IMAGE ENGINEERING

(A scientific and image processing application usage manual for Scion Image for Windows)

General Information

About this Document

This is a "non peer reviewed" guide which attempts to cover the more typical and technical questions that users of Scion Image often need to address.

Image Engineering

Fundamentals of densitometry

It is possible to use a scaling system for pixels which has a one to one correspondence to the concentration of what you are studying. Sample concentrations can be determined using optical, electronic, and most importantly for our purposes, a computer based imaging technique. Densitometric science was described originally by Bouguer and Lambert who described loss of radiation (or light) in passing through a medium. Later, Beer found that the radiation loss in a media was a function of the substance's molarity or concentration. According to Beer's law, concentration is proportional to optical density (OD). The logarithmic optical density scale, and net integral of density values for an object in an image is the proper measure for use in quantitation. By Beer's law, the density of a point is the log ratio of incident light upon it and transmitted light through it.

$$OD = \mathrm{Log}_{10}(\frac{I_o}{T})$$

There are several standard methods used to find the density of an object or a point on an image. Scanning densitometers have controlled or known illumination levels, then measure transmitted light through an object such as a photographic negative. Since both the incident and transmitted light are known quantities, the device can then compute this ratio directly. This is also the case of those who use a flat field imaging technique and capture two seperate images. The first image is of an empty light box and the second is of the specimen to be evaluated. These two can then be used in computing a log ratio.

In the case of a video camera/frame grabber combination, using a non flat field technique, several things are of note. With a camera, you do not measure OD values directly. The camera and frame grabber pixel values are linear with respect to Transmission (T), which is the anti-log of the negative of OD:

$$T = 10^{-O.D.}$$

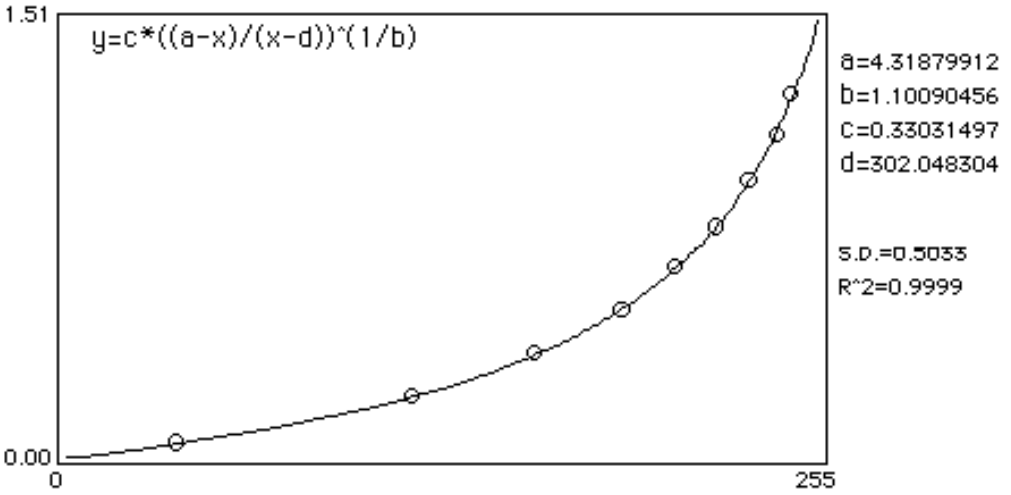
or:

$$O.D. = -\log_{10}(T) = \log_{10}(\frac{1}{T})$$

Since this is often a source of confusion among those designing systems for densitometry you should again note that the camera does not measure T, nor does it measure OD. Camera systems, CCD's, and any frame grabber conversion values (pixel values) have been designed so that they are linear with respect to T. It isn't meaningful to take the minus log of the pixel value since these are not T values.

Nevertheless, you want to do densitometry and need a scale (not pixel values) which correlates to concentration or OD. Further, it may not be convienient to measure the incident light and do a log ratio. Fortunately, you can use an external standard, such as an OD step tablet or a set of protein standards on a gel. Scion Image has the built in "Calibrate..." command to allow you to transform pixel values directly from a scale which is linear with respect to T and into a scale which correlates to OD or concentration. The calibrate command, used with standards, is best done with an exponential, rodbard or other fit since the relationship of OD to T is not a simple linear (y=mx+b) relationship (see equation above) and because the camera may not be perfectly linear with respect to T over the range of density values you use as standards. In other words you have both created a LUT of OD values for each linear to T pixel value and you help compensate for slight nonlinearities of the camera.

