1. Image Optimization

Most blots after scanning will require some adjustments in order to visualize the relevant portions of the images on the computer monitor. This is necessary because the raw images consist of 16 bit tiff images, in which each pixel can range in intensity between 1 and \(2^{16}\) (or 65,536). By contrast, the computer monitors on which these data are displayed can only show \(2^8\) (or 256) different shades of black (or green or red), and these image adjustment tools are designed to allow you to optimize the contrast between your bands and the background of the blot. Importantly, these image adjustments will NOT affect the quantification of the images (the raw 16 bit images are not altered by the software, and these are the images from which quantification information is calculated). Image adjustment is done with either the “Alter Image Display” or the “Adjust Image Display Curves” tools of the Odyssey software.

The “Alter Image Display” tool (shown in Figure 1 on the right) is a series of sliders that allow you to change brightness, contrast, and sensitivity, as well as the ability to hide the 700 or the 800 channel, and/or view a single channel in black and white mode. For details on the other tool (Adjust Image Display Curves), see pages 213-218 in the Odyssey 3.0 User’s Guide.

Note: if the colorized images of the blot contain any white pixels, or if the gray-scale pictures contain cyan (light blue) pixels, these pixels are saturated and beyond the limits of the system to accurately measure. To quantify this blot, the blot MUST be re-scanned using a lower Intensity setting for the channel in which the saturation occurred (see the Odyssey QuickCard on Starting Scans).
2. Set Background Subtraction

For accurate quantification of a fluorescent western blot, you MUST have background subtraction set. This is because the membrane itself will always have some variable level of autofluorescence which will add to the signal obtained from each region of interest on the blot. The background subtraction button is found here:

The background subtraction dialogue box (Figure 2) has several options, but the preferred method is “Median.” This method calculates a local background subtraction independently for each quantification box that you’ll draw on this image, and therefore corrects for spatial variation in the background on the blot. Although the “Average” method also uses a locally generate background subtraction, it is more susceptible to stray high-fluorescence pixels from things such as dust particles, uneven blocking, or nearby bands on the blot. With either the “Median” or “Average” method, the software calculates background from the pixels forming a border immediately surrounding the quantification box. Depending on the proximity of other bands on the blot, this border of pixels can be optionally changed from 1-5 pixels wide, and can also be chosen from all 4 sides of the quantification box, only the top and bottom border of the box, or only the left and right sides of the box.

3. Draw Quantification Boxes

Quantification boxes (or other shapes) can be drawn using the “Add rectangle” (or circle, ellipse, freehand shape) tool on the left side of the Odyssey screen. The shapes drawn by these tools will quantify the relative fluorescence enclosed within them for the channel of data which is currently being viewed. For bands in the 700 channel you will first need to change the viewing mode to “View single channel” using this button: , which will default to the single channel 700 image; for bands in the 800 channel you need to first enter the single channel viewing mode and then press the “View channel 800...” button: . Then click the add rectangle button (see image in the left margin) to add a single box surrounding the left-most band on your blot. The quantification shape that is drawn will be background subtracted according to the
settings chosen previously, which means that the size of the quantification box will have a negligible effect on the data calculation. The box should of course enclose all of the visible fluorescence in the band of interest, but should not enclose fluorescence from bands from adjacent lanes (or from bands of different molecular weights in the same lane).

Although cutting and pasting the same quantification box will allow you to add more of these boxes, a convenient feature to perform the same function is the “Add multiple features” button (Figure 3 on the left), which will open a dialogue box prompting you to enter the number of new features to add, as well as the option of what you’d like to name those features. Change the number to reflect the number of new boxes you’d like drawn (excluding the one you’ve already done) (Figure 4):

After hitting the continue button, the mouse pointer will change into cross-hairs, and double-clicking the cross-hairs on the last band will draw the remaining boxes which will be spaced evenly between the first and last bands on the blot.

To ensure that the imaginary border surrounding each quantification box is an appropriate location from which to generate background values, one can zoom into this region for each individual box using the “Details View”. This border should be relatively free of stray fluorescence or other bands that are visible on the blot. To bring up the details view one must first select a quantification box and then click on the “Details View” button. If unwanted fluorescence occurs in this border, one can either manually change the border boundaries, shrink the border width by 1 or more pixels (in the Background subtraction dialog box), or draw a new box somewhere on the blot and set this as the user defined background.

4. Export Data

The background-subtracted quantification numbers generated in the software are called “Integrated Intensities” (I.I.), and these reflect the relative fluorescence of each band present on the blot. As long as there is no saturation (white pixels) in the image, these relative fluorescence values correlate directly to the relative amounts of IRDye® labeled secondary antibodies on the blot, which in turn will (usually)
correlate in a linear fashion to the amount of primary antibody on the blot, which in turn will be correlated in a linear fashion to the amount of your target protein on the blot. Thus, the I.I. values will serve as good surrogates for the relative expression levels of these targets.

To export these values from the software, first you must select the boxes whose values you’d like exported. The easiest way to do this is to use the hotkey function (Press “Ctrl” and “A” at the same time). Alternatively one can use the mouse and the left mouse button to drag a box around all the boxes whose values you’d like exported. Next, choose the Report View function to obtain a tab-delimited text output of these data (Figure 5).

With the Report Dialogue box opened (see Figure 6), the “Export…” button will save this file as a tab-delimited text file that can be opened with Excel. The “Launch Plug-In…” button (also available from the main software menu) will send these data directly to excel, but on most computer systems the location of the excel software must first be defined in the Odyssey software (see pages 201-202 in the Odyssey User’s Guide). Note that these reports often contain a great deal of unnecessary data, so the “Edit…” button may be used to change the fields that are exported in these reports.

5. Export Images

Images can be exported in a number of formats, but the most utilitarian method is to export them as 8 bit tiff images using the “Export Image View” function, which can be found in the menus as shown in Figure 7. The export image view tool can export images as 300 dpi publication quality images. Note also that this function will export an image of exactly what is being shown in the image view window of the Odyssey software. Thus, to get an image like that shown in Figure 8 (top of next page) that excludes the empty space from the blot, one would need to shrink the viewing window within the Odyssey software before using the export image view function.
There are two other noteworthy tricks for exporting images. First, you may want to toggle off the quantification boxes before using the export image view, using this button:

Second, you may want to export these images in black and white, in which case you will need to switch to single channel mode for viewing the image (button 1, left), and then switch to black and white while in single channel mode (button 2, left).