



Version 6.0

Application Guide: Quantitative Autoradiography

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Quantitative Autoradiography

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Introduction

This document is a supplement to the online *AIS™ Reference Manual*. It describes how to use **AIS** to quantify radiolabeled tissue sections that have been exposed to autoradiographic film or phosphor plates. When images are calibrated appropriately, data may be expressed in isotope concentrations (e.g., nCi/mg tissue wt.) or ligand concentrations (e.g., fmol/mg tissue wt.).

Please note that **AIS** also includes a specialized ‘Study Type’ for receptor binding studies. When enabled, it allows simultaneous sampling of Total Binding and Nonspecific Binding specimens, and automatically calculates a ‘Specific Binding’ and ‘% Specific Binding’ value. If you are performing these kinds of studies, please refer to the *Receptor Binding Application Guide* for instructions. Refer to the current document as a general guide to quantitative autoradiography.

Separate *Application Guides* are also available that describe how to use **AIS** to measure rates of glucose metabolism, protein synthesis, or blood flow from autoradiographic images. Any of these *Application Guides* can be downloaded from our web site, at <http://www.imagingresearch.com/documentation>. If your **AIS** computer is connected to the Internet, simply select *Help > Manuals web site* to connect.

Basic Procedure

The basic procedure for quantifying autoradiographic images is as follows:

1. Adjust camera magnification and focus.
2. Establish a **Flat Field Correction** to correct for uneven illumination.
3. Establish a density calibration.
4. Sample any/all specimens on the same film/plate as the density standards.
5. Repeat Steps 3-4 for all remaining films/plates.
6. Summarize and save the data.

If you are working with phosphor plate images, Steps 1-2 do not apply.

Step 1: Adjust Magnification and Focus

Magnification is controlled by moving the camera up or down. Place a sheet of film on the light box and adjust the camera height while digitizing. With a 55mm lens, you should be able to move the camera close enough so that a coronal rat brain section will almost fill the entire field of view. If the camera is too close to the specimen, you will not be able to generate a clearly focused image.

If higher magnification is required (e.g., for mouse brain or spinal cord sections), use extension tubes. Extension tubes are little barrels that connect between the lens and the F-C adapter. They function like a bellows to increase magnification of the lens. Various

sizes of extension tube give different magnification factors. For example, coronal section of spinal cord can be made to fill the screen when all three tubes are used.

Step 2: Establish a Flat Field Correction

Camera images typically exhibit some intensity variation over the field of view due to uneven illumination. This “shading error” requires correction to less than 1% if accurate densitometry is to be performed. The **Flat Field Correction** compensates for shading error.

TO ESTABLISH A FLAT FIELD CORRECTION:

1. Remove the film from the field of view.
2. Select a pseudocolor look-up table (i.e., SPECT2.VIS)
3. Adjust the illumination intensity on the light box so that a blank field of view appears bluish-pink. Be sure to use a true blank field, not film background!
4. Ctrl-click on the **Digitize** icon and select **Flat Field Correction** from the list of *Input Device Operations*. If the operation is not present, click the **[Customize]** button to add it to the list.
5. A *Flat Field Correction* dialog box will appear (Figure 1). An **[Acquire]** and **[Clear]** button are displayed at the bottom of the dialog box, but only one of them will be active. If the **[Clear]** button is active, it means that a flat field correction is already in effect. Press **[Clear]** to remove it.
6. Select **Digitize** as the correction source and **Pixel by pixel** as the correction method.
7. The **Frame average** option will reduce the contribution of random electronic noise to the shading error. Three or four will usually do, unless you disable the **Smoothing** and **Median** filters (see below).
8. By default, a **Smoothing** and **Median** filter is applied to the shading error reference image (i.e., the blank field image) to reduce spatial variation and improve the signal-to-noise ratio. If you choose to disable these filters, be sure to use the **Frame Averaging** option when establishing the flat field correction, and increase the number of frames to 16 or more.
9. Press **[Acquire]** to establish a flat field correction.
10. AIS scans the blank field and determines how much each pixel deviates from the mean gray level value. Subsequent images are automatically corrected for uneven illumination, based on this error pattern.
11. Click **[OK]** to exit the dialog box.

Please note, the image that you see after you exit the dialog box *is not* a corrected image. The **Flat Field Correction** is not applied until the next time you digitize. If you wish to

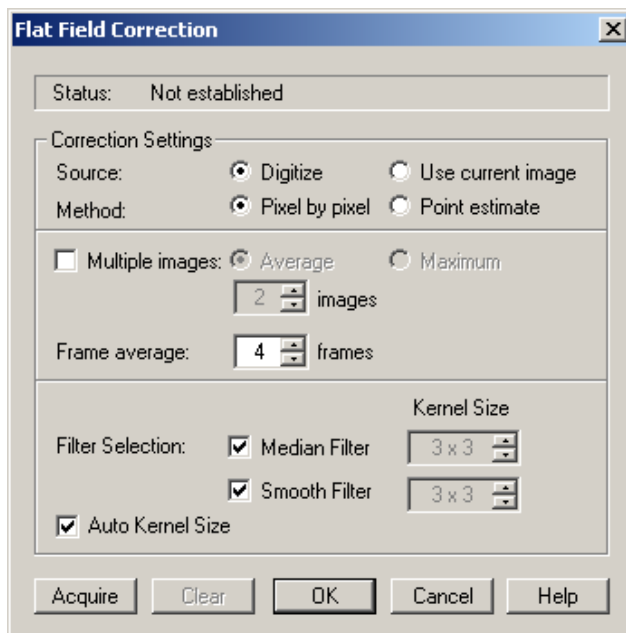
see the effects of the correction, digitize the same blank field again. The image will ‘even out’ as soon as digitization is terminated.

