



Version 7.0

Application Guide: Quantitative Autoradiography

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Quantitative Autoradiography

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Introduction

This document is a supplement to the online **MCID™ Analysis Reference Manual**. It describes how to use **MCID Analysis** to quantify images of 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 **MCID Analysis** 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 **MCID Analysis** 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 **MCID Analysis** computer is connected to the Internet, simply select *Help > Manuals web site* to connect.

Basic Procedure

The basic procedure for quantifying autoradiographic images with **MCID Analysis** is as follows:

1. Load the autoradiographic image file.
2. If necessary, establish a density calibration.
3. Use any **Sample** tool to gather data from tissue section(s).
4. Repeat Steps 1-3 for all remaining image files.
5. Summarize and save the data.

Calibrating Image Files

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.

If you are working with image files that already have a density calibration (e.g., if they were calibrated in **MCID Elite** and saved to disk), retrieve the files with the **Calibrations** option checked. **MCID Analysis** will automatically retrieve any calibration settings (e.g., nCi/mg tissue wt.) that were originally saved with the image. No further calibration is necessary, and all density data will be expressed in calibrated units.

If you are working with image files that do not have an appropriate density calibration, they can be calibrated in **MCID Analysis**. All that is required is an image file that contains an appropriate reference scale (e.g., Microscales). Once the calibration is established, it can be used to generate data from any object in the same image, or it can be applied to any other image that was acquired under the same optical conditions as the reference scale (i.e., same sheet of film, same camera height, illumination level, etc).

Instructions for establishing a density calibration in uncalibrated images are presented below. You can skip this section if you are working with images that are already calibrated.

Establishing a Density Calibration

The goal of a density calibration is to create a standard curve that relates the system's internal density measurement units (e.g., ROD, PSL/mm²) to a set of standard values (e.g., isotope or ligand concentrations). 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, **MCID Analysis** 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.

The general procedure is as follows:

1. Retrieve the image file that contains the concentration standards.
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 **MCID Analysis** what to do with pixels that are outside the range of calibration values.

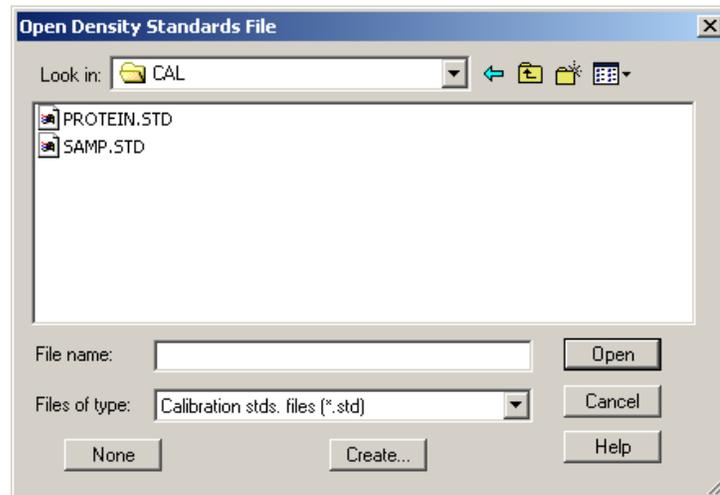
Building the Calibration Table

Click the **Calibration** shortcut icon to open the *Calibration* operational window (or select *View > Go To > Calibration*):



Open the *Calibrations* 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 1). 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 1: When you select a calibrated density unit (e.g., Cal stds), MCID Analysis asks for a file that contains the values of the external standards.



