Discovery-1™

Analyzing a Translocation Assay on the Discovery-1 System

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Overview

This document describes the procedures for measuring a molecular translocation assay using the Review Screen Data on the Discovery-1 System, versions 6.2r4 and higher. There are two versions of this application module available for Discovery-1: Translocation and Translocation-enhanced. Both versions are discussed in this application note.

The translocation application modules provide quantitative measurements of a probe inside and outside of a cellular compartment. The compartment and the probe are identified using fluorescent markers either a fluorescent protein homologue, immunofluorescent staining, or FISH.

There are two application modules that may be used for analysis of translocation assays – Translocation and Translocation–Enhanced.

Translocation makes several assumptions about your experiment and requires only a few user settings. Because of its ease-of-use, this version requires the least amount of user input.

However, it is possible that the intrinsic settings included in the Translocation application module are not ideal for the compartments of the cell type(s) in your image. In these instances, the Translocation–Enhanced application module makes fewer assumptions about your experiment and provides greater control over the parameters of the analysis algorithm. Because more parameters can be customized, the Translocation–Enhanced application module might require additional steps to configure it optimally, however, it can potentially provide more accurate results than the basic Translocation assay.

Note: For more information about the procedures and dialog boxes described in this document, refer to the Discovery-1 online help system. Press [**F1**] to access help for the active dialog box.

This document describes the following procedures:

- Configuring the Assay and Saving Settings
- Running the Assay

Note: Configuring and saving translocation settings must be completed before you run the assay for the first time. After settings are saved, they can be loaded as needed without further configuration.

This document assumes the following:

- The Translocation and/or Translocation-enhanced drop-in has been loaded using the Meta Imaging Series Administrator.
- The images on which to perform the assay have been acquired using the Discovery-1 Screen Acquisition dialog box.

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Note: Refer to Artifacts and Controls for more information about proper image collection and possible preprocessing necessary for proper analysis.

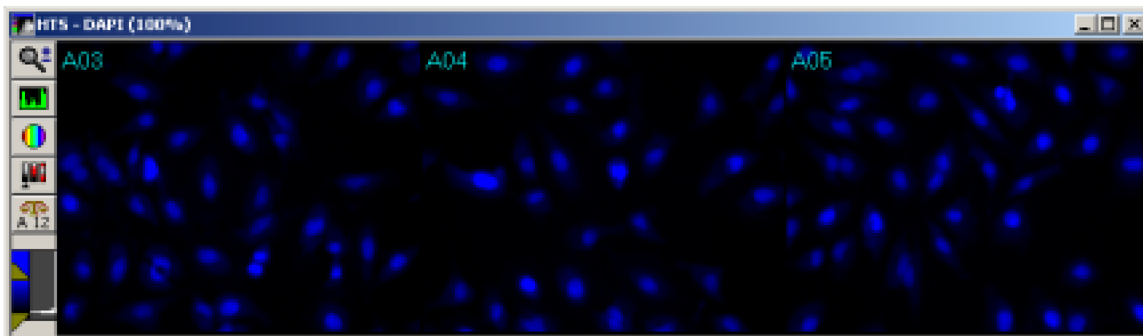
Configuring the Translocation Assay

The next section assumes that the data set you are interested in is open in the Review Screen Data or Review Screen Data [DB]. For instructions on how to do this see the documentation for Screen Data Utilities.

Complete the following steps to configure the assay in the Review Screen Data dialog box:

1. On the Wavelengths tab, click the check box next to the two wavelengths that you want to use for your analysis. One wavelength should represent the fluorescently-marked compartments and the second wavelength should represent the fluorescent probe to be analyzed.
2. Click *Apply* to view montage windows ("HTS-Wavelength") of the selected sites.
3. Click a site within the montage window to open new image windows of the selected site, as shown in Figure 1:

Figure 1
Opening an Image of a Site



In this example, thumbnail A04 in the montage was clicked and the following image window opened:

