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Toolbox

How to Convert a Traditional Electron Microscopy Laboratory to Digital Imaging: Follow the 'Middle Road'

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Today, electron microscopy (EM) is increasingly confronted by the revolution in image-processing technology provoked by modern computers. Digital cameras are fast replacing film-based cameras in EM, as elsewhere, and the procedures for digital image-archiving, image-analysis, and image publication are rapidly evolving. To take advantage of these advances, we have chosen for the moment a 'middle road', in which film remains our basic recording medium in the electron microscope, but immediately thereafter, all film-based images are converted to digital files for further analysis and processing. The rationale behind this approach is that film still offers far greater sensitivity and resolution (providing an image equivalent to >10000 pixels per inch in a 1-s exposure), and film is still far easier to organize and archive than digital images of comparable resolution. However, digital manipulation of EM images has become mandatory. Hence, we explain here, in some detail, how we convert from film to digital.

Key words: Digital imaging, electron microscopy, resolution

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Overview of the Following Essay

All electron microscopists are today facing the question of whether or not they should mount a digital camera in their electron microscope and stop taking electron micrographs on film. Here, we present our rationale for why we have not gone this route, despite the tremendous advances that have occurred in digital CCD cameras and the tremendous advances in computer–based acquisition of images from such CCD cameras. We will argue that the prudent course for at least the next few years will be to 'follow a middle road': namely, to continue to record EM images on 3.25×4 inch film while working at the electron microscope, but immediately thereafter convert these films to digital files for analysis and dissemination. We will indicate how this conversion to digital can be made rapidly and effectively, and in a manner that allows microscopists to confront their 'raw' data almost as freshly as they first confronted it during the initial microscope session. Additionally, we will explain how live TV viewing can be a tremendous aid at the time of first viewing samples in the EM. However, this is not for image acquisition, but rather for public display on a TV monitor so that others in the EM room (or others at great distances from the microscope, observing 'remotely' as has become the fad these days) can see for themselves the 'live' EM image—the image that heretofore was the private domain of the lone microscopist gazing intently at the dimly phosphorescent screen inside the electron microscope.

Introduction to the Problems and Challenges Facing Microscopists Today

At issue today are basic questions of how best to 'handle' EM images: how to organize them by experiment and date, how to archive them for long-term storage and retrieval, how to analyze them and view them after the session in the microscope, how to handle them quantitatively, and ultimately, how to select, crop, and otherwise photographically manipulate them for final publication or other public dissemination. (Indeed, this final issue—of publication *vs.* other forms of public dissemination—is itself evolving rapidly these days.) Already, journals generally prefer to receive EM images as digital files, or go ahead and convert any submitted EMs to digital files themselves. Hence, it behooves all compulsive electron microscopists to generate their own digital files in a form that they consider to be optimal, just as earlier microscopists used to go into the darkroom and make their own 'very best prints' for publication. Furthermore, the whole new world of on-line publication, plus the advent of a variety of efficient protocols for image-dissemination and transfer directly over the Internet, has already changed the nature of scientific exchange at its very roots. This will increasingly affect the biomedical electron microscopist as well. In this world, digital files are a 'must', of course, and issues of how to convert EMs into an optimal form for general Internet dissemination intersect with issues of how to prepare digital EMs for more traditional publication in printed journals. Our opinion for how best to accomplish these separate but overlapping goals will be offered in this 'Toolbox' as well.

Back to Basics: Why Stick with Film as the Primary Recording Medium for EM?

Basically, film still offers vastly greater sensitivity and resolution than CCD cameras. A simple way to appreciate this is to consider that standard EM film provides a resolution equivalent to roughly 10000 pixels per inch. (Note: since film is not a planar array of ~7 μm square photon-counting transistors that can be described as 'pixels', as is found on a CCD chip, but is instead a relatively 3-D layer of overlapping, light-sensitive, ~0.05 μm silver-halide crystals, its equivalency to 10000 pixels per inch may not be immediately obvious. The reader is referred to standard texts on photography published by Kodak, from which the Appendix provided at the end of this Toolbox has been exerpted.)

Accepting for the moment the notion that film offers a resolution of roughly 10000 pixels per inch, the second unassailable advantage of film is its sheer size: 3.25×4 inches in current usage. In comparison, the largest and highest-resolution CCDs currently available on the most advanced (and expensive) electron microscopes are roughly 2×2 inches in size and have roughly 2000 pixels per inch, yet these cost hundreds of thousands of dollars! The more typical CCD chips available at a 'moderate' cost for electron microscopes today are generally 1×1 inch and roughly 2000 pixels per inch. Comparing these various formats, we can see that one piece of sheet film creates an image equivalent to 32500×40000 pixels, or some 1300 megapixels, while the most expensive CCD chips create images of 16 megapixels and the standard CCD chips create images of roughly 4 megapixels (versus the oft-touted 1 or 2 megapixel chips in today's popular digital home cameras).