Once established, the flat field correction becomes part of the camera’s default configuration. Unless disabled or modified, **AIS** will apply the same correction each time the particular camera is used, even if you have exited and re-started **AIS**. As such, you should establish a new **Flat Field Correction** at the beginning of each analysis session.

One should also re-establish a **Flat Field Correction** whenever there is a change in ambient lighting, camera position, focus, camera gain and offset, light box position, or any other major factor that might affect the illumination pattern. Small changes in illumination level do not usually need a new correction, however, providing that optical system components are not moved relative to each other.

To remove or modify the current flat field correction, click the **[Clear]** button on the *Flat Field Correction* dialog box. Press **[Acquire]** to establish a new one.

Figure 1: The Flat Field Correction dialog box.



Step 3: Establish a Density Calibration

To express autoradiographic data in units of isotope or ligand concentration, you must co-expose a set of isotope concentration standards (e.g., Amersham™ Microscales™) with your tissue sections. A standard curve is then constructed, that relates the system’s internal density measurement units (e.g., ROD, PSL/mm²) to the standard values. This is achieved by entering the standard values into a calibration table and reading the density of each corresponding standard. As each standard is sampled, **AIS** plots the standard curve (density vs standard value) next to the calibration table. The standard curve should span

the entire range of densities found in the specimens under study. At least one set of standards should be also present on each film or phosphor plate.

For film autoradiography, the general procedure is as follows:

1. Digitize the standards. Adjust the illumination level so that film background appears pinkish-blue with the SPECTRAL.VIS or SPECT2.VIS look-up table. This step ensures that you are maximizing the dynamic range of the system.
2. Construct a calibration curve. Enter the standard values into the calibration table and sample the standards.
3. Select a curve fitting function.
4. Select an extrapolation function. This step tells **AIS** what to do with pixels that are outside the range of calibration values.

For phosphor plate autoradiography, Step 1 is unnecessary.

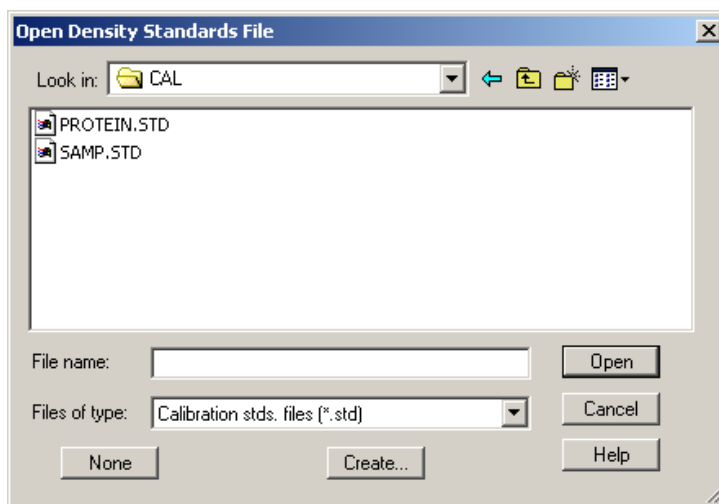
Building the Calibration Table

Click the **Calibration** icon to open the *Calibration* operational window:



Open the *Establish* menu and select the *Density* option. Change the density unit to **Cal stds**. A dialog box now appears, asking you to select a density standards file (Figure 2). To load a file from disk, select the file and click on **[OK]**. If you want to type the values into the calibration table, click on **[None]**. To create a standards file, click on **[Create]** (see the [Advanced Details](#) section for instructions).

Figure 2: When you select a calibrated density unit (e.g., *Cal stds*), AIS asks for a file that contains the values of the external standards.