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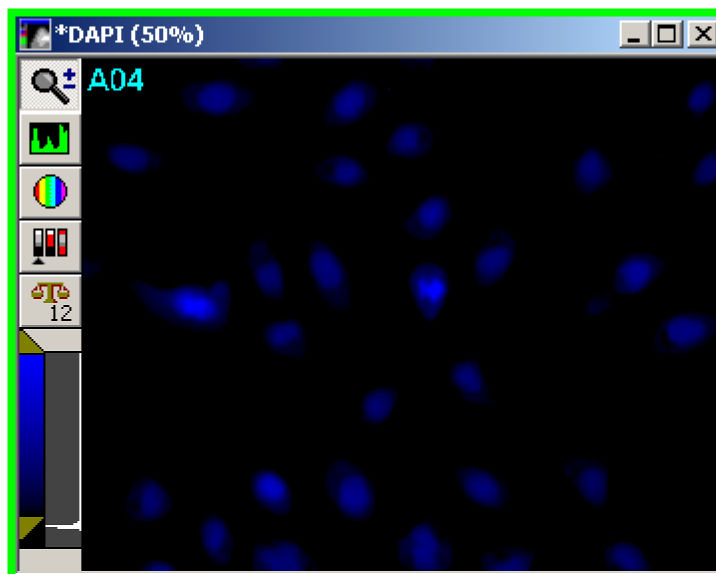


Note: Although only one image window is shown opening in the above example individual wavelength images will open for both of the checked wavelengths.

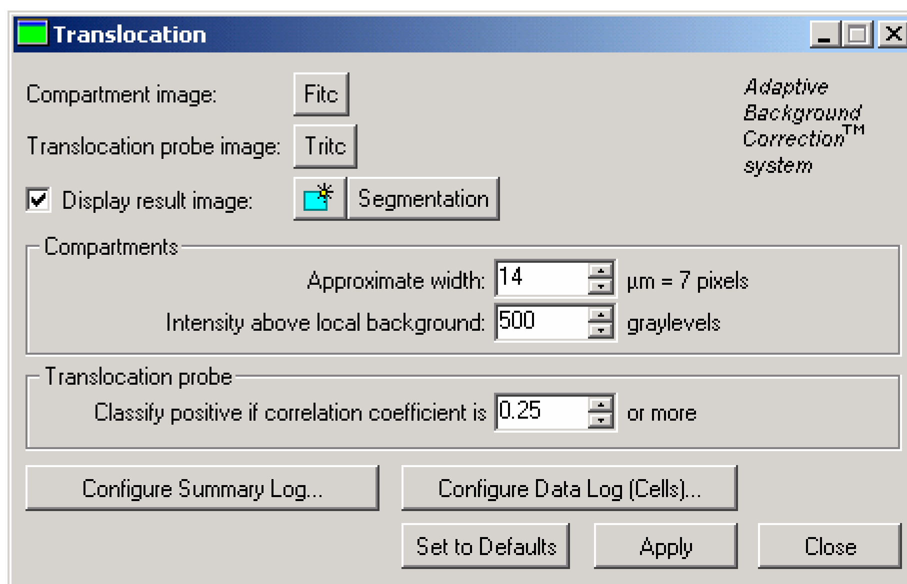
Configuring the Translocation Application Module

To configure the Translocation application module, complete the following steps:

1. In the Review Screen Data dialog box, click the Assay tab.
2. Select *<Translocation>* from the Assay drop-down list.
3. If you are not running the database version of the software click *Location* and choose the folder for storing and retrieving assay settings.

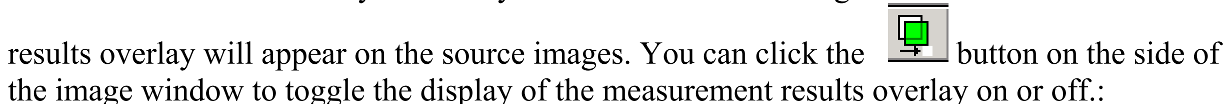
   If you have already configured and saved translocation assay settings, or have been given translocation assay settings skip to the **Running the Assay** section of this document, if not, continue this procedure.
4. Click *Configure Settings*. The Configure Settings for Translocation dialog box opens, as shown in Figure 2:

Figure 2
Configure Settings for Translocation dialog box



5. Click *Compartment Image* and select the wavelength image representing the compartment. This wavelength was checked in step 1.
6. Click *Translocation probe image* and select the wavelength image representing the probe you wish to measure.

Note: Steps **7** through **9** involve the configuration of the dialog for your specific sample. Because each sample is typically different, this document provides only application module configuration recommendations for your cell type. As you try different settings, you can use the measurement results overlay to check your results. After clicking *Test Run* the measurement



results overlay will appear on the source images. You can click the button on the side of the image window to toggle the display of the measurement results overlay on or off.:

Note: Follow the instructions for the Online help topic ***Translocation***. The instructions will guide you through setting all of the parameters for the Translocation dialog box.

7. Define the approximate width of the compartment. Enter the intensity above the local background for the compartments to be detected. For this version, the approximate width is used to split multiple touching compartments.

   To estimate the width of your compartments:

   a. Draw a region of interest around a typical compartment.
   b. From the Measure Menu, click Show Region Statistics. The Show Region Statistics dialog box opens.
   c. Uncheck "Entire Image."
   d. Read the Width measurement.