A sample calibration curve fit:



There are several other points of note which you should adhere to in performing your densitometric analysis. Your standards should always exceend the range of data which you want to image and perform density measurements on. You should not use curve fit data (or the pixel values) which extend beyond the last, or before the first calibration point. Additionally, there is a point at which the camera can no longer produce meaningful output when additional light is input (saturation). You could also have a low light level condition where the camera or CCD can not produce a measureable output (under-exposure). You will notice that these data points do not fit well into an end of the curve, or could basically ruin the fit of the data. You should remove these points from your calibration data and not use the density values for these pixels in your measurements.

Camera signal to noise (SNR) and significant bits

Camera signal to noise ratio is defined as the peak to peak signal output current to root mean square noise in the output current. Although this sounds confusing, the value describes whether the camera is one which can give you 256 significant gray levels or not. A formula to convert camera SNR into number of significant digits is very useful. The formula to do this is defined as :

$$SNR\ (dB) = 6.02n + 1.8$$

Where n will be the number of significant bits. You will need 50 or higher dB for the potential to convert a signal to 8 bits. In practice you will need a better camera than this to get 8 bits since it is likely most conditions for imaging are not ideal.

In order to get a true 8 bits, you will also need to capture the video using a frame grabber that does not introduce error. A frame grabber which has an analog to digital converter (ADC) with less than one half bit of differential non-linearity, specified at the video bandwidth, is needed to capture 8 significant bits.

Designing your own optimal threshold

If you do not like the automatic threshold which Scion Image picks for your data, you can implement alternative thresholding with a macro. If you want a consistently picked threshold point based on specific image parameters you might try the following technique. Find an appropriate threshold by trying a multiple of the number of standard of deviations past the mean. Running the macro below will help you do this. Once you are satisfied with the right multiple (can be decimal), code the number into your macro and execute it on all the images you want to analyze. If you have a border or other artifact which is not in all images, be sure to exclude this by using a ROI.

```
Macro 'Std Dev Threshold [T]';
 VAR
   Count, Threshold:integer;
   StdDev,TheMean,MultFactor:real
BEGIN
  ResetGrayMap;
{Pick a multiplication that works for your dataset}
  MultFactor := GetNumber('StdDevs past the mean to threshold?',1.0);
{
Hardcode the number in the macro when you find a good value
that seems to work well with your dataset
}
{MultFactor := 1.0;}
  ResetCounter;
  Measure;
  StdDev := rStdDev[rCount];
  TheMean := rMean[rCount];
{Apply the multiplication and threshold}
  Threshold := TheMean+ round(MultFactor*StdDev);
  SetThreshold(Threshold);
  Showmessage('Threshold level =',Threshold,'\\Using ',MultFactor:4:2, ' standard of
deviations','\from the mean');
```

```
END;
```

Cell Colony Counting macros

The technique described below relates to the cell colony counting macros.

Digitize cell colony.
If needed perform flat field and dark current correction to image.
Digitize ruler
Spatially calibrate image
Enter into software a minimum and maximum area for a single object to count. (i.e. anything less than min is dust or junk, anything greater than max is an overlap).
Perform background subtraction on image- use "Rolling ball" technique.
Convolve image with a Gaussian (5x5) kernal.
Threshold image-have software find optimal threshold via iterative histogram technique.
Count all particles >min and <max (singular particles), each particle is found using an automatic outlining technique (reproducible).
Find the average area of each particle >min and <max (singular particle average area)
Find the total area of particles >max- (overlapping particles).
Divide this area by the average area of singular particles.
Add the number of singular particles to the calculated value from the previous step step yielding the total cell colony population.

Convolution

From salzman@Athena.MIT.EDU posting on nih-image@soils.umn.edu

>Does anyone know what a good reference for determining how a given
>convolve filter will effect an image? Also the other way around- how to
>determine the proper array to achive a specific effect. Here I am talking
>about effects more suited artistic type stuff than image analysis.

What you see on the screen is called "2D direct space," while the representation of an image in terms of its frequency components is called "2D Fourier space."
Convolution of an image by a filter (in direct space) is identical to multiplication of the Fourier transform ("FT") of the image by the FT of the filter, followed by retransformation back into direct space.