What these numbers mean in terms of effective resolution, which is of course what the electron microscopist cares about, will be discussed in detail in the next section. However, think first of these comparisons in terms of relative file-sizes. The 1300 MP image on a piece of film, even if recorded as a black-and-white image with only a moderate 8-bit of depth of contrast (256 grays) would be over 10 GB, larger than the entire hard drive of most current computers! Such an image would take probably an hour to 'open' in Photoshop and many hours to transfer on the even latest 100 MHz LAN Internet lines. By comparison, the highest-resolution digital images from the most expensive cameras would be roughly 128 MB, and the lower-resolution ones from more standard EM-type CCDs some 32 MB, only 1/300th of the file size provided by a single piece of film! (Even 30 years ago, Kodak was proud to boast that an entire 24-volume encyclopedia could be stored on a single photographic plate just 2.5 inches square.) These considerations indicate why film is, and will continue for some time to be, the optimal form of data-storage for information-packed images like electron micrographs.

How Post-Magnification of Film Brings it Down to the Range of CCD Resolution

One caveat in the above comparison is that EMs are generally shot at lower than final magnification, e.g. final EM images are usually generated by photographic enlargement of the original EM negative (see Figures 1–3). A $3\times$ enlargement of an EM negative, typical for generating an 8×10 inch print, reduces its effective pixel size to 3000 pixels per inch, close to the range of current digital cameras, while a 10x enlargement (as is often used in single-particle analyses or as is used to make high-magnification 'insets' in published electron micrographs) reduces the film's effective pixel size to 1000 pixels per inch, well within the range of current digital cameras. (Still, the effective area of a 1000 pixel-per-inch CCD chip is only 1 or 2 inches square, while a 10x enlargement of an EM negative would create a 'mural' almost 3×4 feet across!) In any case, the clear advantage of film over CCD images becomes apparent when the microscopist goes to enlarge their image after the microscopy is done (see Figures 1–3).

Practically speaking, electron micrographs are rarely shot at greater than $100000\times$; more commonly, they are shot at $\sim 5000\times$. At $100000\times$, a single, ~20-nm macromolecule, would measure 2 mm on the final photographic negative. This 2 mm would be roughly 400 silver grains in diameter (assuming 10000 silver grains per inch; cf. Appendix, below). Hence, a 'standard' 2000 pixel per inch CCD in an EM could capture just as 'clear' an image of a 20-nm molecule if the primary microscope magnification was increased until the macromolecule filled roughly a quarter of the CCD chip. That would require projecting a 1 cm image of the molecule onto the CCD chip, which would require a primary microscope magnification something like 5 times higher than that used for film (e.g. around $500000\times$; derived from magnifying a 2-nm object to a 10-mm image). Indeed, modern electron microscopes are capable of achieving such high magnifications; but with even the brightest of electron guns the image is extremely dim at this point, and, hence, the duration of exposure (with even the most sensitive of current CCDs) would be several seconds—enough time for serious specimen drift, or even for frank physical deterioration of the sample from beam-damage. Still, even if one did manage to capture a digital image at $500000\times$, one would end up with a single 50–100 MB image of one molecule, versus an image of hundreds of molecules on a single 3.25×4 inch piece of film shot in 1 s at $50000\times$.

When (and How) to do the Necessary Post-Magnification of Film

The moment for EM magnification to the molecular level is thus clearly not inside the electron microscope, but is, instead, later when post-processing the film. Here, to do this magnification, we strongly advocate the use of a top-end digital SLR camera (one with a 2–4 MP CCD chip) mounted on an old-fashioned photographic copy-stand. Devices such

as an old Bessler copy-stand, outfitted with a high-quality copy lens such as a Rodenstock 'Rodagon' and a bellows-type magnification tube, all mounted over a trans-illuminating base, allows one to enlarge an EM negative typically in the range of 3–10 × (Figure 4). Looking through the eyepiece of a quality digital SLR camera mounted on such a copy-stand provides the microscopist with a second 'primary' experience with their sample, much like the original experience of scanning the sample for good areas to photograph in the first place. Thus, he or she can search once again for good areas on the negatives, can appreciate structural contexts and interrelationships by eye, and then can select relevant areas for magnification and for instantaneous conversion to digital, just by a 'click of the shutter' on the SLR camera.