   To estimate the intensity above local background:

   a. Place your cursor over the edge of a compartment.

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b. Read the gray value at the bottom of the screen.

c. Place your cursor next to the compartment.

d. Read the gray value at the bottom of the screen.

e. Subtract the second gray value from the first gray value.

8. Specify the correlation coefficient threshold value needed to classify the compartment as positive. This value between negative one (-1) and one (+1) represents the Pearson's Correlation Coefficient between the two fluorescent channels in the region surrounding and including the compartment. The detected value must be greater than or equal to the specified value in order to classify the compartment as positive.
9. Open a summary log and, if applicable, a data log from the Log menu of Discovery-1.
10. .Configure the Summary and/or the Data Log. Click Configure Summary (and/or Data Log). Within Configure Log dialog Confirm that the measurement parameters you wish to measure are checked. Click *OK*.
11. Click *Test Run* to process the images and check your settings. Adjust your settings if necessary. Click *Save Settings* to name and save your settings.
12. Close your summary log and data log, if applicable, then click *Close* to exit the Configure Settings for Translocation dialog box.

Running the Assay

Complete the following steps to run the assay:

1. From the Assay tab of the Review Screen Data dialog box, select the setting from the *Settings* drop-down list.
2. Open a summary log and possibly a data log from the Log menu of Discovery-1.
3. Click *Run Assay for All Positions* to run the assay for all position on the plate.

   OR

   Click *Run Assay for Selections* to run the assay for the selected sites

   Note: You can select sites by right-clicking on different data points.

   OR

   Click *Run Assay for Site* to run the assay for the current active site. The image is processed and the data is displayed in your log (s).
4. Click *Close* when finished.

Configuring the Translocation-Enhanced Assay

To configure the Translocation-Enhanced application module, complete the following steps:

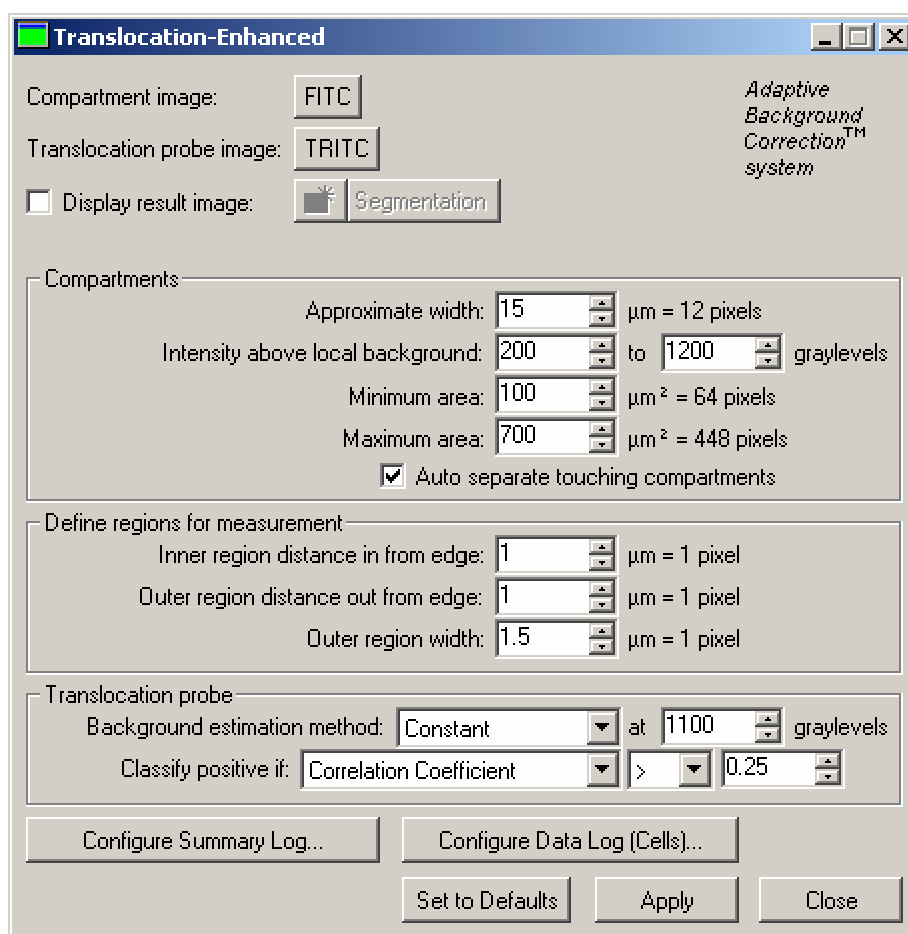
1. In the Review Screen Data dialog box, click the Assay tab

2. Select *<Translocation-Enhanced>* from the Assay drop-down list.
3. If you are not running the database version of the software click *Location* and choose the folder for storing and retrieving assay settings.

