with respect to the object. If we keep the distance from this lens to L4 the same (which in practice we will), then that space will remain a pure infinity one with respect to the object, so the focus shift will actually be on the object side, which is what we want.

In fact, if the objective pupil is at the rear focus of L1, it will be reimaged here, and in practice the image will at least be somewhere nearby. It can be seen from the Figures that the tube lens L2 and the relay's collimating lens L3 provide a telecentric relay for the objective pupil, from which one can see that the magnification of the pupil image is given by the L3/L2 focal length ratio, regardless of the pupil's actual position. Although the pupil doesn't directly enter into the calculations, the accompanying spreadsheet optionally allows its size to be entered, so that the size of its image can be displayed if required, although the calculation is simple enough to do by hand! Knowing the position of the pupil image is useful in any case, as the formation of such an image defines the beam diameter there, where it is likely to be at a minimum. That is often useful to know.

TELECENTRIC REFOCUSSING?

We now have a system that is telecentric overall in object space, and we also know where in principle we can place corrector lenses within it in order to change the focus without affecting the magnification. However, before going further with the analysis it will be useful to consider the various practical configurations, as it turns out that the position and focal length of L4 are unlikely to matter. Why is this?

We are going to be considering two alternative multifocussing possibilities. The easier one to understand is where we have two or more cameras. In this case there will be one or more beamsplitters in the infinity space beyond the relay's collimating lens L3. There will then be a separate refocussing lens L4 for each pathway. The alternative is that of our image splitters, where the individual pathways are recombined to be refocussed by a common lens L4. This recombination occurs after the location of the corrector lenses, allowing a separate one to be used in each pathway, which of course the multicamera implementation provides by default. Please see our image splitter and camera adapter literature for further details and diagrams.

The important general point is that once we get beyond the corrector lenses, *we no longer need to be telecentric*. That is to say, the front focus of L4 doesn't have to be conjugate with the relayed image of the rear focus of L1, which we previously named the “telecentric position”, and is where the corrector lenses need to go. Once a system has been set up, the distance between the camera(s) and L4 is going to be fixed, so in practice it isn't going to matter if any variation here affects the magnification. That's especially so for the image splitter case, where all channels share the same pathway and hence path length from L4 to the single camera. And even in the multicamera case, where the positions and/or focal lengths of the individual L4 lenses may differ between channels, the effects of the corrector lenses with respect to the equivalent shifts in object position will be the same.

A better way of looking at this is to view each camera (or portion thereof in the image splitter case) and its associated L4 lens as a single unit. The camera/L4 combination is viewing a collimated beam of certain specific characteristics, and we choose the focal length of L4 with respect to L3 such that it delivers the required field of view from the microscope image onto the camera sensor. There may well be a size mismatch here! In practice we therefore match the focal length of L4 relative to that of L3 according to the size of the camera sensor, and we can make the distance between the corrector lenses and L4 pretty much whatever we like as long as the beam doesn't overfill L4. However, to keep things simple our Figures always show L4 as having the same focal length as L3, and positioned with its front focus conjugate with the rear focus of L1. To summarise, L4 affects the overall system magnification for *viewing* purposes only, rather like a microscope eyepiece. This is in contrast to the relay's collimating lens L3, which does directly affect the magnification, according to the ratio of its focal length relative to that of the tube lens L2 and other system parameters, as well as the location and effects of the corrector lenses.

THE FOCUS-SHIFTING CALCULATIONS

We have already shown that placing a further lens at a location that is conjugate with the rear focal plane of the objective cannot change the magnification of the relayed image, because the rays that leave from any position on the object at the same angle as each other will intersect somewhere on this conjugate plane (which is going to be at or near the image of the objective pupil). However, the additional lens will change the focus. Since the focus of the camera stays the same, this shift must be with respect to the object side of the relay, which is provided by the primary microscope image.