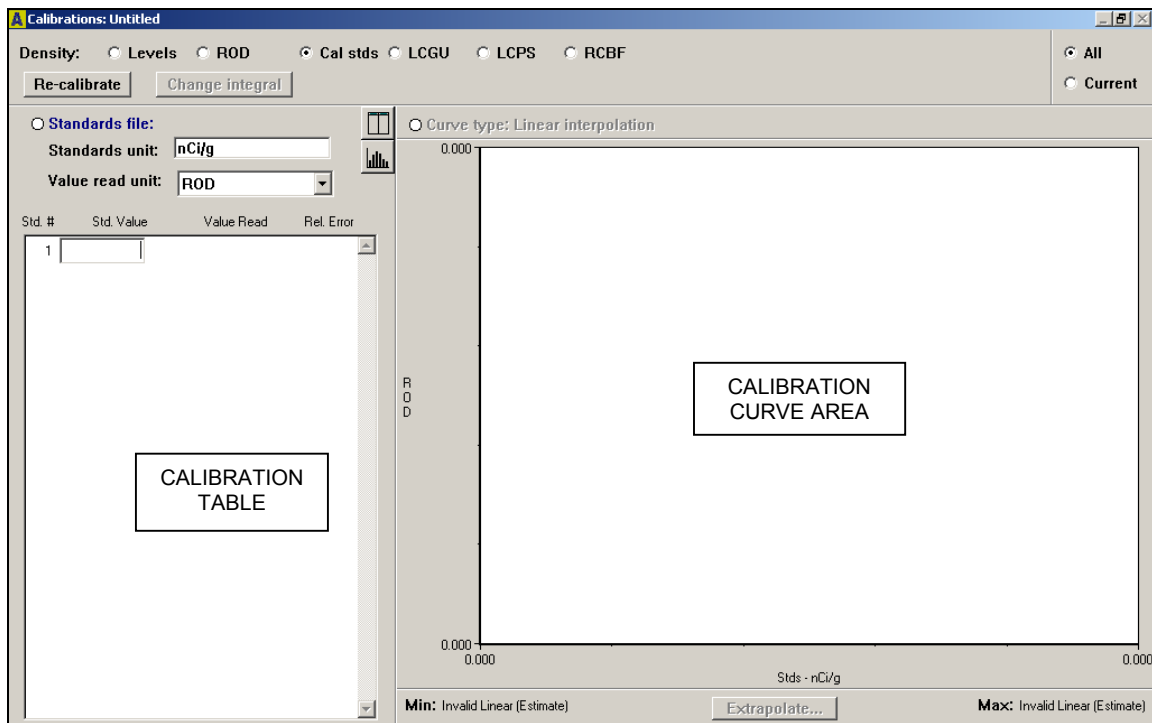
Entering the Standard Values

When you click on [OK] to leave the *Open Density Standards File* dialog box, the *Calibration* window appears as it does in Figure 3. On the left side of the window is a table with four columns labeled **Std #**, **Std Value**, **Value Read**, and **Rel Error**. Just above the table are two entry fields, one for the **Standards unit** and one for **Value read** unit. To the right of the table is the region where the calibration curve (e.g., ROD vs standard value) will be plotted.

If you are using a density standards file, the standard values are displayed in the **Std Value** column. Otherwise, type the values of the standards into this column and enter the appropriate concentration units (e.g., nCi/g, fmol/mg) into the **Standards unit** entry field. We recommend using a “0” as the lowest concentration standard. When you have entered all of the standard values, click the cursor in the **Value Read** column next to the first standard value.

Note: When entering the standard values, make sure you use ‘tissue equivalent’ values and that they are appropriate for the thickness of your tissue sections.

Figure 3: The Calibration window.



Sampling the Standards

Select a pseudocolor look-up table (SPECTRAL.VIS or SPECT2.VIS) and digitize the standards. A pseudocolor display makes it easier to see the appropriate light intensity settings for background and standards. It is important to optimize the lighting of your specimen. Optimal lighting varies with film density but, ideally, there is a range of less than 1 ROD between the darkest and the lightest areas of the film. In this case, blank film would read about .05 ROD in an 8-bit image (appears pinkish blue in the pseudocolor

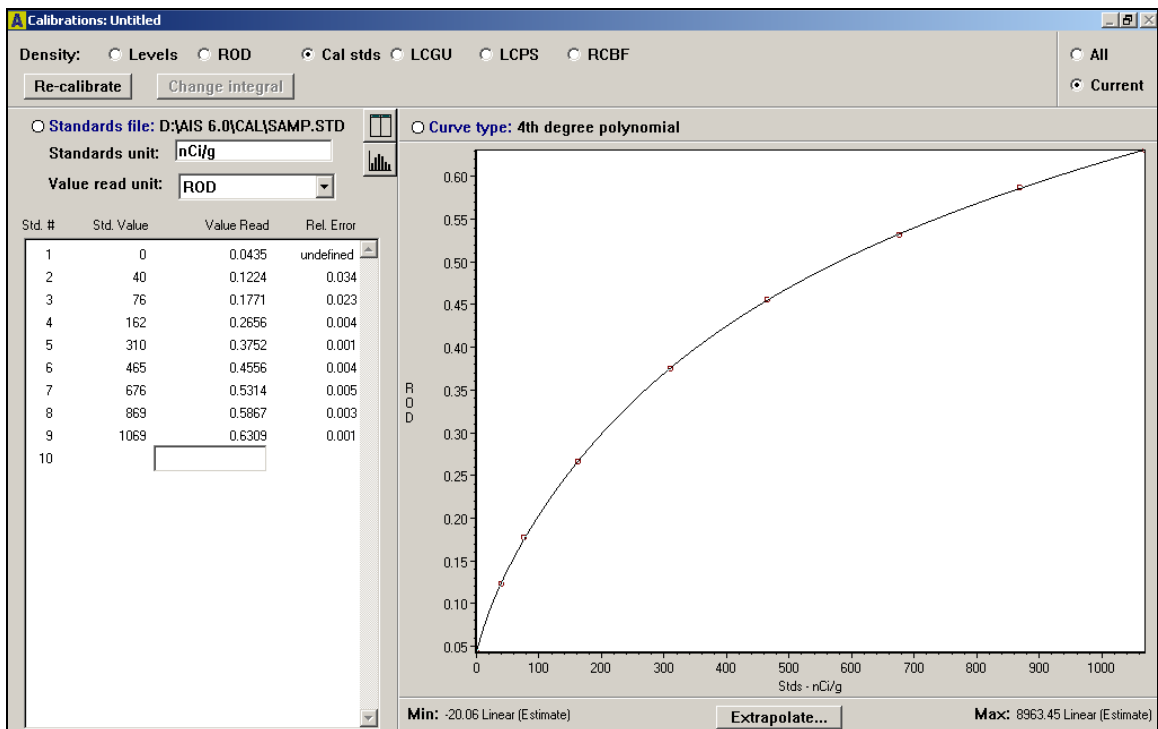
image), while the darkest areas of the autoradiograph read about 0.9 ROD (appear dark red). Do not read from film areas that are so light that AIS sees a maximum light value (white). If the film background is pink with many white speckles (saturated pixels), turn down the illumination a bit.