Note: If you have already configured and saved *Translocation-Enhanced* assay settings, or have been given *Translocation-Enhanced* assay settings, skip to **Running the Assay.** If not, continue with this procedure.

4. Click *Configure Settings*. The Configure Settings for Translocation-Enhanced dialog box opens, as shown in Figure 2:

Figure 3
Translocation-Enhanced Configure Dialog Box



5. Click *Compartment Image* and select the wavelength image representing the compartment. This wavelength was checked in step 1.
6. Click *Translocation probe image* and select the wavelength image representing the probe you wish to measure.

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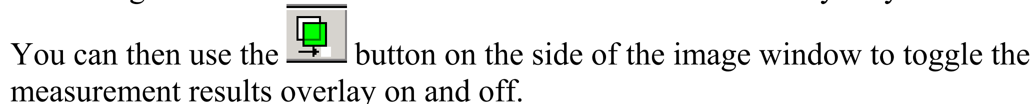
- **Note:** Steps **7** through **11** guide you through configuring the dialog box for your specific sample. Since each sample is different, it is possible to only recommend application module configuration settings for your cell type. As you try different settings, click *Test Run* to log measurements and see a measurement results overlay on your source image.

  You can then use the button on the side of the image window to toggle the measurement results overlay on and off.

Note: Follow the instructions for the Online help topic ***Translocation-Enhanced***. To view these instruction, press the **F1** key when the Translocation-Enhanced dialog box is open and active. The instructions will guide you through setting all of the parameters of the dialog box. Use the following steps for additional guidance.

7. Define the approximate width of the compartment, the minimum and maximum areas for compartments. Enter the minimum intensity above the local background for the compartments to be detected. For this version, the approximate width is used to split multiple touching compartments if Auto separate touching compartments is checked.

   To estimate the width and areas of your compartments:

   a. Draw a region of interest around a typical compartment
   b. Bring up Show Region Statistics (Measure Menu)
   c. Uncheck "Entire Image"
   d. Read the Width and Area measurements.

   To estimate the intensity above local background:

   a. Place your cursor over the edge of a typical compartment.
   b. Read the gray value at the bottom of the screen.
   c. Place your cursor next to the compartment.
   d. Read the gray value at the bottom of the screen.
   e. Subtract the second gray value from the first gray value.

8. Enter the maximum intensity above the local background for the compartments to be detected. This maximum intensity can be used to eliminate debris or compartments not of interest.

9. Define the regions for measurement. These values depend upon the areas of the compartment and the area of the cell. The outer region width dictates how far from the compartment to go for quantization. Do not go too far or you will have a substantial part of your area outside of the cell and in the background. Alignment of the two wavelengths is not greater than 1 pixel. Thus greatest difference between a signal inside vs. outside of a compartment may not be at the very border of the compartment. It may be worth going in by at least a pixel from the edge of the compartment and out by at least a pixel from the edge of the compartment in order to get accurate measurements of intensity inside and outside of the compartment.

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10. If applicable determine the use of Background for your images. If you are using the correlation coefficient a background is not necessary and will not affect your results. Background will affect measurements of intensity inside and outside of the compartments.. The Constant estimation method assumes that your background values do not change. Auto Constant assumes the computer can calculate a background from the gray values in the image. If the staining is consistent or if the cells sometimes form a complete monolayer use Constant and enter a gray level. Otherwise Auto Constant is suggested.
11. Specify the measurement and values to be used to classify a compartment as positive for translocation. Choose either the correlation coefficient or the outer-to-inner compartment intensity to classify the compartment. Set a limiting value. The correlation represents the Pearson's Correlation Coefficient between the two fluorescent channels in the region surrounding and including the compartment. A correlation coefficient has values between negative one (–1) and one (1). An intensity ratio is greater than zero (0).

    Note: If you are analyzing nuclear translocation you should use the correlation coefficient measurement for better reproducibility.