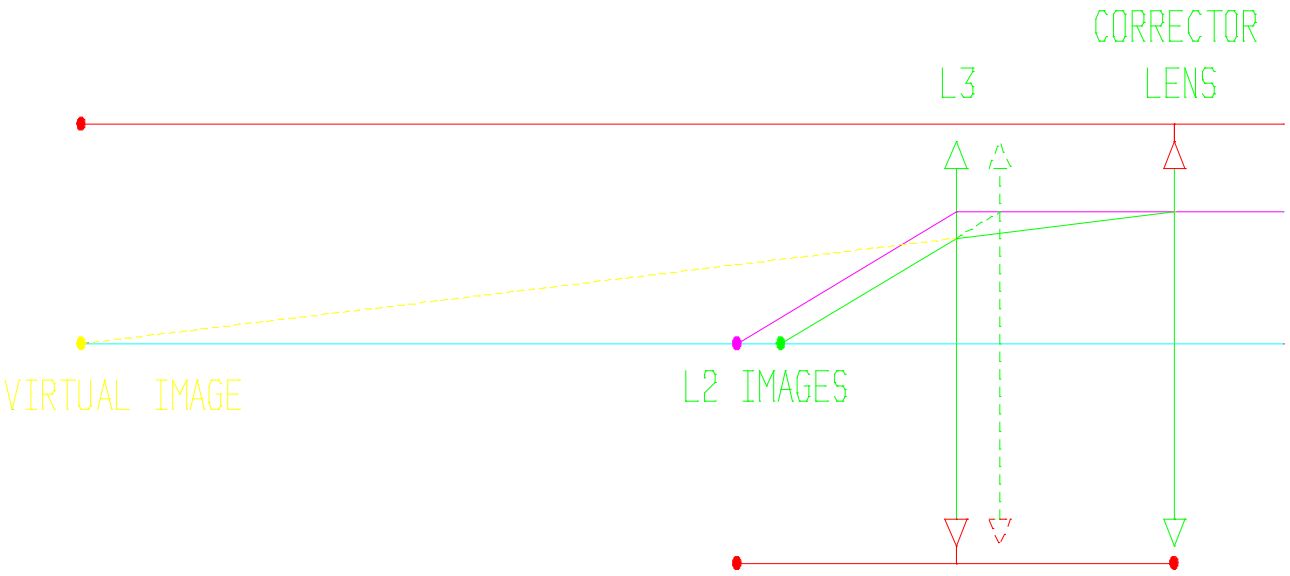

FIGURE 4

What is going on here is best appreciated when both the microscope and the relay are individually telecentric, for which the situation shown in Figure 4 refers. Here we need concern ourselves only with the image position rather than the image height, as we know that the system is fully telecentric in the absence of the corrector lens, and the corrector lens can't affect the magnification of the image. For clarity we therefore just show a point image on the optical axis.

Without any corrector lens, the primary microscope image will be at the front focus of L3, and from which L3 will generate a collimated beam. This is shown by the purple on-axis object and ray. However, once the corrector lens is introduced, the beam will only be fully collimated beyond it, so the additional focussing effect will move the effective focus of L3 closer to that lens, as shown by the green on-axis object and ray.

We can calculate the focus shift from the corrector lens by noting that since it produces a collimated beam, the output beam from L3 must appear to have come from the front focus of the corrector lens, so we can extrapolate it back to that point as shown by the dotted yellow line. It forms a virtual object for L3, and we can now calculate the new effective object position for L3 as an image from this object, using the standard lens equation.

One can replace any number of lenses in this type of situation by a single lens of equivalent focal length, as we already did for analysis of the objective. We can do so here by extrapolating the still-diverging ray going into the corrector lens until it intersects with the collimated one that would have been there in the absence of the corrector lens. This is the location for the equivalent single lens, which has been shown dotted, and its focal length is given by the distance to the shifted image, and which therefore marks the front focus position.

Clearly the extent of this focus-shifting effect will depend on both the relative size and position of the corrector lens, but Figure 4 shows the special case where the corrector lens is at the rear focus of L3. In this case the shift in the position of the equivalent single lens is equal to the shift in the object position (or that is to say, the object position that will generate a collimated beam beyond the corrector lens). Therefore, the system remains fully telecentric, and with no change in the

magnification.

However, in the practical situation, the rear focus of L3 has probably been displaced from the corrector lens position in order to restore the overall telecentricity of the system, so this may no longer seem to work. In fact it does, because we are fully telecentric at the corrector lens position, which means that the system is exactly equivalent to the rear focus of L3 *being* at that position. This is confirmed by optical design software.