Note: Small changes in illumination level do not usually require a new **Flat Field Correction**, providing that optical system components are not moved relative to each other.

The lightest standard should be a film background reading, entered as a zero concentration in the **Std Value** column. The darkest standard read should lie within a fairly linear region of film response. Starting with the lightest standard, sample each one in order of ascending concentration. Re-digitize and re-position the standards as needed (do not change the camera height). Use the **Box** sample tool, and adjust its size so that it fits just within the edges of the standard. As each standard is sampled, its density value appears in the calibration table in the **Value Read** column, and the entry field automatically shifts to the next position on the list. A calibration curve is plotted to the right of the calibration table (Figure 4).

If you are calibrating a phosphor plate image, simply sample the standards in the order that they appear in the calibration table. The lightest standard should be a plate background reading, entered as a “0” concentration in the **Std Value** column.

Figure 4: A completed density calibration.



Curve Fitting

Once you have constructed the calibration table, you need to fit a curve to your data points. Use the **Curve type** option to select an interpolation or approximation function that calculates density values that lie between the steps provided by the calibration reference (Figure 5).

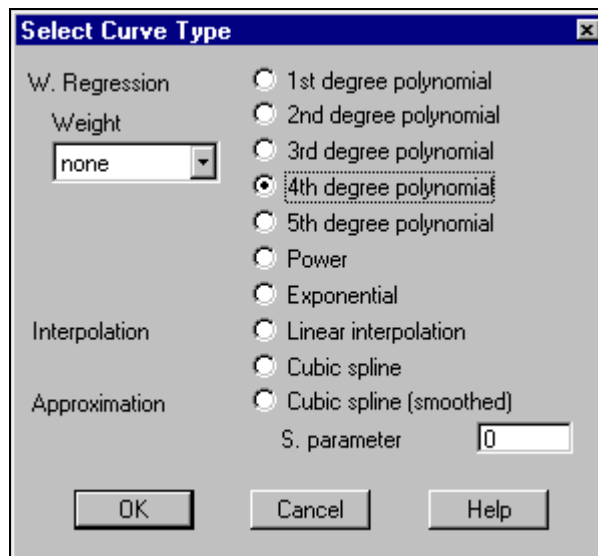
The graph shows how well a selected fitting function fits the concentration values of the reference. A numerical estimate of goodness of fit is available from the **Rel Error** column in the calibration table, where:

$$\text{Relative Error} = (\text{standard value} - \text{curve value}) / \text{standard value}$$

With any good fit, the error proportions will be low (< 0.1).

Experiment with all of the fitting functions. If you are using highly linear devices, such as phosphor imagers, you may prefer one of the linear regression models. Our own favorite for both linear and non-linear scanners is the smoothed spline, which offers significant advantages relative to the unsmoothed spline. The unsmoothed spline is a cube root function that goes through all the points. Because it accommodates to standards that do not fit a cube root polynomial, the unsmoothed spline may contain abrupt changes in slope. Smoothing the spline allows it to deviate from all of the points, except for the lightest density reference (the X,Y origin of the calibration). The smoothed spline is forced through the lightest reference.

Figure 5: Interpolation or approximation functions are used to calculate concentration values that lie between those contained in the standard set.

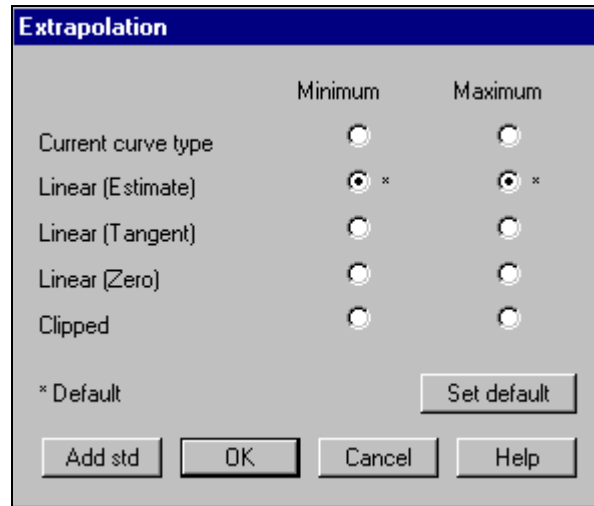


Extrapolation Functions

By default, **AIS** extrapolates calibrated values for pixels that are out of range (i.e., that do not fall within the calibration curve). A variety of extrapolation functions are available, and they can be applied to either end of the calibration curve. Click on the **[Extrapolate]** button located at the bottom of the calibration curve. This displays the *Extrapolation*

dialog box, in which you can either select different forms of extrapolation or turn extrapolation off (i.e., clip pixel values to the minimum or maximum standard value).

Figure 6: Density values beyond the range of standard values are estimated by extrapolation of the standard curve. You can apply different functions to the maximum and minimum ends of the curve.



Validity of Density Calibrations

Density calibrations are valid only under a very narrow range of conditions. ROD values, for example, will fluctuate in response to changes in illumination, lens aperture settings, camera height, camera settings (gain, offset, exposure time) and even to changes in ambient lighting conditions. ROD values will also fluctuate from one sheet of film to the next. A density calibration is valid for any specimen that was exposed on the same sheet of film as the reference scale and imaged under the same optical conditions that were in effect when you established the calibration curve. Re-calibration is necessary whenever any of these conditions change.

If you are calibrating phosphor plate images, density calibrations are valid for any specimen that was exposed on the same plate as the reference scale.