Black-and-white digital images from such cameras are typically imported via Firewire to a desktop PC as 5-10 MB TIFF files (or ~2 MB JPEG files, if so desired). As such, they are extremely easy to manipulate, catalog, store, transfer to other computers, etc. They become the microscopist's 'bread and butter': the images from which all further analysis and processing will be done. Perhaps dozens of such digital images can be shot from one single negative before the negative is 'retired' to an archival file. However, the EM negative will always remain available as a permanent, fully contextual record of the entire digital data-set.

Why Convert to Digital with a SLR Camera Rather than a 'Flatbed' Scanner?

It is important to stress that we do not advocate 'scanning' EM negatives on modern flatbed scanners to obtain digital records of their entire contents (unless, of course, the microscopist simply wants to publish a 1× view of the entire negative). Again, this is because the file-size from a high-resolution scan of an EM negative (that is, a scan that would be sufficient to permit its later enlargement or cropping in Photoshop) rapidly becomes unwieldy. The typical maximum

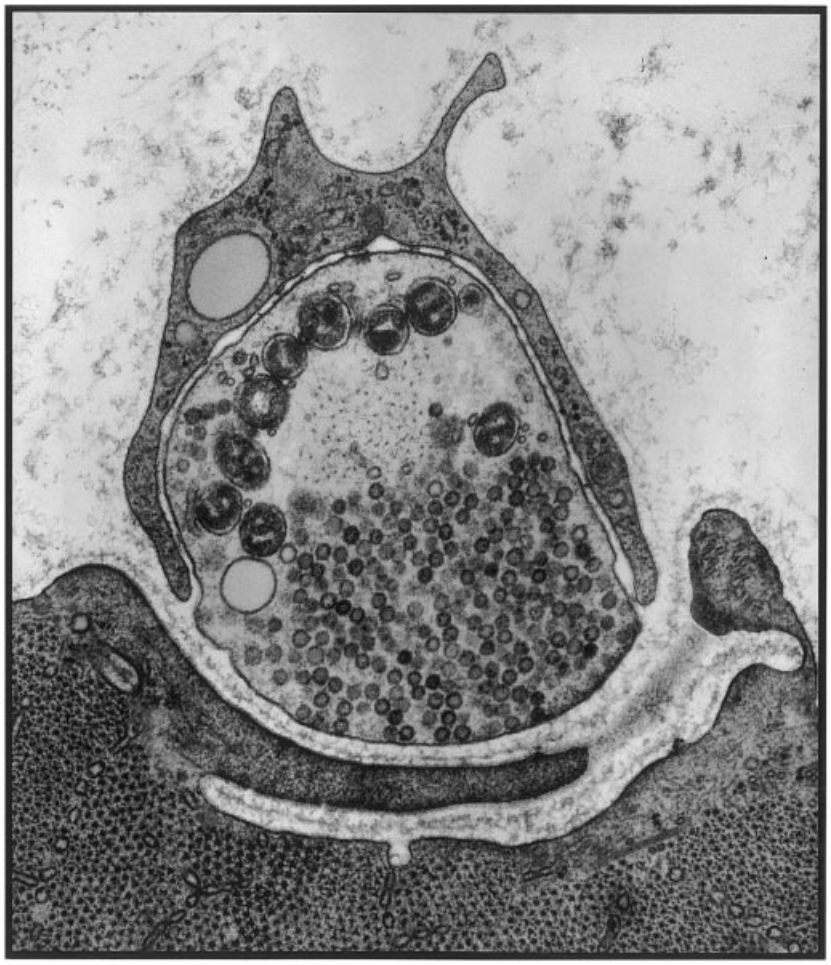

Figure 1: A typical field of a routine electron micrograph. In this instance, we see a 1.5 μm dia. nerve terminal filled with synaptic vesicles and mitochondria from a frog neuromuscular junction. This was originally shot at a primary magnification of 20000× in order to fill a standard 3.25 × 4 inch sheet of EM film. To generate this Figure, the original EM negative was contact-printed at 1× to fill the column. Comparable fields had to be shot on 1000 and 2000 pixel per inch CCD cameras at 6000x, due to their smaller 1 × 1 inch recording size. This made proper focusing of the image in the EM that much more difficult.