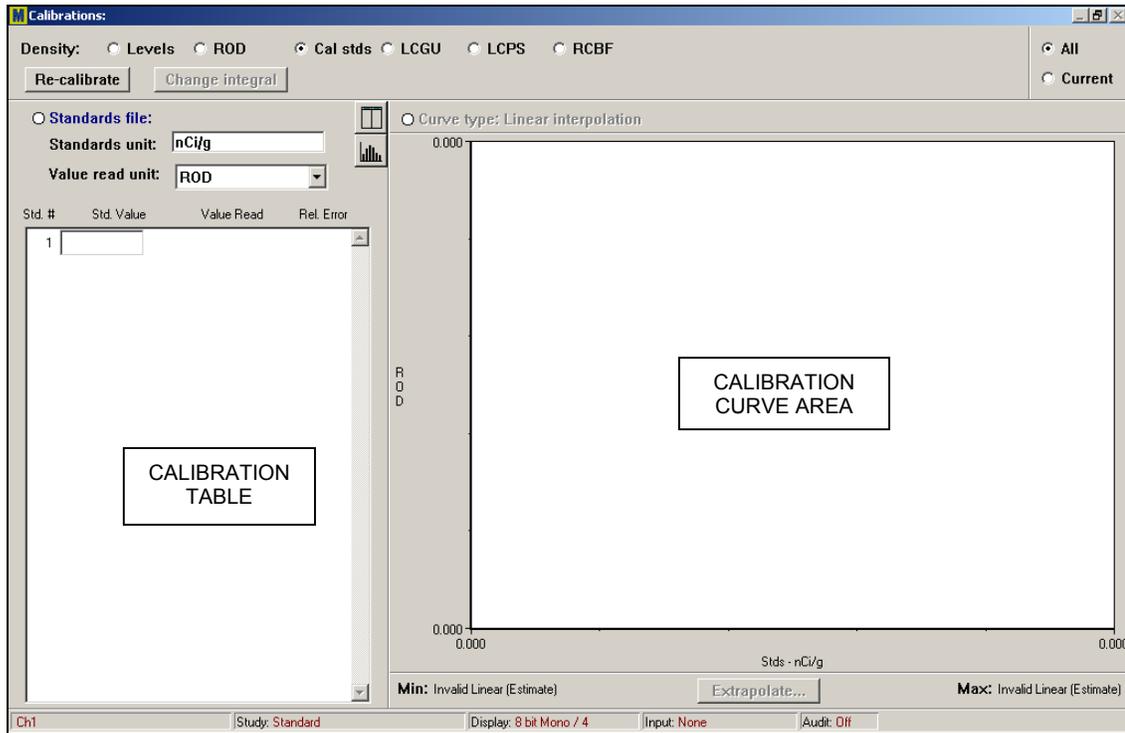
Entering the Standard Values

When you click on [OK] to leave the *Open Density Standards File* dialog box, the *Calibration* window appears as it does in Figure 2. 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 2: The Calibration window.