Step 4: Sample the Specimen

To gather density data, move to the *Sample* operation window, select a **Sample** tool, and click it on the image. See the online *Reference Manual (Chapter 4: Collecting Data)* for complete descriptions of **Sample** tools and instructions for their use.

If necessary, use *Visuals* controls to make features of interest more visible when sampling. You can change the way an image looks by selecting a different look-up table (LUT), by enhancing contrast, or by changing the LUT mapping function. Although these *Visuals* manipulations change the way an image looks, please remember that none of them change the actual density values of the image pixels. See the online *Reference Manual* for details (*Chapter 12: Altering Image Appearance*).

Calculating the Density Value

Density data are reported in the data table in calibrated units. **AIS** calculates the density value of a sample by first calibrating each pixel and then taking the mean of the calibrated pixel values. This is called “integrated optical density”, or IOD:

$$\text{Integrated Optical Density} = \text{Sum of the calibrated pixel values} / \text{number of pixels}$$

This is the most accurate way to calculate density values and is appropriate whether you are working with internally calibrated units (e.g., phosphor plate imagers) or with calibrations to external density standards.

When Density Data are Displayed in Red

Occasionally, calibrated density data may be “flagged” in red. This means that some of the pixels in the sample have density values that do not fall within the range of values established for the calibration curve. See the [Advanced Details](#) section for more information.

Labeling Data

AIS organizes data in a hierarchical fashion as they are gathered:

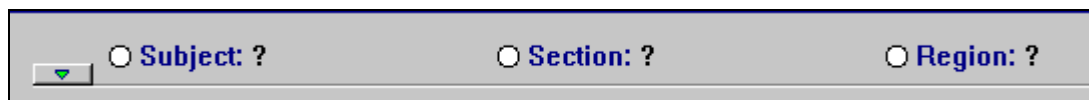
- Subject** Animal/subject used in study.
- Section** Tissue section from subject.
- Region** Anatomical region within tissue section (e.g., “thalamus”, “cortex-left”)
- Target** Area of an image sampled under a discrete Region label. A target is outlined or defined by a Sample tool.

Enter the appropriate **Subject**, **Section** and **Region** labels as you gather data. “Targets” are numbered automatically.

TO CREATE LABELS:

1. Click on one of the **Subject**, **Section** or **Region** headings. A label dialog box appears on the screen. Each label type has a specific dialog box.
2. Enter the **Subject** (optional), **Section** (optional) and **Region** label in the entry field of the relevant dialog box.
3. Sample the region corresponding to this label.
4. Enter a new **Region** label and continue until you have all read regions in the section.
5. Repeat steps 2 - 4, updating the subject and section labels as required.
6. Click on the **[Done]** buttons to close the dialog boxes.

Figure 7: The basic set of labels for managing data from sectioned specimens.



For more information, see *Chapter 10: Data Management* in the online *Reference Manual*.

Redirected Sampling

Anatomical landmarks are often difficult to visualize in autographic images, or they may be absent altogether. If this is the case, use the **AIS channel linking** feature to perform re-directed sampling from a counterstained tissue section. Re-directed sampling allows you to define regions of interest in one channel and gather data from the same coordinates in another. For example, you could load an image of a counterstained specimen into Channel 1 and its corresponding autoradiographic image (aligned with the counterstained specimen) into Channel 2. To perform redirected sampling, use the *Sample > Advanced > Channel Linking* command to direct **AIS** to report data values from Channel 2 only. You then view Channel 1 and place your target outlines there using the counterstain to define anatomical boundaries. **AIS** replicates the target outlines from Channel 1 and reports density data from the autoradiograph in Channel 2.

See the [Advanced Details](#) section for more information about re-directed sampling and aligning images.

Step 5: Calibrating and Sampling Remaining Films/Plates

A set of concentration standards should ideally be present on every sheet of film or phosphor plate used in the study. Re-calibration is necessary whenever you change films or load a different phosphor plate image. To establish a new density calibration, go to the *Calibration* operation window and click the **[Re-calibrate]** button. **AIS** clears the existing calibration and is ready to receive a new one. Repeat the procedure outlined in [Step 3: Establishing a Density Calibration](#), above.

Once you have established a new calibration curve, **AIS** will convert the density value of each pixel that you *subsequently* sample to a value that corresponds to the new calibration. Data gathered under previous density calibrations are unaffected.

Step 6: Summarize and Save the Data

AIS contains a number of sophisticated data tracking, reporting and management facilities. If you label your targets as you gather data, you can create a variety of different data display formats that allow you to summarize data across and/or within Subjects, Sections and/or Regions. The **AIS** imaging system's data tracking capabilities can even show you where a given number comes from in an image. You can save data to disk as a proprietary, binary "log" file (*.lg2), or as standard text or spreadsheet files.

It is beyond the scope of this *Application Guide* to present all of the **AIS** imaging system's data management features. A few particularly useful summary configuration and saving options are presented below, however. For a complete description, see the online *Reference Manual* (see *Chapter 10: Data Management*).

Summary Data Displays

Two sets of controls are used to create data summaries. The first is a set of radio buttons underneath the **Subject**, **Section** and **Region** label indicators. The second is a set of four ‘fly-out’ icons at the top right of the data table. Only one of these icons is visible at a time, and it is labeled as **Select table**.

All Targets

The AIS imaging system’s default summary format is the **Target Table**:



The **Target Table** is the most detailed form of data display, as it shows a discrete line of data for each individual target. By default, the AIS data table lists only the data from the **Current Subject**, **Current Section**, and **Current Region**. To view all of the data you have gathered in one table, change the radio buttons to **All Subjects**, **All Sections**, **All Regions**. Every sample is subsequently displayed by its **Subject**, **Section**, **Region** and **Target** label (Figure 8).