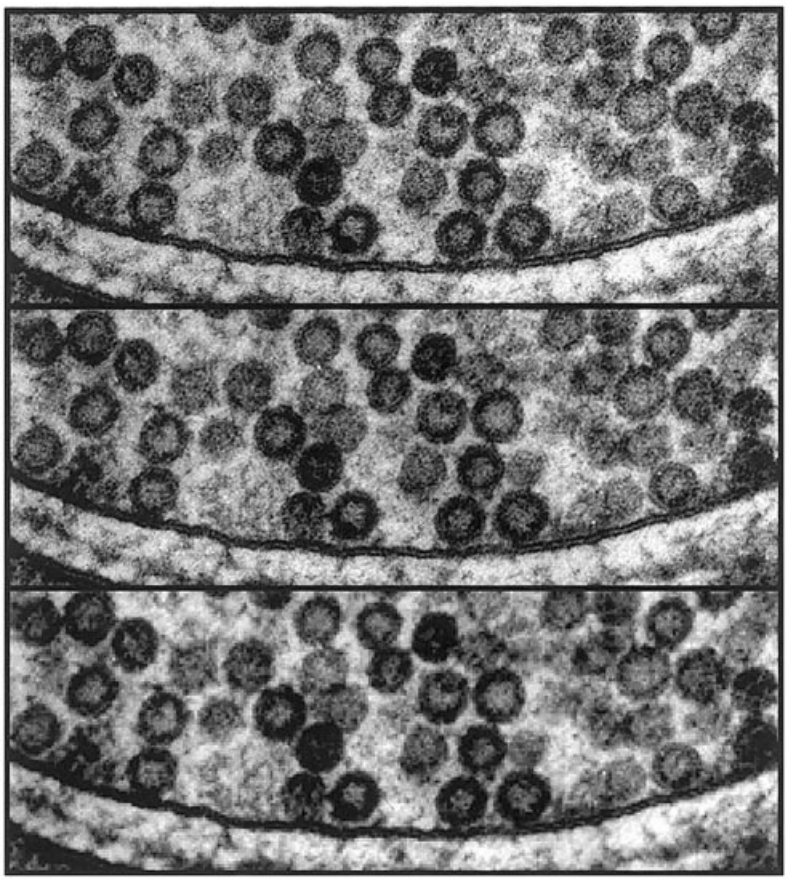

Figure 2: Enlargements (3x) of the original film and CCD images shown in Fig. 1. At this magnification, the loss in image quality in CCD images just begins to become apparent, mostly by the loss in clarity of cellular membranes. Top panel is the film image; center panel is a 2000 × 2000 pixel CCD image and lower panel is a 1000 × 1000 pixel CCD image.

flatbed scanning resolution of 1200 dpi will create a ~150 MB black-and-white file from a 3.25 × 4 inch negative, and will take at least several minutes to complete (versus the instantaneous 'click of the shutter' in the digital camera). In most desktop PCs, such a 150 MB file is opened rather slowly by Photoshop. (Furthermore, only four such files can be stored on an entire CD!) Even then, 1200 dpi file would permit selected fields to be taken from the negative and printed at no more than a 4× magnification (assuming the microscopist is trying to achieve the standard optimum for print resolution, which today is generally considered to be 300 dpi). But 4× is at the low end of the magnification range of the copy-stand/digital-SLR combination proposed above, and today's digital SLRs produce images that measure about 6 × 7 inches at 300 dpi, quite large enough for most journals (or measuring a whopping 2 × 2 ft at standard computer-screen resolution of 72 dpi). Hence, flatbed scanners are useful only for unmagnified conversions of EM negatives. At 300 dpi, a 1–2× flatbed scan of an EM negative creates a file in the range of 10–20 MB, comparable to the digital-SLR's image file-size.

How to Handle Digital EM Files once they are Acquired

Once EM negatives have been converted from real film to digital files, a whole new world is opened, in terms of what can be done with them. We will not attempt to deal with this topic in any depth, except to say that the following computer-manipulations are basic to any 'digital' EM laboratory:

1. Photoshop® operations to manipulate the images.
2. A cataloging program such as Cumulus® or Extensis Portfolio® that will rapidly and seamlessly create 'thumbnails' of each and every image and catalog them (with storage and source-data that will make them instantly retrievable).
3. A CD-burning program such as Toast®, plus a CD burner to off-load the images from the computer as soon as experiments and data-sets are completed and image files are organized. (No amount of hard drive memory will suffice to store the vast numbers of digital images generated by the typical EM laboratory!) Indeed, we have 'burned' over 300 CDs filled with EM images in just the 3 years since we have converted to the 'middle of the road' approach advocated here. This is almost 200 GB of data.

(Of course, Kodak reminds us that this amount of data could theoretically be 'crunched' onto just 20 sheets of EM film!)