All you need to do to understand the influence of a filter is to understand what its Fourier transform looks like. For instance, a piece of dust is a tophat function-- 1 at the center, falling suddenly to zero at some radial distance-- and its FT looks like a sombrero (actually, an Airy function, sometimes called the Sinc function)-- near 1 at the center, but vibrating around zero as the distance from the center increases. That is why you sometimes see concentric rings in high-resolution images: interference due to the wave-nature of photons.

There are some subleties due to the difference between complex amplitude and real intensity, but you can ignore them for the purposes of this discussion.

Useful Fourier duals, read either left-to-right or right-to-left, include
Tophat (Sin(X))/X [Sinc]

Exp(-AxX^2)	Exp((-X^2)/A) [Gaussian]
Delta function	constant
Cos(X)	DeltaFn(X/2)+DeltaFn(-X/2)
Sin(x)	DeltaFn(X/2)-DeltaFn(-X/2)
Sawtooth	((Sin(X))^2)/(X^2)

To perform the convolution on an image in direct space, you multiply a discrete representation of the filter, called a kernel matrix, by a stencil around each element of the image, sum, and assign the result to the coordinates in the image. Any real matrix can serve as a convolution kernel, but to conserve information, the sum of all the elements of the convolution kernel should equal 1. For instance, if the convolution is represented as the 3x3 kernel

```
A11 A12 A13
A21 A22 A23
A31 A32 A33
```

and the image is designated by the 10x10 array of pixels

```
B00 .. B90
 .. .. ..
B90 .. B99
```

then the convolution produces a 10x10 array of pixels C00..99, and

```
C62 := (  A11xB51 + A12xB52 + A13xB53
        + A21xB61 + A22xB62 + A23xB63
        + A31xB71 + A32xB72 + A33xB73
       )
```

and analogously for the other 99 pixels Cii. (When the stencil overhangs an edge, the overhanging elements are ignored (e.g. use a 2x3, 3x2, or 2x2 stencil as appropriate), and the remaining kernel elements should be renormalized to sum to 1.)

The FT of the FT of the function is the original function again, which means that the FT of a sombrero is a tophat. So, a reasonable "sharpening filter" is the convolution kernel

```
.25 -.5  .25
-.5   2  -.5
.25 -.5  .25
```

The FT of a thin bell curve (Gaussian) is a fat bell curve and vice versa. So, a good smoothing ("unsharpening") filter is the convolution kernel

```
.05 .10 .05
.10 .40 .10
.05 .10 .05
```

An good edge-emphasizing kernel sums to zero, not to one. Try

```
-1 0 1
-1 0 1
-1 0 1
```

for Western edges, and 90 degree rotations of it for the other compass directions.

From Norm Hurst norm_hurst@maca.sarnoff.com posting on nih-image@soils.umn.edu

What convolution does is add the image to itself at many different

displacements (as many as there are numbers in the kernel). The position of each number relative to the number in the center determines the [X,Y] displacement of a shifted image, and its value determines its relative contribution to the sum (that's why it's called a "weight"). The weight in the center determines the amount of the "main" (unshifted) image that comes through.

If a tap weight is negative, you get an inverted, shifted image added into the total.

For weird effects, use large kernels, sparsely populated (mostly zeros). Large displacements clearly appear as distinct images; smaller displacements just look like blurring (or sharpening, depending on the values).

Here's a 9x9 kernel with just a few values set to non-zero:

3	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	-4	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	5	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	4
0	0	0	0	0	0	0	0	0

This will give you a main image (the "5") and three other images, one of them a negative.

Even with this large matrix the displacement is slight. You need bigger kernels to get bigger displacements, but computation time goes up as the square of the displacement you are trying to acheive. I tried a 39x39, and you have to be patient.

Try cascading convolutions (convolve the result of a convolution).

At very large kernels you will notice that Scion Image automatically makes a selection of a subset of the picture; this is because when you get closer than half the kernel width to and edge, some weights "fall off" the edge, and it's not clear what to do.

I don't think the kernel must have and odd-by-odd size (but it must be square for some reason... but if your kernel is even-by-even, there is no "center", and your output image will be shifted a little (1/2 pixel).