Clearly it's nicer to come up with a formula for the focus shift rather than having to calculate the effective object and image positions for L3 and the corrector lens in turn. For this it's easier to run the system the other way round, so we start with light coming from infinity (from the right in Figure 4), which first is focussed by the corrector lens, and then more strongly by L3. In the absence of L3, the corrector lens would focus the light at a distance f_{corr} from it. However, as shown in Figure 4, this forms a virtual object for L3, at a distance $f_{corr} - f_{L3}$ from it. From the standard optical equation, the revised image distance v' from L3 (equivalent to the object distance in Figure 4), instead of just being given by f_{L3}, is now given by

$$1/v'_{L3} = 1/f_{L3} - 1/(f_{corr} - f_{L3})$$

By multiplying through to give a common denominator, we can rewrite this as

$$1/v'_{L3} = [f_{corr} - f_{L3} + f_{L3}]/[f_{L3}(f_{corr} - f_{L3})]$$

Hence

$$v'_{L3} = [f_{L3}(f_{corr} - f_{L3})]/f_{corr}$$

This compares with the image (in practice object) distance v for L3 without a corrector lens of just f_{L3}. The focus shift $v' - v$ produced by the corrector lens is therefore

$$v' - v = f_{L3}\{1 - [(f_{corr} - f_{L3})/f_{corr}]\}$$

Note the polarity! A convex corrector lens reduces the focus distance, which corresponds to an increase in the microscope's image distance, and hence a focus distance slightly less far into the sample. This equation also works for concave corrector lenes, which will therefore cause opposite shifts. That's well worth exploiting, as it doubles the focus range before the objective's point spread function is going to be significantly affected. In order to convert the focus shifts given by this calculation into equivalent shifts into the object, we then just apply the longitudinal magnification relation derived previously. The accompanying spreadsheet does all this automatically, but the overall calculation is easy enough to do by hand. There is even a rumour that in the predigital era, people could do this sort of calculation in their head, but we see no need to enter into such fanciful speculation here.

But if you are uncomfortable with this derivation, there is no need to take it on trust! The focus controls on microscopes are generally calibrated, or if in doubt you can do the calibration yourself, by focussing onto objects of known different depths. You then see by how much you need to change the focus to compensate for the addition of any given corrector lens. In fact, we very strongly recommend that you do this anyway, as it is *the* definitive test. The whole point of this document is rather to explain *how to change the focus without changing the magnification*, for which the optical conditions described here must be satisfied.

We can now think in practical terms. The focal lengths of the corrector lenses are likely to be

relatively long, in the thousands of mm, so they are unlikely to contribute any signifcant aberrations. Standard singlet lenses will therefore work perfectly well. Again in practice one is likely to use a range of “stock” lenses of defined focal lengths, so the most straightforward approach is to choose a focal length for the corrector lens, calculate the resulting focus shift on the object side of L3, and then use the longitudinal magnification relationship to calculate the equivalent shift in the object position.

To summarise, for a given corrector lens focal length, the other things we need to know are:

The magnification of the objective

The distance between its rear focal plane (use the seating plane if not exactly known) and the tube lens

The focal length of the tube lens (L2)

The focal length of the collimating lens in the relay optics (L3)

We can also optionally enter the size of the objective pupil in order to calculate the size of its image

Note that this simple calculation doesn't take the telecentricity of the system into account, but the effect of that on the focus shifts is relatively small. The reason that there is one at all is that as previously shown, the square-law relation for the longitudinal magnification is only absolutely true at the limit for a nontelecentric system, whereas it is exactly followed for a fully telecentric one. But it should also be borne in mind that the ultimate limit for the focus shifting is set by the inevitable deterioration in the objective's point spread function as it is asked to operate at object distances that increasingly differ from the one for which it has been designed. For a high-magnification objective, the useable distance range may be just a few microns, but that can nevertheless be quite significant compared with its z axis resolution, so this is still a potentially very powerful technique.