4. An Ethernet link and a visible Ethernet 'site', such as a homepage or an FTP site, where some (or all) of the digital EM files can be accessed, viewed by other investigators at remote sites, and if desired, downloaded to their own computers by a standard file-transfer protocols such as Fetch®. (Personally, we maintain a constantly available, public FTP site where EM images are 'mounted' for transfer to our colleagues during the active stages of a collaboration, or for transfer directly to journal publishers when a paper is ready for press. (Although we advocate this last link in the chain of working with digital EMs, we must admit that we have not properly addressed the potential problems of pre-publication publicity or copyright protection that free access to our images may be creating.)

The Advantages of Converting to 'Live' TV Images While Working at the Microscope

Finally, although we eschew the capturing of 'primary' EM data by digital means for all the above reasons, we cannot stress strongly enough the great utility of creating live video images while one is actually sitting at the microscope. This not only eliminates the neck-strain and eye-strain of peering for long hours at the dimly phosphorescent screen through the dark porthole or through the binoculars of an EM, but—by bringing the image up to a nice, big, bright TV monitor—it allows colleagues and students of all ages to share the experience of EM viewing right as it is happening. Several people can sit in a room (or even at a great distance) and work together to scan a sample, to discuss what it represents, and to choose fields for photography. Indeed, the entire session can be videotaped for subsequent playback to others at a later time, allowing others to enjoy vicariously the original scanning and choosing (and more seriously, allowing

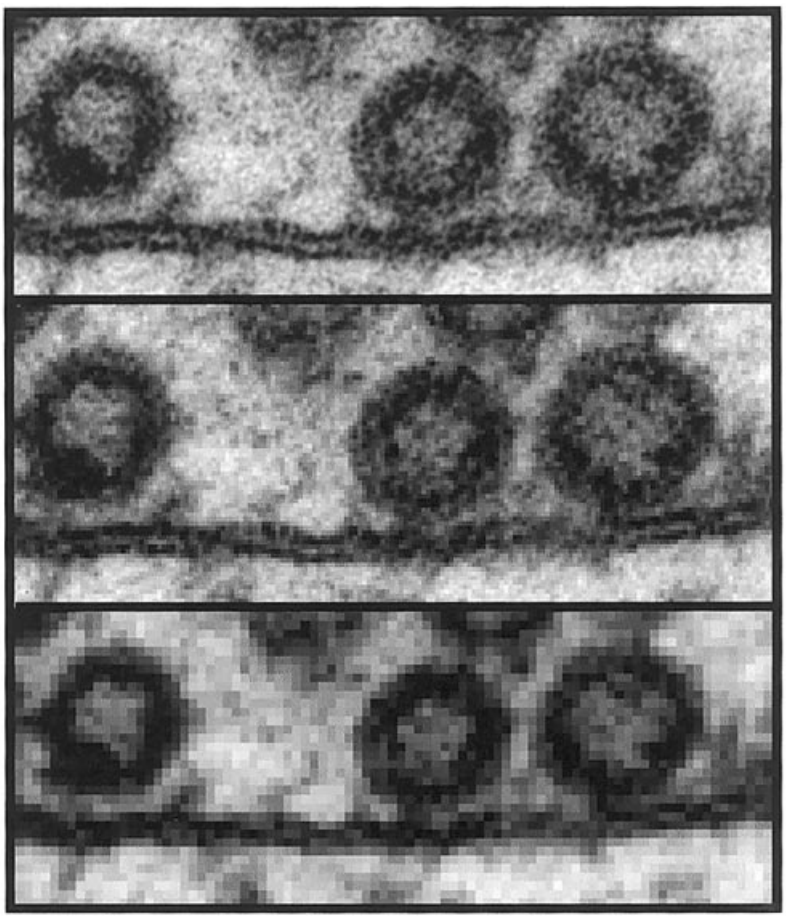

Figure 3: Enlargements (3x) of the film and CCD images shown in Fig. 2. At this magnification, the loss of image quality due to pixelation of the CCD images becomes immediately apparent. Indeed, the bilayer structure of the cell membrane is completely obscured in the 1000 × 1000 pixel CCD image at the bottom. In contrast, the film image at the top retains its full clarity with no apparent graininess even at this magnification.