For kernels whose taps add up to zero, Scion Image automatically adds a gray-level offset to the filtered data (because half of the pixels will be negative, and Scion Image only diplays positive numbers). No one knows how to display negative light :-)

Cross correlation

From russ@mat.mte.ncsu.edu reply on nih-image@soils.umn.edu

past questions and answers:

>> >Isn't plain old 'filtering' (ie convolution) fairly similar to
>> >cross-correlation, except for a constant multiple?
>>
>> I do not think it is the same. Spatial convolution is the same thing as
>> multiplication in the frequency domain. Cross-correlation, on the other
>> hand, is performed by convolution in the frequency domain.
>>
>correlation is multiplication by the complex conjugate in the frequency domain

Cross-correlation can be carried out in either the frequency or spatial domains with identical results. In the spatial domain, you would use the brightness pattern of one image as the filter, and apply it in the usual way to the second image. In the frequency domain, you just multiply as noted above. The spatial domain method works fine, but it is very slow when the target pattern gets large. If the things you are trying to track are small (less than about 7x7 pixels), the filtering approach will work fine. A word of caution - no matter how it is done, the cross correlation method assumes that the target has not changed, and in particular has not rotated as it moved. In many cases, such as surveillance tracking or recognition, it is necessary to use many different targets corresponding to all possible orientations of the object.

Background subtraction notes

From A. Eke <ekeand@kisk.sote.hu> posting on nih-image@soils.umn.edu

> Try multiple smoothing of the image, which effectively works as defocusing it. Then subtract the defocused image from the original. This yiled an image of no backgroung gradient. I do not know about some other disturbing details in the background that may bother you.

From Edward J. Huff huff@mcclb0.med.nyu.edu posting on nih-image@soils.umn.edu

Since you have the opportunity of collecting some more images from the sample, you should try to get an image that reflects the illumination intensity. Then this image can be subtracted from, or better, divided into, the original image. The best algorithm I have found so far is the flat field correction. You have to collect a dark image (no light on the camera, and adjust the gain and offset so that this image does not give all zeros) and a bright image (out of focus may do the job, or move the in focus sample around a lot while collecting the image) which reflects the amount of illumination at each pixel. The calculation is (sample - dark) * coef / (bright - dark). The integration time for the each pair of images being subtracted must be the same. If your bright image is collected for a different time than the sample, then it needs its own dark image.

For fluorescence microscopy images, this calculation is absolutely essential, because it converts the image from a record of how much light struck the camera at each pixel into a record of how much dye was present at each point of the sample. The assumption is that the amount of excited light from each dye molecule is proportional to the amount of illumination.

Using background subtraction is inadequate if the illumination varies by more than a few percent. For instance, if the illumination is twice as strong at the center, then after background subtraction, a spot of dye at the center will look twice as bright as the same spot moved to the edge. But after flat field correction, the two spots will look the same brightness.

Noise removal

From jvanheld@dbmdec5.ulb.ac.be (Jacques VAN HELDEN) posting on nih-image@soils.umn.edu

One way to remove noise from a gray scale image is to apply a median filter. This filter replaces each pixel from the source image by the median value of its neighbours. The effect is to remove all points that are darker or brighter than their neighbours, and thus to remove noise. By fixing the X and Y size of the median filter (the number of neighbours to take into consideration is X*Y), you determine the size of the noise you want to remove.

From (russ@mat.mte.ncsu.edu) posting on nih-image@soils.umn.edu

>A gray scale microscopic image of cells. In certain cells in the field there is
>a darkly stained structure of irregular shape that I want to measure the area
>of. When I threshold for the stained material I get a lot of non structure
>pixels througout the image (noise). The area of the noise is 6 pixels or less
>while the structures are much larger. I would like to filter out the noise. What
>I have tried is making a duplicate of the image and make it binary and then
>erode with a coefficient of 5 several times which gets rid of almost all of the
>noise and then AND the eroded image with the density sliced image, but I find
>that I have lost a significant portion of the strutures of interest. I think a
>filter based on size might solve my problem - or does anyone have other
>suggestions.

Two approaches may be useful:
1. as you say, measure everything and discard features based on some size criterion (area, length, etc.) This is probably the fastest unless the presence of too many little things bogs down the measurement process
2. use erosion with a suitable coefficient, but then follow it with an equivalent number of dilations. (Opening). This will restore the area of the larger features, more or less, but it will smooth their shapes somewhat.