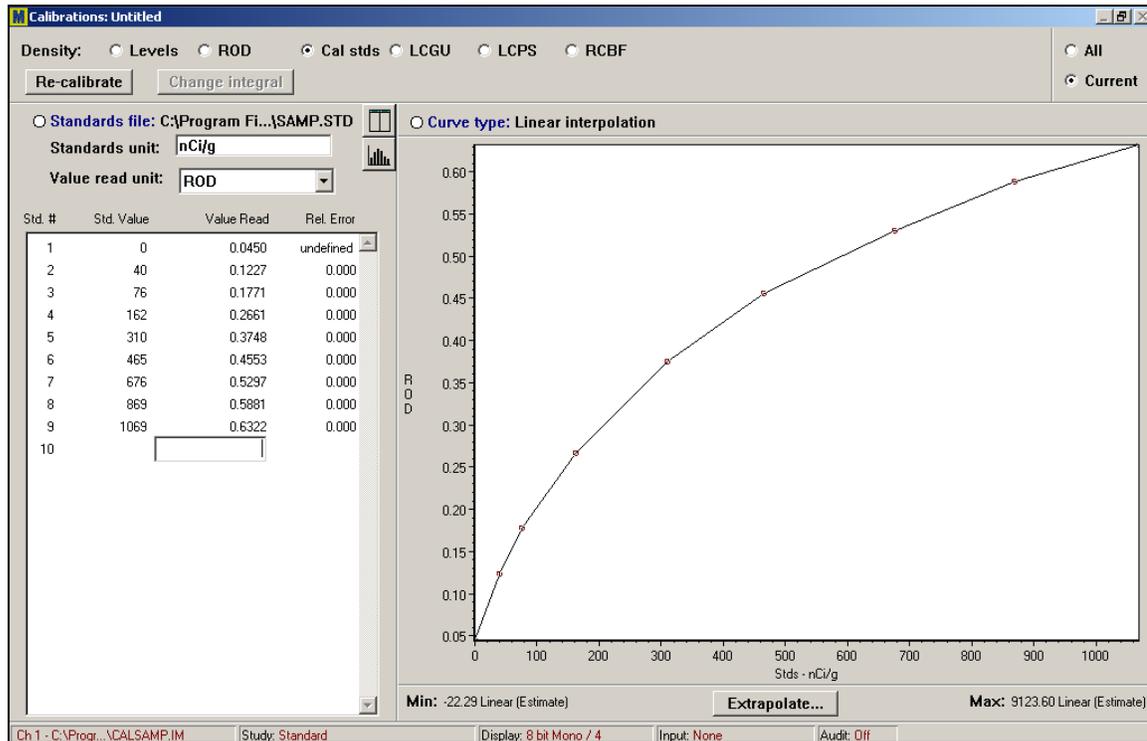
Sampling the Standards

In the Image View, sample each of the standards (for the “0” standard, sample the plate or film background). Keep to the same order as the table. As you sample each standard, the density value appears in the **Value Read** column and the entry field automatically moves to the next position on the list. A calibration curve is plotted to the right of the calibration table (Figure 3).

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.

Note: If your standards are contained two images (i.e., if you could not fit the entire range of standards into a single field of view while maintaining a suitable level of magnification), you will have to sample the standards in two steps. First, load the first image and sample whatever standards are visible. Then load the second image *with the **Calibrations** checkbox un-checked* and sample the remaining standards.

Figure 3: 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 4).

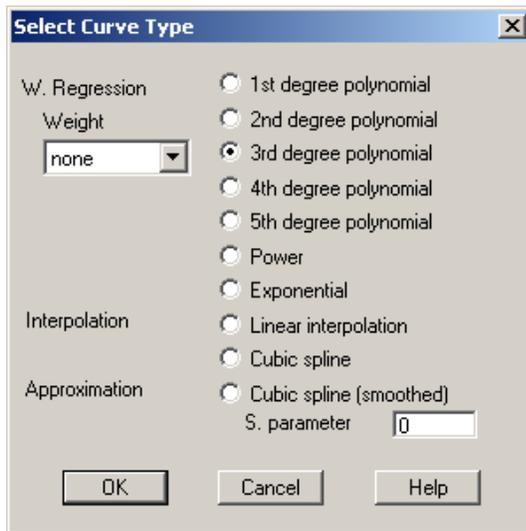
The graph shows how well a selected fitting function fits the concentration values of the reference. A numerical estimate of goodness of fit is available from the **Rel Error** column in the calibration table, where:

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

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.

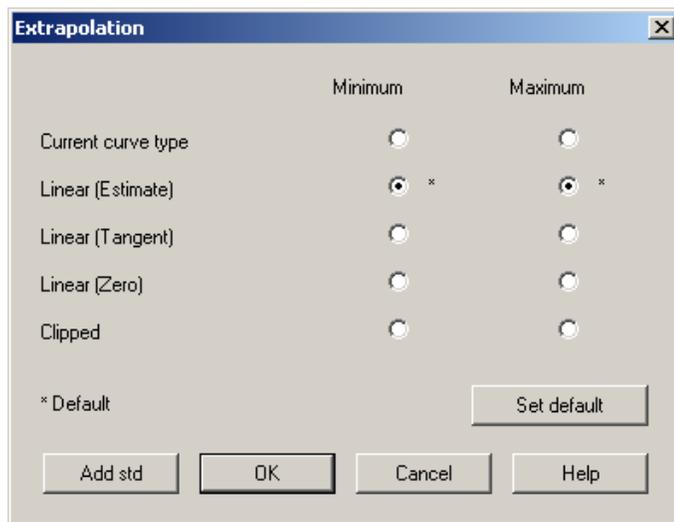
Figure 4: Interpolation or approximation functions are used to calculate concentration values that lie between those contained in the standard set.



Extrapolation Functions

By default, **MCID Analysis** 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 5: 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.



Applying the Density Calibration to Other Image Files

Once the density calibration is established, it can be used to quantify any object in the image. If you are working with a phosphor image file, the calibration is valid for any specimen that was exposed on the same plate as the density standards.

TIP: If you are using *Image > Cut* to cut a large phosphor plate image into smaller files (subsections) calibrate the large image first. That way, each subsection can be saved to disk in fully calibrated form.

If you are working with X-ray film, the density calibration is valid for any image that was acquired under the same optical conditions that were in effect when you imaged the standards (i.e., the same sheet of film, lens aperture settings, camera height, camera settings, ambient lighting conditions).