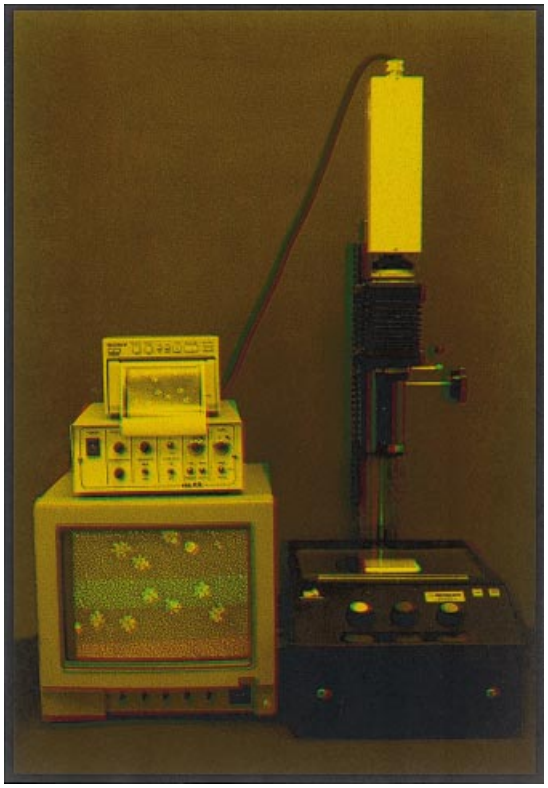

Figure 4: Recommended copy-stand set-up for converting EM negatives to digital or TV files. This is an anaglyph stereo image; see (1), for an explanation of this viewing procedure. (Anaglyph glasses available in Traffic 2000; 1) In the configuration shown here, images on EM film are converted to television for storage on optical memory disks and for instant generation of small prints with a Sony Model UP860 B&W thermal printer. For generation of computer-based digital files, the Newvicon camera and TV monitor shown here are substituted by a Kodak Professional DCS 520 SLR camera (the digital version of the Canon EOS-1 SLR camera), and images are imported instantly to a Macintosh G3 computer and monitor by a Firewire connection. Subsequently, digital images are printed with a Hewlett Packard Color 4500N LaserJet printer.

them to assess the overall condition of the sample and the degree of subjectivity that went into selecting the final image-choices, which has always been the biggest problem with electron microscopy). Our mentor, Sir Bernard Katz, once said publicly: 'If you look long enough in the electron microscope, you can find anything you wish.' (Actually, we don't fully agree with that dictum because there are plenty of things that we have 'wished' to find in the EM, but never could.)

A further advantage of substituting 'live' TV for direct viewing of the electron microscopic screen is that modern TV cameras are sufficiently sensitive that illumination levels can be kept very low, radiation damage to the sample can be minimized, and the microscope can be pushed to very high magnifications (or very thick samples can be viewed in energy-loss mode), when formerly, such samples would be too dim to see with the naked eye. (It is important to stress here that the actual resolution of these 'live' TV images or of the videotapes made of them is far too low for them to be a source of permanent still images.)

Here, we advocate the use of very standard analog (Newvicon) or digital CCD cameras with high sensitivity and good grayscale resolution, but only 640×480 resolution. These we suggest should be mounted external to the microscope and focused (via a standard 35 mm 'macro' lens from an SLR attached to the camera by a standard C-mount) directly through the porthole and onto the phosphorescent screen normally used for viewing. We have designed such a camera-mount so that it can be easily swung out of the way for direct viewing of the screen, when that is deemed necessary (Figures 5 and 6). Such a rig, including all its components—the camera, 35 mm lens, TV monitor, VCR, and some simple machined parts for the camera mount—should cost under $10000. Yet it totally obviates the need for an expensive internally mounted TV camera or a camera coupled by expensive fiber optics to a special screen inside the vacuum of the EM. Despite the fact that the true resolution of such TV images (or the videotapes of them) is far too low to be a source of decent still images, we do still attach a Sony thermal printer to our TV setup so that we can create little paper images that we can stick into our laboratory notebook. However, these serve as nothing more than thumbnail reminders of what we saw (c.f. Figure 4).

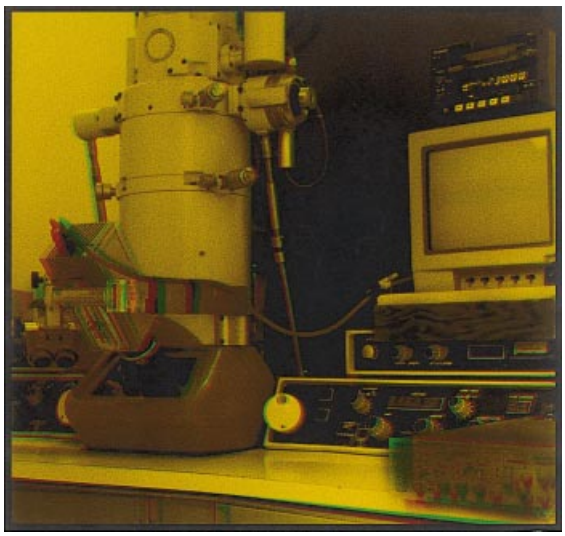

Figure 5: Anaglyph stereo-view of TV equipment mounted on a standard JEOL 200CX electron microscope. This set-up provides a TV monitor well-positioned for displaying the screen image to many viewers simultaneously, plus a control unit for live, real-time reversal of deep-etch EM images to 'negative contrast' for optimal depth perception, plus a digital video tape recorder for continuous acquisition of EM images as the sample is scanned. This tape deck can be connected by Firewire to a computer for storing low resolution 'thumbnail' images of areas worth further scrutiny and higher resolution film-based photography.