In practice therefore, the relations given here should be adequate for normal use. However, it is also possible in principle to calculate through the entire system on a lens-by-lens basis, but it will of course only be as accurate as the parameters that are entered into it. An alternative approach is to model the system using optical design software, which uses a “brute force” optimisation approach for obtaining the focus shifts. We have used this to verify the results given here. But again to repeat, the advantage of full telecentricity is that the images will all be of the same magnification as each other. Our image splitters and camera adapters support a variable-length coupling downstream of the collimating lens L3 to allow the all-important infinity distance within the relay to be correctly set. And in spite of all the technical detail here, there is nothing wrong in setting this distance by trial and error!

SYSTEM SIZE ISSUES

A potentially important practical issue is the overall size of the system, especially that of the relay. Since the microscope is likely to be less than fully telecentric, the length of the infinity region in the relay needs to be correspondingly greater. However that is not necessarily a problem in itself, as the length increase is with respect to the infinity space before the corrector lens position, where the beam diameter (defined by the size of the objective pupil image) will be the same as for a fully telecentric microscope. Instead the potential optical penalty is that in a less telecentric microscope the beam divergence going into the relay's collimating lens L3 is going to be greater, requiring it to

be of larger diameter, as already shown in Figure 2. This means that it may contribute greater aberrations if not designed with sufficient care. However, in the real world this is the situation with which we are confronted in the first place, so we must make the best of it.

As shown in Figure 3, the relative length of the relay optics compared with the microscope optics can be reduced by using lenses of shorter focal length, and this also applies to the relative increase in the length of the infinity section needed to restore telecentrity. Although it reduces the required size of L3 in aperture terms, it also increases the relative field size compared with its focal length, so we may not be much if any better off in terms of an aberrations tradeoff. It is assumed that we would reduce the focal lengths of L3 and L4 together in order to retain unity magnification through the relay with respect to the size of the microscope image, but as previously noted we are free to change L4 to match the sensor size to the required field of view if (as may be the case) the sensor size is different from that of the microscope image.

To make the system telecentric overall for any given microscope, it is therefore going to be necessary to adjust the distance between the relay's collimating lens L3 and the supplementary lens location, in order for that location to be conjugate with the rear focus of the objective. As previously noted, our image splitters and camera adapters can be fitted with “trombone” couplers to allow this condition to be achieved. It should be clear from the preceding analysis that the focal length of L3 significantly affects this distance, so in principle it can be changed in order to bring the adjustment to within a convenient range, but at the expense of making a possibly undesirable change to the overall system magnification. However, for *viewing* purposes, the focal length of the camera focussing lens L4 could be changed to offset that.

In general it should be possible to reach some reasonable compromise here, but this discussion would not be complete without covering an alternative possibility, which is that of introducing a *field lens* at the rear focus of the tube lens L2, namely at the primary microscope image. In practice we're likely to want to reduce the distance to the corrector lenses, in which case this lens is going to be a convex one. An ideal lens at this position cannot affect the image itself, or the magnification or position of the subsequent camera mage. However, it will clearly affect the relayed position of the rear focus of L1, and hence the supplementary lens position, so such a lens is potentially useful. Optical modelling confirms that there are no theoretical complications, but the calculation of the change in position is sufficiently straightforward to be done by standard optical analysis, rather than being gone into in any detail here.

Although there may be no theoretical complications, there is a practical problem in having a lens exactly at an intermediate image position, since any dust (or worse) on the lens will also be perfectly refocussed onto the camera. It is therefore good practice to offset the position of this lens by some reasonable amount. Since it is going to be in the relay system, it will need to be on the L3 side of the microscope image. Moving it away from the microscope image means that it will also affect the relaying of that image, but just as we did in Figure 4 we can treat L3 and the shifted field lens as a single lens of a correspondingly shorter focal length. Clearly a whole variety of intermediate effects can be achieved, culminating in just reducing the focal length of L3 while leaving the corrector lens position unchanged if this extra lens were moved all the way to the L3 position. However, one cannot help but feel that this sort of approach to moving the corrector lens position is best avoided unless other reasons strongly favour it. Simpler solutions are always preferable! While we nevertheless recognise this possible approach, we do not explore it further here.