There are two ways to apply or ‘transfer’ the calibration to other image files (where appropriate):

- **Save the calibration** – Any calibration can be saved to disk as a calibration file (*.ca). The calibration file can then be re-loaded after you have retrieved another image file.
- **Retrieve subsequent images without calibrations** – When you retrieve an image with the **Calibrations** option checked, any calibration in the channel is replaced by the image calibration. If **Calibrations** is not selected, the existing channel calibration is maintained.

Once you have applied an existing calibration to another image file, you can preserve the link by saving the image with the **Calibration** option checked.

Sampling 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 11: Altering Image Appearance*).

Calculating the Density Value

Density data are reported in the data table in calibrated units. **MCID Analysis** 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:

Integrated Optical Density = Sum of the calibrated pixel values / 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

MCID Analysis 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 6: The basic set of labels for managing data from sectioned specimens.



For more information, see *Chapter 9: Data Management* in the online *Reference Manual*.

Re-directed Sampling

Anatomical landmarks are often difficult to visualize in autographic images, or they may be absent altogether. If this is the case, use the **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 **MCID Analysis** 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. **MCID Analysis** replicates the target outlines from Channel 1 and reports density data from the autoradiograph in Channel 2.

Note: The counter-stained specimen should be aligned with its corresponding autoradiographic image during the image acquisition phase of the experiment and saved to disk.

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

Calibrating and Sampling Images from 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.

If you need to establish a new density calibration (i.e., if you are working with uncalibrated images), go to the *Calibration* operation window and click the [**Re-calibrate**] button. **MCID Analysis** clears the existing calibration and is ready to receive a new one. Repeat the procedure outlined in [Establishing a Density Calibration](#), above.

Once you have established a new calibration curve, **MCID Analysis** 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.

Summarizing and Saving Data

MCID Analysis 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 **MCID Analysis** 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 **MCID Analysis** 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 9: 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 **Table** toolbar (*View > Table bar*) also lists a variety of data summary configurations.



All Targets

The 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 **MCID Analysis** 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 7).

Figure 7: 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, select the *Subject x Region table* option from the toolbar's drop-down list:



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 **MCID Analysis** can only display one measure at a time in this summary format. **MCID Analysis** 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 8).

Figure 8: A Subject x Region data summary. Notice the Subject labels arranged in rows, Regions labels as columns. MCID Analysis can display one measure at a 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

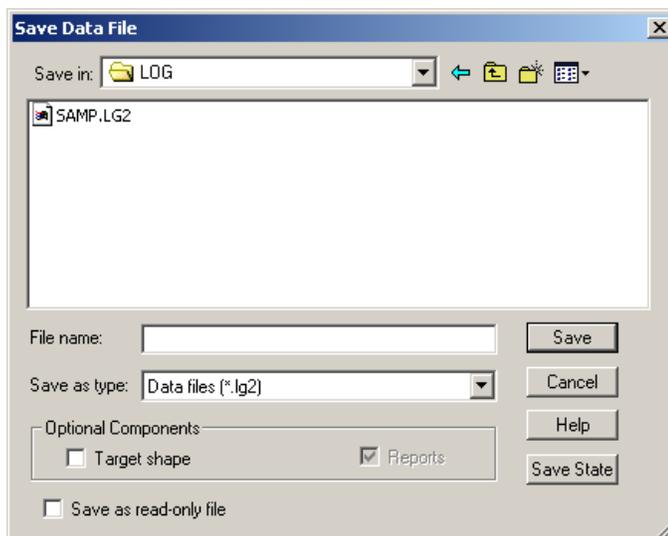
Saving Data

MCID Analysis 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 9).
3. Type a filename into the entry field (e.g., Experiment 1). There is no need to type a file extension; **MCID Analysis** always attaches the appropriate extension whenever it saves a file.
4. Press the [Save] button. **MCID Analysis** stores the file to disk under the specified filename.

Figure 9: The Save Data File dialog box.



Re-Opening a Log File

When you open a log file (*File > Open*), **MCID Analysis** 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. **MCID Analysis** 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 **MCID Analysis** 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 **MCID Analysis** 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.

