Pictures, puzzles, and patches

Researchers are using digital montage systems to clarify image analysis.

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What is an image? Most dictionaries define an image as the counterpart of an object produced by an optical device. For example, an image of this page is formed on your retina, and your brain interprets this image so that you can read the text. If you want to extract additional information about this image, your biological imaging system needs help from additional image analysis tools. A digital counterpart to your eyes is the charge-coupled device (CCD) camera. Information from digital camera images is stored in pixels in the form of a matrix, and each pixel contains information about light intensity and X, Y coordinates.

Analyzing and making quantitative measurements from images are important parts of the scientific discovery process, covering the range from simple densitometry measurements of gels to the complex application of analysis algorithms for cellular assays in drug discovery. In fact, many scientists use images and need to extract information from them. The simplest form of image analysis can be in the form of making measurements such as distance, area, or intensity. More complex analysis may involve identifying and separating various objects from each other and from the image background. Many mathematical filters aid in this object enhancement, separation, and identification.

The metastructure image

Biologists are accustomed to viewing cellular detail at high magnification. To gain an appreciation of larger-scale structure (metastructure), they move the slide around while comparing images of different aspects of structure. Metastructure is more easily appreciated and communicated, however, when a single image shows large areas of the specimen.

Metastructure images can be made by reducing magnification so that the field of view is larger. Although this method is sufficient if cellular detail is not required, there are limitations. Resolution is reduced with low-numerical-aperture objectives. Low-power objectives tend to introduce geometric distortion. Each camera pixel represents a larger portion of the specimen, reducing image resolution. Even a 1× objective may not accommodate the metastructure of interest. This last problem occurs especially with digital cameras, which see only a small part (~30%) of the eyepiece field of view.

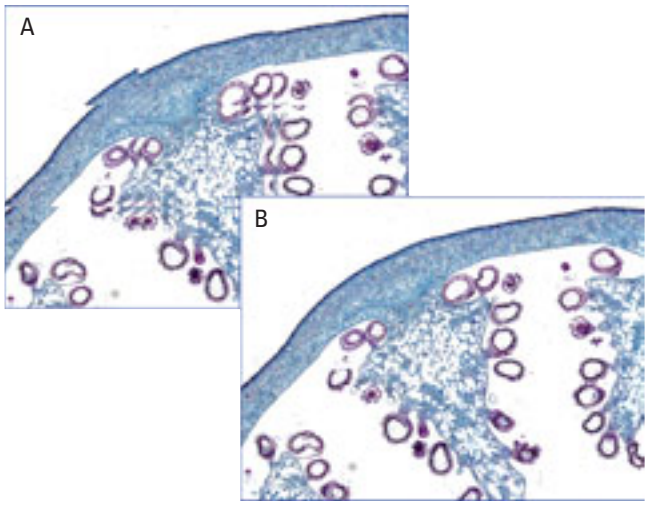

Figure 1. Alignments. Nine microscope fields of a transverse section of *Papaver nudicaule* montaged with a 4× objective and a Sony DXC970 color video camera. In panel A, no attempt has been made to align the edges of each individual tile. The resulting image misalignment is clearly seen. Panel B shows the same set of nine fields, acquired using alignment algorithms. (Image courtesy of Imaging Research.)

To get around these problems, scientists have developed systems that generate image montages. A basic montaging system drives a motorized microscope stage to contiguous microscope fields and acquires an image at each field. The user can then stitch these fields (also called tiles) together on the computer by using many types of software. Basic systems usually limit the number of fields (e.g., 16) that can be montaged, so that errors in stage positioning do not accumulate to a serious degree.

Basic requirements

More advanced montaging systems automate the process and yield much better images, relying on the actions of several key components. Motor stage control algorithms correct positioning errors from field to field so that these errors do not accumulate (Figure 1). Similarly, alignment algorithms perform precise edge mapping of the tiles and construct the montage automatically. The best systems do this during the montaging process so that the user can see the large image build in real time.

An autofocus system acquires large areas without user attention. Some montaging systems also include image combination (yielding great depth of field) and image deconvolution (digital confocal) functions (Figure 2). Intensity and color correction algorithms remove spatial variations in microscope illumination and camera detection. Left uncorrected, these variations tend to result in a patchwork montage, with each tile visible. Multimodal capabilities are also important because alignment, intensity, and color correction algorithms must be able to accommodate fluorescence and various bright-field modes of operation.