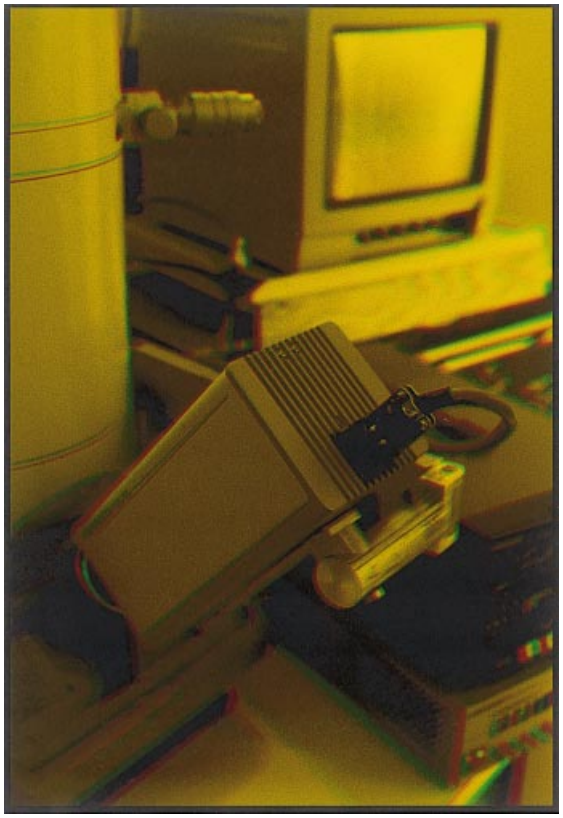

Figure 6: Close-up anaglyph stereo-view of television camera mounting and positioning on the electron microscope. A modern Hamamatsu 2400 Newvicon camera is aimed at the standard viewing screen of the electron microscope via a Nikon F1.4 35 mm focal length SLR camera lens coupled to it via a standard TV camera C-mount. The images provided by such cameras are far superior in gray scale and tone to standard EM-mounted CCD cameras, plus the camera is a fraction of the cost. The camera can be swung out of the way for normal viewing of the EM screen via the simple hinged arm visible immediately below it. This can be easily constructed in any University machine shop from plain aluminum.

'Live' TV Contrast-Reversal for EM Laboratories that do Freeze-Etching

The final advantage of 'live' TV viewing for EM laboratories that do freeze-etch microscopy, in particular, is that the TV-rendering allows a contrast-reversed image to be presented on the TV monitor, right during the actual observation period. We have long advocated contrast-reversal for freeze-etched and platinum-replicated samples, simply because it makes the platinum-coated elevations of the sample look 'highlighted' (rather than dark as they appear on the screen, since platinum is the electron-scattering part of the sample) and it makes the non-platinized 'depths' of the sample look dark, as if they were truly 'in the shadows'. This greatly assists the viewers' ability to discern the correct 3-D topology of a sample, right while they are viewing it. Formerly, this had to be performed by making contact-printed inter-negatives of each and every plate shot in the electron microscope, and then by printing these inter-negatives on photographic paper: a highly laborious and expensive process. Now, this can be performed by a direct 'contrast reversal' in Photoshop (although the digital SLR enlargements advocated above are made directly from the EM negatives, so they are already contrast-reversed).

In any case, doing a contrast-reversal 'live', right while several people are viewing a platinized sample together, greatly assists the microscopist to share with others the wonderful experience of 'the search'. This is what makes electron microscopy such a fun 'distraction,' as Keith Porter used to call it.