Figure 8: Part of a Target Table display showing data gathered from All Subjects, All Sections and All Regions.

Subject	Section	Region	Target	Dens - umol/100g/min
SUB1	SEC2	REG1	1	32.65
			2	42.08
		REG2	1	180.19
			2	196.11
		REG3	1	126.16
			2	132.84
	SEC3	REG1	1	37.45
			2	43.91
		REG2	1	165.54
			2	196.42
		REG3	1	37.25
			2	42.73

Subject x Region Summary Table

Autoradiography data is frequently exported into statistics packages for analysis by Analysis of Variance. The preferred summary format is consequently a table that displays data from each individual subject by each individual anatomical region.

You can display data in tabular format, with **Subjects** as rows, **Regions** as columns. To see the **Subject x Region** view, click on the **Select table** icon to see a fly-out icon bar. Select the **Subject x Region** icon:



To pool data across all sections, select the **All sections: Summary** radio button. To display data within each section, select **All sections: Detail**.

Please note that **AIS** can only display one measure at a time in this summary format. **AIS** displays the current measure above the **Subject** label indicator on the left side of the data table. If you have made more than one type of measurement, double-click in this field to scroll to another measure (see Figure 9).

Figure 9: A Subject x Region data summary. Notice the Subject labels arranged in rows, Regions labels as columns. AIS can display one measure at time in this format.

Dens - nCi/g	Area1	Area2	Area3	Area4	Area5
rat1	426.13	228.83	33.99	309.71	191.91
rat2	472.98	219.63	32.64	324.83	142.13
rat3	459.74	244.92	39.07	313.11	267.16

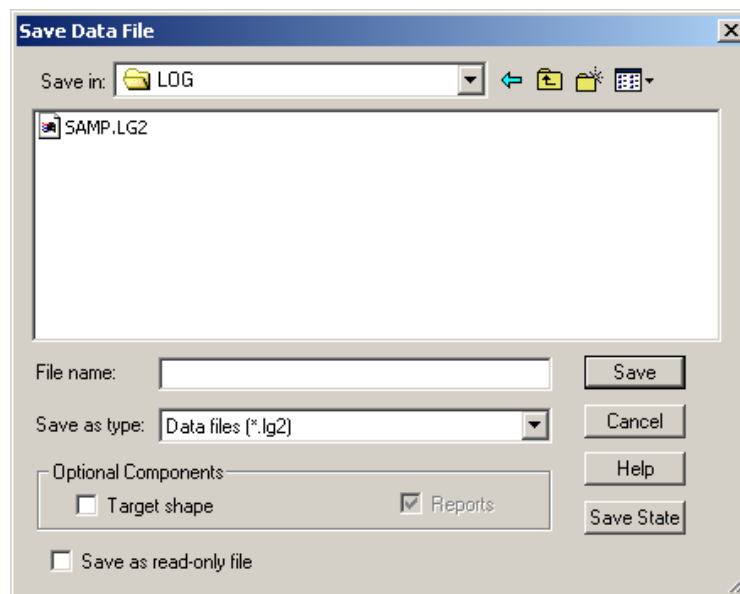
Saving Data

AIS stores data in a proprietary, binary “log” file format (*.lg2). Log files contain more than just numerical data. They contain data, label lists, channel tracking information, and the calibration that was in effect when the file was saved. You can also save target outlines in log files.

TO CREATE A LOG FILE:

1. Go to the *Sample* window and open the *File* menu.
2. Select the *Save as* option. The *Save Data File* dialog box appears (Figure 10).
3. Type a filename into the entry field (e.g., Experiment 1). There is no need to type a file extension; **AIS** always attaches the appropriate extension whenever it saves a file.
4. Press the [**Save**] button. **AIS** stores the file to disk under the specified filename.

Figure 10: The Save Data File dialog box.



Re-Opening a Log File

When you open a log file (*File > Open*), AIS loads its data, labels (*Subject, Section, Region*), and the last density calibration. This is almost like going back to the analysis session when you created the file. You can edit data within the log file, create different data summary configurations, and add new data if you wish.

Note: The calibration contained in the log file is the one that was in effect when you last saved the log file. AIS saves calibrated data, but only the most recent calibration. If you intend to continue sampling, we recommend you establish new calibrations.

Saving Target Outlines

To save target outlines with the log file, check the **Target shape** option in the *Save Data File* dialog box. With target outlines saved, you can click on any numerical value and see the target outline from which it came.

Please note that target outlines are linked to channel coordinates, not to specific images. For this feature to be useful (i.e., to be able to re-display the target outline on the *image* from which it came), you have to be able to display both the sample window and the image associated with each numerical value. Images should consequently be saved to disk after they have been sampled. We recommend using the Subject and Section label entries as image filenames for easy indexing. You want to be able to identify the image associated with each numerical data point.

To see the origin of a data point, retrieve the log file that contains it. Now load the image file that contains the data point that you wish to check. When you click on the data point, the sample window saved with it appears on the image.

Exporting Data

The AIS log file format (*.lg2) cannot be read by other programs. To format data for use with other software (e.g., word processing, spreadsheet, database programs), select the *File > Export* command. A variety of text file formats are available, including tab delimited (*.txt), comma delimited (*.csv) and space delimited (*.prn). **Lotus® 1-2-3** (*.wks) and **Microsoft® Excel Workbook** (*.xls) files can also be created. Please note, to create *.xls files from your data, Excel must be installed on the AIS computer.