From "Norm Hurst" <norm_hurst@maca.sarnoff.com> posting on nih-image@soils.umn.edu

Here's a macro for "coring" an image. First the image is high-pass filtered, then these highs are "cored", which means values near zero (you tell it how near) are set to zero, and values outside that range are moved closer to zero by the amount of the coring width.

A low-pass image is formed by subtracting the highs from the original, and the cored highs are added to the lows.

This approach works well if your image has edges separated by noise-infested flat areas.

Filters for color imaging

From "Tarja Peromaa" <peromaa@gra-gw.hut.fi> reply on nih-image@soils.umn.edu

>However, I am not sure of the best filter combination
>for capturing "real life" color images. Intuitively, one could think that
>the best filters are those with spectra close to the eye color pigments. But
>if I understood, red and green pigment are largely overlapping.
>Alternatively, one could choose three totally non-overlapping filters, but
>would the image then look like what we see ?

The absorption spectra of cone pigments are indeed overlapping. In addition, the spectra is weak at the blue end of spectrum. See for example Cornsweet (1970) Visual Perception, s. 171 for psychophysically derived estimates of the spectra.

One possibility is to use CIE color matching response functions (or "tri-stimulus curves") as reference. (However, the spectral response of your camera is probably non-ideal, so although Your filters were ideal, the camera would distort the measures.) These functions are used when perceived color is modelled (CIELab, CIELuv). I think that there are some scanners (or they are under development) that have filters with this sensitivity.

Color separation (used when color pictures are printed using halftoning) is performed using approximately gaussian filters (I don't know whether ideal profiles were better), with some overlap and the average spectral absorptions being 440, 540 and 620 nm. Color separation filters are narrowish compared to tristimulus filters.

If the spectra overlap considerably, there leads to a situation, where saturated colors are difficult to reproduce, because tight and wide spectra of original colours appear similar to the filters. For saturated colors, tight transmission spectra of the color separation filters are requested, but then the system does not distinguish all the colors (those remaining between the filters.) (Saarelma & Oittinen 1994: Fundamentals of printing technology. Helsinki University of Technology, Laboratory of Graphic Arts Technology, s. 95). (Probably this book also handles these questions, Hunt 1987: The reproduction of color. Fountain Press: England.)

The SBIG ST-6 ccd-camera performes color imaging with a color filter wheel, where the passbands are 585-680, 496-585, <380-502 nm. This instrument, however, is ment for astronomical purposes, so the bands may not optimal for

other purposes.

From russ@mat.mte.ncsu.edu reply on nih-image@soils.umn.edu

>Does anyone know how to choose the best spectra for the R, G, and B
>filters ?

"Best" is a tough word. As a practical matter, I have for a long time been acquiring separate RGB planes through Kodak Wratten filters 25=Red, 58=Green, and 47 or 47B=Blue, and then combining them as color planes in Photoshop. One advantage of using these filters is that you can get them at practically any camera store, they are cheap, and available as plastic sheets that can be cut to fit. Much simpler than trying to find custom dichroic filters, or getting glass filters in the right size.

Many of my images are "real world" rather than microscope, and so the criteria for judging the truth of the colors are probably more critical (visual observers are used to seeing by "white" sunlight, but microscopists know that the color values are easily changed by the color temp of the light source, or filters, etc.). In most cases, the simple combination of the three planes produces a quite viewable and acceptable color balance that observers find realistic. In cases where the color balance appears to be "off" it usually appears just a little too blue, and a simple adjustment with the Photoshop controls can be made.

From Nick Safford (NSafford@scigen.co.uk) reply on nih-image@soils.umn.edu

The eye has dreadful sensors in it, as far as red-green discrimination is concerned. [If you look at the spectral sensitivity of the various cells (measured in a rather indelicate experiment involving a liquidizer and a darkened room) the raw data give a distribution of spectral sensitivities such that, in some cases, you'd be hard pushed to decide whether to class a given cell as red or green sensitive]. The brain (or retina) sorts this stuff out, but if you have a free choice of filters to use, you'll benefit from better separated channels.