The best montaging systems create seamless, focused, and large-scale images com-

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pletely automatically. There should not be any limitation on the number of fields or on the type of microscopy being performed. For example, the montaging system developed by scientists at Imaging Research Inc. (*www.imagingresearch.com*) is used for everything from demonstrating neural organization to making multicolor fluorescence images of entire tumors.

Montages can be visually spectacular. The eye is seduced by what looks like an impossibly sharp photomicrograph, showing large-scale organization far exceeding what we are used to seeing from a microscope. Although digital montages are created for their scientific value, they are also a joy to behold, and many can be appreciated as art.

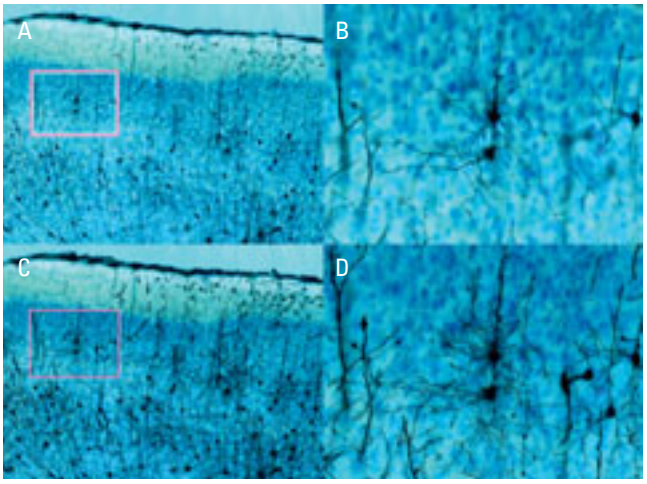

Figure 2. Montage mania. Twenty-five microscope fields (A, C) montaged with a 20× objective (counterstained Golgi preparation, section thickness ~60 µm). High-resolution views of the inset areas are shown in panels B and D. The montage at the top contains a single focal plane, whereas the one at the bottom uses both autofocus and image combination to increase depth of field, which is useful with this thick specimen. (Image courtesy of Imaging Research.)

Digital dilemma

Compare a digital microscopy image with a photomicrograph. The photomicrograph contains more detail than the digital image. Because most electronic cameras use a relatively small number of pixels (a few million is typical), they cannot resolve all of the detail supplied by the optics. In contrast, 35-mm film has far higher resolution (equivalent to about 12 million pixels in a single frame). Therefore, digital images tend to compromise resolution, whereas film photomicrographs are very close to what we see through the eyepiece. If a digital imaging system is to match photomicrography, it must represent the specimen with an adequate number of pixels, and there are several ways to increase the pixel count.

High-resolution cameras (e.g., 4096 × 4096 pixels) are becoming more cost-effective. As such cameras mature, we can expect digital photomicrographs to more closely approximate film. However, the camera is limited to the portion of the microscope field projected onto the detector. It cannot go beyond a single microscope field. There are also a limited variety of cameras (color and low-light capabilities can be difficult), they function slowly (transferring all those pixels from the camera to the computer takes time), and they tend to be costly.

Higher magnification is also useful because if we magnify the specimen, each part of it occupies more pixels and resolution is improved (Figure 3). Of course, this strategy has a negative aspect in that it curtails the field of view. Alternatively, we can use higher magnification to improve resolution, and retain field of view by montaging. This approach is cost-effective and allows the use of a broad variety of cameras.