File > Export uses the WYSIWYG (what you see is what you get) principle. Only the data in the current display will be exported, just as it appears on your monitor. You should consequently select all the data display options (e.g., within and across Regions, Sections, Subjects), and the summary options (e.g., **Region** or **Target Summary** table) *before* exporting your data.

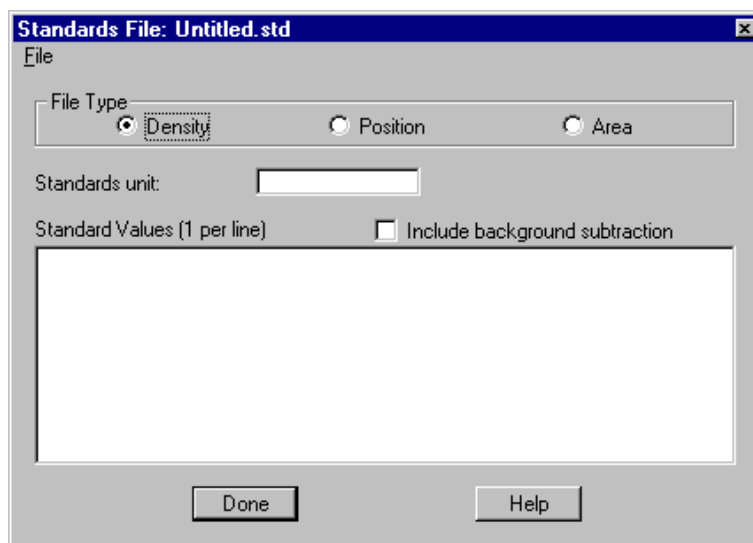
Advanced Details

Creating Standards Files

Most laboratories use a limited number of standard sets as external references. To avoid repetitive typing, you can save the values for each set and retrieve them when you need to calibrate. To create a standards file, either click on the **[Create]** button in the *Open Density Standards File* dialog box (Figure 2) or open the *Establish* menu and select *Edit accessory file > Calibration standards*. This calls up a dialog box into which you can type the values of your standards, one per line (Figure 11). Enter the standards unit (e.g., fmol/mg) in the **Standards unit** entry field. Then open the *File* menu in the dialog box and save the file. The file is saved with an *.std extension.

Note: This dialog box has no scroll bars. Use the arrow keys to navigate up and down the list of standard values.

Figure 11: Creating a file containing the values of external density standards.



Background Subtraction

For **Density** calibrations, you can use an unexposed part of the film or plate area as the background standard. This represents zero concentration, but the density standards also contain this background value and calibration should take care of the background contribution to standard values.

If you prefer, **AI**S can subtract a background value from each standard before calibration. To do this, click on the **Include background subtraction** checkbox. A **Bkgd** term appears in the *Std Value* column. Now sample a background region.

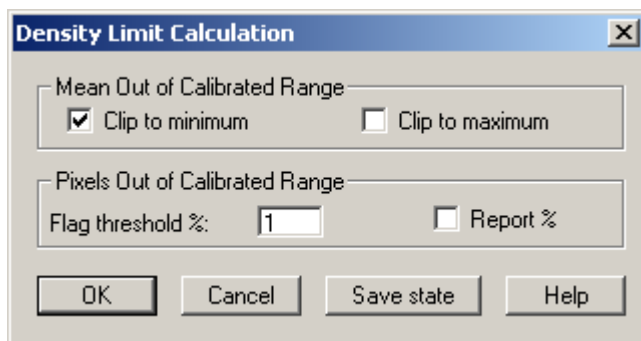
When Density Data are Displayed in Red

Occasionally, calibrated density data may be “flagged” in red. This means that some of the pixels in the sample have density values that do not fall within the range of values established for the calibration curve. **Treat such data with caution.** Your decision to

accept or reject the data should depend on a) how many pixels are out of range, b) how far out of range they are, and c) what you've told AIS to do with pixels that are out of range.

To find out how many pixels are out of range, open the *Sample* menu and select *Advanced > Density limits*. The *Density Limit Calculation* dialog box controls the display of data that is out of calibrated range. Select the **Report %** checkbox and AIS will report the proportion of out-of-range pixels in the sample. The proportion is displayed in the data table, next to the flagged data.

Figure 12: The *Density Limit Calculation* dialog box controls the display of data from samples that contain pixels that are out of range. Such data is displayed in red.



By default, AIS will flag data when 1% or more of its pixels are out of calibrated range. If you are willing to accept a higher percentage of out-of-range pixels in your samples, enter a higher **Flag threshold**. For example, if you are willing to accept data from a sample with less than ten percent of its pixels out of range, enter a “10” in the **Flag threshold** entry field. AIS will then only flag data when 10% or more its pixels are out of range.

By default, AIS extrapolates calibrated values for pixels that are out of range. A variety of extrapolation functions are available, and they can be applied to either end of the calibration curve. Alternatively, AIS can “clip” out-of-range pixels to the maximum or minimum standard value. For access to extrapolation options, go to the *Calibration* window and press the **[Extrapolate]** button displayed beneath the density calibration curve.

Regardless of whether you choose to extrapolate or clip pixels that are out of range, be advised that these operations are meant to correct for minor excursions beyond the calibration curve. They should not be used as a correction for poor exposures.