Irritatingly, I'm not going to make specific recommendations, but I'd suggest you could look at what filters would be best at separating the colors you're trying to distinguish. There are tricolor filters available (have a look in the Wratten catalog from Kodak). These are optimised for interacting with film, but they do a good general purpose job. I don't know if you can readily get them in a form suitable for putting in you microscope. On the other hand, if you're trying to distinguish (say) two stains, you could just pick a filter that blocks one of them and not the other. I guess I'd call it pseudocolor microscopy (sorry, I just made it up, but I'm sure it's been done before and has a name). Then you might need only two exposures (or even one) to do the job. The results wouldn't be unlike the infra-red satellite images you sometimes see.

PS - I don't have the exact reference for spectral sensitivity of the human eye, but I think it was one of two books called "Human Color Vision" (published in New York) and "Colour Vision: physiology and psychology" (or something of the like, possibly published in Britain, given the spelling).

Polarising filters with CCD's

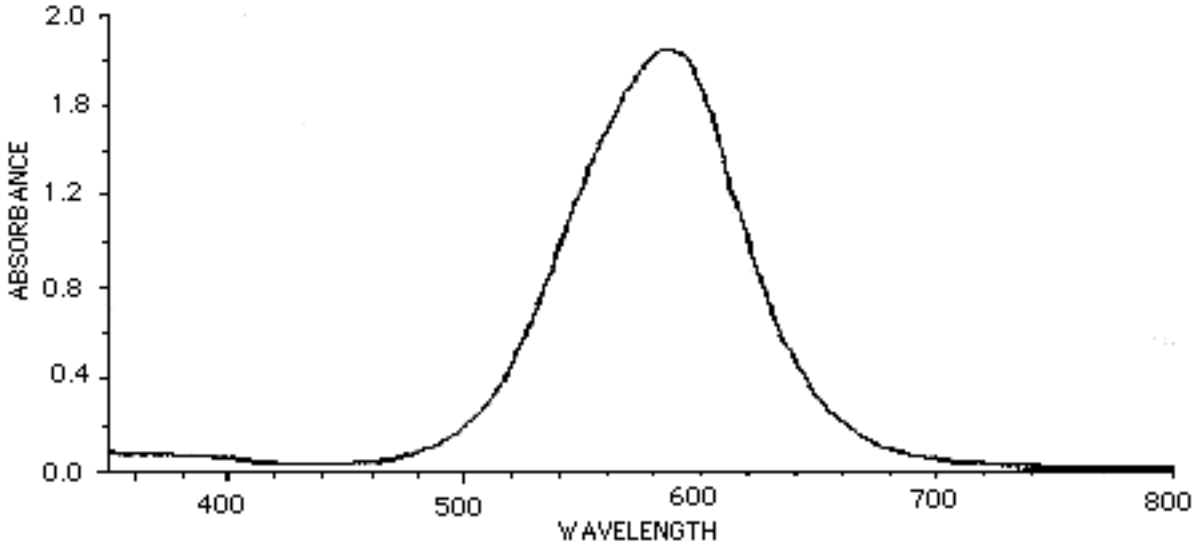
From Don Seltzer <Don_Seltzer@qmlink.draper.com> reply on nih-image@soils.umn.edu

Specular Reflections
>Try a polarising filter over the camera lens. Rotate this until the
>specular reflections are cut out.

Warning: Most CCD cameras are responsive to near IR wavelengths. Many polarizing filters, particularly the plastic type, do not polarize in this range. I learned the hard way. After setting up filters on both the light source and camera, and verifying by eye that the glare was eliminated, I was still getting a camera image with a glare reflection. My solution was to insert an additional blue or yellow filter in front of the camera, passing only the polarized visible wavelengths.

Infra-red & Coomassie blue filters

There are filters you can place in front of a camera lens to give complete rejection of the IR (>700nm) wavelengths. This is often crucial in video acquisition using CCD cameras. Filters which show 0% transmittance above 700 to 800 nm are available and recommended over those which show 10% or more transmission at these wavelengths. There are also several filters that compensate for the poor video response in the wavelengths associated with Coomassie blue stains. The graph below represents a pass of Coomassie brilliant blue in typical solution through a spectrophotometer. The peak absorbance is about 585 nm.



Useful handheld debounced pushbutton

The circuit below is a useful handheld external trigger for frame grabbers or other devices. It can be adapted in a number of ways to debounce the signal from an instrument to the quickcapture or other frame grabber. The top half is for battery power, the bottom half does the debouncing.