Acknowledgments

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Appendix A

The statement that EM film resolution approximates 10000 dpi implies that it has an effective resolution (or grain size) of ~3 μm. Of course, a developed black-and-white photographic image consists of silver particles suspended in gelatin. The size and distribution of these particles, together with the thickness of the emulsion layer, contribute to the image-structure properties of any given film. However, in describing these properties, the terms 'granularity', 'resolution', 'sharpness', etc., etc. can be very confusing and misleading. To help the reader think more clearly about these matters, the following statements have been exerpted and paraphrased from old Kodak publications. Some of these old publications plus more current explanations of film characteristics can be obtained from Silver Pixel Press, 21 Jet View Drive, Rochester, NY 14624. Their website address is: http://www.saundersphoto.com/html/books.htm

'Granularity'

While the densities in a photographic film may appear to the eye to be homogeneous, microscopic examination will reveal a collection of discrete particles of metallic silver. These 'grains' form a pattern that becomes visible when a photographic image is enlarged. Two terms are used to refer to this pattern: 'graininess' and 'granularity': 'graininess' is the subjective impression of it, while 'granularity' is a true objective measure of it (typically obtained by scanning film with a microdensitometer having a 48 μm circular measuring aperture).

'Resolving power'

Resolving power refers to the ability of a film to record distinct images of small, nearly contiguous objects in a scene (cf. 'American National Standard Method for Determining the Resolving Power of Photographic Materials,' PH2.33-1969). Typically, this is defined as a film's ability to maintain separate images of parallel bars whose relative displacement is very small, which is generally described in lines/mm.

Importantly, the resolving power of film is a function of the relative contrast of the parallel bars or 'target elements' used in a resolution test-pattern. It also depends upon a number of other factors. In general, the higher the contrast of the film, the higher its resolving power. (This is simply because higher contrast films are finer-grained.) Resolving power is also affected by exposure, peaking at a certain optimum exposure level and then falling off at higher levels, largely due to diffusion or scatter of light within the photographic emulsion. In addition, processing conditions and different types of developer can exert a measurable effect on resolving power.

'Modulation transfer characteristics'

The 'Modulation Transfer Function' (MTF) describes the ability of a film to more or less accurately reproduce varying inputs of light, as a function of spatial frequency. It specifically measures the effects of light scatter in the emulsion during exposure and the chemical dynamics that occur during the development process. To measure the MTF, patterns with sinusoidal variation in luminance are projected onto film. The spatial frequencies of these sinusoidal patterns extend from well below to well beyond the maximum resolving-power of the film, though generally do not exceed 400 cycles/mm.

During exposure, scattering within the emulsion results in a reduction in the image modulation, which is increasingly obvious at higher spatial frequencies. This can be quantitated by scanning the processed photographic image with a micro-densitometer. The ratio of the actual modulation of densities in the film, compared to the original optical image projected onto the emulsion, is called the 'modulation transfer factor.' Plotting this factor as a function of spatial frequency (expressed in cycles per millimeter) results in an MTF curve. The Fourier transform of such an MTF curve represents the 'spread function' of the film, the most objective value of its image-capturing characteristics.

Although emulsion-scattering and film-processing effects combine with grain size to determine both the MTF and resolving power of any film, these two measures of are not numerically related. For this reason, it is not possible to determine classical resolving power (in lines/mm) by simply identifying the spatial frequency (in cycles/mm) at which the MTF drops to some arbitrary response, e.g. 5%. The published resolving powers of two different films might well be identical, but their imaging properties could still be very different. Resolving power is a function of the granularity and 'micro-densitometry' of a film as well as its MTF. More detailed information and help in working with the MTF (sine-wave response) of film can be found in numerous technical papers referenced in the SPSE Handbook of Photographic Science and Engineering.

'Sharpness'

Although resolving power is a measure of the ultimate ability of a photographic material to record fine detail such as double-stars or fine parallel lines, it is often not the most important factor in microscopy. Generally of more importance is the 'sharpness' with which an image is reproduced. Although resolving power is one important indication of a film's ability to produce 'sharp' pictures; it does not follow that a series of photographs will necessarily be ranked in the same order for 'sharpness' as for resolving power. By using certain combinations of lenses and films, it is possible to make two photographs, one of which has a higher resolving power but a lower 'sharpness' than the other. Moreover, some developers reduce 'sharpness' markedly while affecting resolving power very little.

The resolving power versus 'sharpness' of a photographic material are conditioned primarily by two factors: the turbidity and the inherent contrast of the emulsion. First, considering the turbidity of an emulsion, it is a product of two conflicting variables: its light-scattering power versus its light-absorbing power. Turbidity is measured as follows: when a film is exposed to an image of a point or an extremely narrow line in a series of increasing doses, image size increases with increasing exposure level, and does so at a rate that directly reflects emulsion turbidity. Second, the inherent contrast of an emulsion depends primarily upon the range of grain sizes and grain shapes within it. Like turbidity, these film characteristics are fixed by the method of manufacture. On the other hand, the contrast and 'sharpness' of a particular image can be altered drastically by varying its development conditions, although this generally exerts only a minor influence upon its actual resolving power.