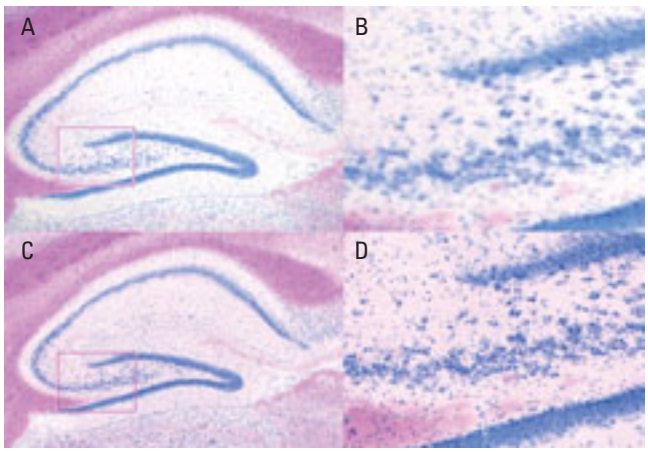

Figure 3. Resolving our differences. (A) Images of rat hippocampus acquired with a Sony DXC970 color video camera and a 4× objective (top) or montaged with a 10× objective (24 fields, bottom). Full-resolution views of the inset areas are shown at right. (B) Notice that individual cells and subtle colors are poorly rendered by the 4× objective. (C) The 10× montage is much better. (Image courtesy of Imaging Research.)

Various tools

One option for reducing the cost of true high-pixel-count cameras is to use standard 1.3-megapixel CCD chips that can interpolate and/or pixel-shift to increase the total pixel count and thus improve resolution. Cameras such as the Professional and Penguin lines from Pixera (*www.pixera.com*), AxioCam from Zeiss (*www.zeiss.com*), DXM 1200 from Nikon (*www.nikon.com*), and ProgRes C14 from Jenoptik (*www.jenoptik.com*) all work in this fashion. When used at the base image sensor resolution mode, these cameras take a single image. Color is achieved by placing a color mask over the CCD chip, with each pixel seeing only one of the three colors (red, green, or blue). Software-based interpolation algorithms fill in the remaining colors. The image resolution is equal to the number of pixels physically present on the CCD chip, typically 1300 × 1030 pixels.

For higher resolutions, up to 3900 × 3090 pixels, the camera goes into a scanning mode in which multiple frames are taken, and either the sensor array is shifted between frames or the incoming light is redirected by microlenses. This shift causes each physical pixel to be presented with different spatial location information. The resultant image is thus of a higher resolution than the physical size of the array. This approach solves the acquired image resolution but does not overcome the single field of view as seen through the microscope eyepiece.

With motorized stage control software, such as the Scope Pro from Media Cybernetics (*www.mediacy.com*), the researcher relies on the precision of movement in the X, Y direction of the motor stage to create a set of image tiles. These can then be stitched together with either third-party software or custom algorithms written by

the researcher.

Alternatively, in systems such as the Turboscan real-time mosaic imager system from Objective Imaging (*www.objectiveimaging.com*), the motor stage movement is synchronized to video camera acquisition, resulting in fast scanning speeds. Although the result is a scanned image that may need some additional alignment to remove any visible seams, a complete scan of a structure of interest containing more than 1000 images can be completed in less than 60 s at a 20× objective magnification.

Image stitching

Spatial errors accumulate as a motor stage moves over larger distances. The edges of two adjacent fields might be within a pixel, but the error will be multiple pixels three fields away. To account for this problem, researchers at Imaging Research developed an advanced montaging system, the image stitching system, as part of their MCID Elite and Basic image analyzers. The image stitching system includes algorithms that perform automated alignment of the discrete fields, and feedback regulation of stage precision, as the montaging proceeds.

Cameras, microscope optics, and illumination systems all contribute to intensity variations (~20%) across a field of view. There are also color variations that might not be obvious in a single field, but any intensity or color variation that repeats from tile to tile will form a pattern that is obvious in a montage. Thus, it is important that both intensity and color errors be corrected automatically. Similarly, residual tilt in the microscope stage is not noticed within a single field but is obvious across multiple fields of view. Point-to-point variations in specimen depth are present both within and across fields.

The image stitching system includes automatic corrections and accommodations that achieve sharply focused images. A tilt correction function adjusts for stage misalignment. Software autofocus makes several exposures and calculates the best plane of focus for each tile. Image combination convolves any number of planes of focus into a single sharp image. The image combination feature is particularly useful for thick specimens. The system uses any of the monochrome or color cameras that the MCID image analyzers support, including those suitable for low-light imaging of fluorescent or luminescent samples.

The big picture

Anyone who has spent hours making dreaded photomontages, in which multiple photomicrographs are cut and pasted together, tends to look for other methods. This has led to widespread adoption of digital montaging systems and the increasing desire for image stitching systems.

Further reading

Russ, J. C. *The Image Processing Handbook,* 4th ed.; CRC Press: Boca Raton, FL, 2002.

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