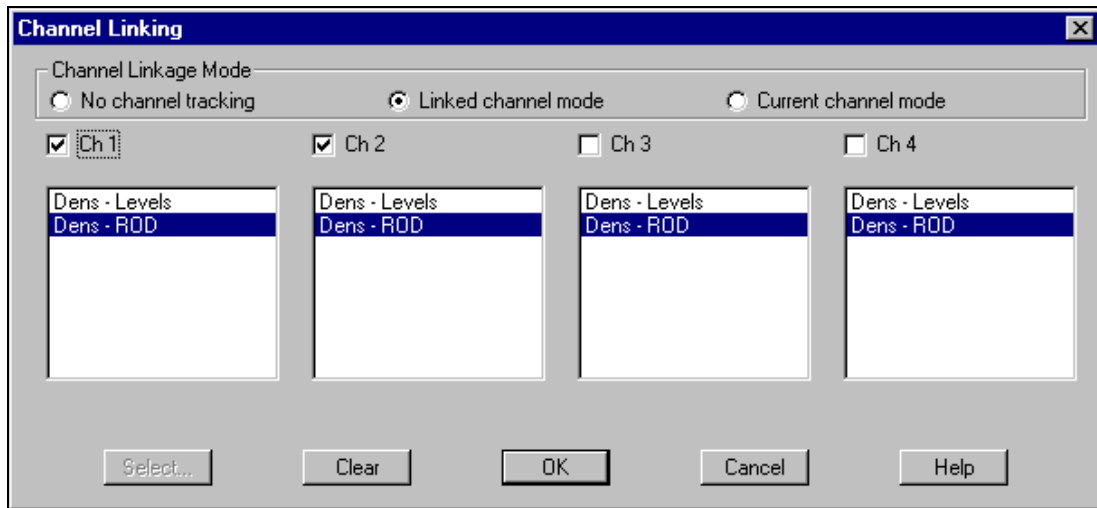
Re-Directed Sampling

Re-directed sampling allows you to define regions of interest in one channel and gather data from the same coordinates in another (i.e., to define anatomical boundaries in a counterstained tissue section and gather data from the same its corresponding autoradiographic image).

TO ENABLE CHANNEL LINKING:

1. Open the *Sample* menu and select *Advanced > Channel linking*. The *Channel Linking* dialog box will appear.
2. Set the **Channel Linkage Mode** to **Linked channel mode**.
3. The **Channel** checkboxes (labeled “Ch 1” and “Ch 2” in Figure 13) indicate which channel(s) AIS will gather data from. Put a checkmark in the channel containing the autoradiographic image, and select the measures(s) you wish to make.
4. Click **[OK]** to exit the dialog box.

Figure 13: The Channel Linking dialog box.

**TO PERFORM RE-DIRECTED SAMPLING:**

1. Digitize the autoradiographic image into “data” channel specified above.
2. Align the counter-stained specimen with the autoradiographic image (see blow). Place the aligned image into another channel.
3. Use any **Sample** tool to define anatomical boundaries in the counter-stained section.
4. AIS automatically gathers data from the same coordinates in the autoradiographic image and reports the data in the data table.

Aligning Images for Re-Directed Sampling

TO ALIGN THE IMAGES:

1. Digitize the autoradiographic section into Channel 1 (or whatever was designated as the “data” channel in the **Channel Linking** dialog box)
2. Click the **Alignment** icon:



3. Two shadowy images are now visible, one on top of the other. One is the autoradiographic section, which is “frozen” in place. The other is a live image, which is actively digitizing. The live image may be moved (by hand) into alignment with the stored image.
4. Remove the film from the field of view. Move the counterstained section into the field of view and position it until it lies directly over the frozen autoradiographic image. If a density calibration is in effect, do not change the focus, camera height or illumination level!
5. Press the **[OK]** button. The autoradiographic image remains in Channel 1, and the aligned counterstained section is automatically placed into Channel 2.

If you are working with phosphor plate images, it is still possible to perform re-directed sampling so long as you have a camera with which to digitize the tissue section. The procedure is a little cumbersome, given that the camera produces a smaller image than the phosphor imager and has a lower bit density. The trick is to get the images to match in terms of size, magnification level and bit density.

TO ALIGN A SECTION WITH AN IMAGE FILE:

1. Cut the phosphor image into smaller files (*Image > Cut*) that match the size of the camera output (e.g., 1280 x 1024 pixels).
2. Set the display format (*Settings > Display format*) to the bit density that matches the camera.
3. Retrieve a cut image file into Channel 1. Let **AIS** convert the image file to a bit density that matches the display format.
4. Click the **Alignment** icon:



5. Two shadowy images are now visible, one on top of the other. One is the image file, which is “frozen” in place. The other is a live image, which is actively digitizing.
6. Move the counterstained section into the field of view. Adjust the camera height and focus until the tissue section(s) is aligned with the section(s) in the image file.
7. Press the **[OK]** button. The cut image file image remains in Channel 1, and the aligned counterstained section is automatically placed into Channel 2.
8. Save the aligned counter-stained image to disk. Use a filename that you can easily associate with the cut image filename.
9. Repeat the procedure for all remaining cut images. The procedure is not as difficult now that the camera magnification matches the phosphor image.

TO PERFORM RE-DIRECTED SAMPLING:

1. Enable the **Channel linking** feature as described above.
2. Load a cut phosphor image file into the “data” channel. If necessary, change the display format to match the image bit density.
3. Load the corresponding tissue image into any other channel. Let **AIS** convert the image to a bit density that matches the display format (i.e., that of the phosphor image file).
4. Use any **Sample** tool to define anatomical boundaries in the counter-stained tissue image.
5. **AIS** automatically gathers data from the same coordinates in the autoradiographic image and reports the data in the data table.

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