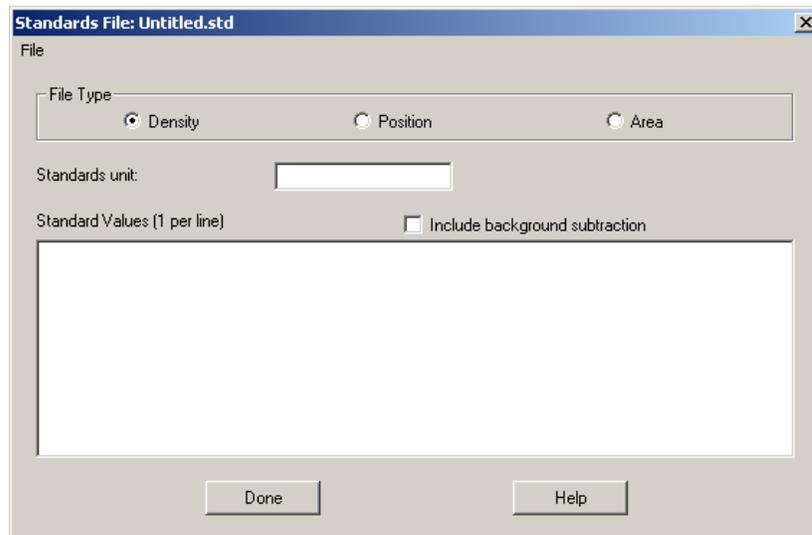
Advanced Details

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 1) or open the *Calibrations* 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 10). 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 10: 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, **MCID Analysis** can subtract a background value from each standard before calibration. This procedure can reduce the relative error at the lower end of the calibration curve when weighted regression curves are used (i.e., $1/\text{read}$, $1/\text{read}^2$). To use this feature, click on the **Include background subtraction** checkbox.

If **Include background subtraction** is enabled, a **Bkgd** term is added to the list of standard values when you open the standards file. Sample an unexposed part of the film as the **Bkgd** standard. When the calibration curve is constructed, this background density

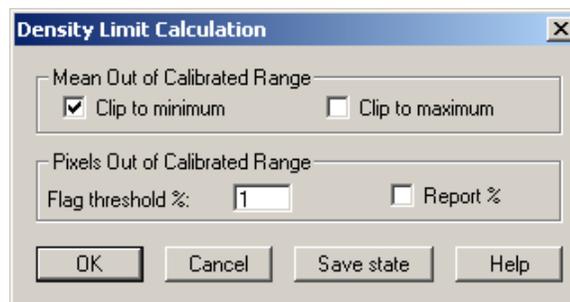
value is subtracted from the **Value Read** for each standard. The resulting values are used to derive the parameters used for **1/read** and **1/read²** weighted regression curve types.

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 **MCID Analysis** 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 **MCID Analysis** 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 11: 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, **MCID Analysis** 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. **MCID Analysis** will then only flag data when 10% or more of its pixels are out of range.

By default, **MCID Analysis** 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, **MCID Analysis** 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.

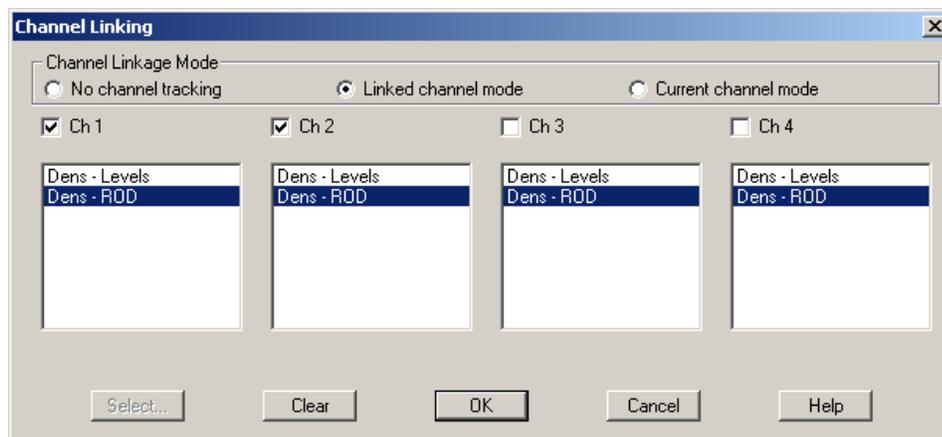
Re-Directed Sampling

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 12) indicate which channel(s) **MCID Analysis** 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 12: The Channel Linking dialog box.



TO PERFORM RE-DIRECTED SAMPLING:

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

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