    To estimate a value for classification, complete the following steps:

    a. Open a Data Log from the Log menu.
    b. Return to the Configure Settings for Translocation Enhanced dialog box.
    c. Click *Configure Data Log*. Confirm that the measurement parameter you want to use for classification is checked. Click *OK*.
    d. In the Montage window, click a site thumbnail that is positive for translocation to open new image windows of the selected site.
    e. Click *Test Run*.
    f. In the Montage window, click a site thumbnail that is negative for translocation to open a new image windows of the selected site.
    g. Click *Test Run*.
    h. Inspect the Data Log to determine a good cutoff point for the classification.
    i. Close the Data Log.
12. .Configure the Summary and/or the Data Log. Click Configure Summary (and/or Data Log). Within Configure Log dialog Confirm that the measurement parameters you wish to measure are checked. Click *OK*.
13. Open a Summary Log and, if applicable, a Data Log from the Discovery-1 Log menu.
14. Click *Test Run* to process the images and check your settings. Adjust settings if necessary.
15. Click *Save Settings* to name and save your settings.
16. Close your summary log, if applicable, then click *Close* to exit the Configure Settings for Translocation dialog box.

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17. Open your Summary Log and, if applicable, your Data Log from the Discovery-1 Log menu.
18. Click *Test Run* to process the images and check your settings. Adjust your settings if necessary.
19. Click *Save Settings* to name and save your settings.
20. Close your Summary Log and, if applicable, your Data Log then click *Close* to exit the Configure Settings for Translocation-Enhanced dialog box.

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Running the Assay

Complete the following steps to run the assay:

1. From the Assay tab of the Review Screen Data dialog box, select a setting from the *Settings* drop-down list.
2. Open a Summary Log and if applicable a Data Log from the Log menu of Discovery-1.
3. Click *Run Assay for All Positions* to run the assay for all position on the plate.

   OR

   Click *Run Assay for Selections* to run the assay for the selected sites (select sites by clicking on different data points with right mouse).

   OR

   Click *Run Assay for Site* to run the assay for the current active site. The image is processed and the data is displayed in your log (s).
4. Click *Close* when finished.

Choosing the proper measurement

The translocation applications provide the following three measurements for translocation:

- correlation coefficient
- outer-/-inner intensity ratio
- compartments classified positive

The ***correlation coefficient*** is the Pearson's correlation coefficient of the two fluorescent signals used to mark the compartment and the second probe. When the second probe is excluded from the compartment, there is a negative correlation coefficient. When the second probe is not excluded from the compartment, the correlation coefficient is more positive. This measurement is relatively insensitive to the background in the image but can be skewed by changes in cell shape or size that cause the measurement region to overlap the background. In many experiments this measurement is more reliable than the *outer-to-inner intensity ratio*.

The ***outer / inner intensity ratio*** measures the average fluorescent intensity from the Translocation probe image in the defined regions outside and inside the compartment areas defined from the compartment image. This measurement is very sensitive to the background and can also be skewed by changes in cell shape or size that cause the measurement region to overlap the background. If you are analyzing nuclear translocation you should use the correlation coefficient measurement for better reproducibility.

The ***compartments classified positive*** uses either the correlation coefficient or the outer-to-inner intensity ratio to determine the fraction of the compartments exhibiting translocation. The validity of this measurement depends on which measurement is used for the classification and the cut off chosen. Since compartments are classified as either translocated or not translocated; there is no middle ground and changes in subpopulations of compartments are more readily discerned.

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Artifacts and Control Settings Errors

Several different types of artifacts and potential control settings errors can lead to false measurements of translocation. These artifacts and control settings errors can include but are not limited to the following:

- Bleed through
- Background autofluorescence
- Misalignment of the wavelengths
- Changes in cell morphology or loss of compartment integrity

Bleed through

Bleed through of signal from the compartment fluorescent probe into the second fluorescent probe wavelengths will appear as positive co-localization of the second probe in the compartment. This will increase the correlation coefficient and decrease the outer / inner intensity ratio. To test for bleed through take images of the compartment wavelength fluorescent probe without the second fluorescent probe. If substantial bleed through is present change your fluorophores, your filter sets or use arithmetic pre-processing to correct for the problem.

Background autofluorescence

Background autofluorescence of the cell, the media, or the plastic of the plate should be removed from the image if you are logging measurements of intensity. The outer / inner intensity ratio will get closer and closer to one (1) as the fraction of the total signal from background increases. If the background is due to cellular autofluorescence, the Auto Constant method of background correction will not remove the background. The Constant Estimation method should be used to address cellular autofluorescence.

Misalignment of the wavelengths

Misalignment of the two wavelengths will decrease the amplitude of the signal between positive and negative translocation. The correlation coefficient will be closer to zero (0) and the outer / inner intensity ratio will be closer to one (1). Test for this artifact using multicolor beads. If there is shift between the wavelengths it is often due to rotation of the filers and you should try to correct the hardware. If a hardware correction is not possible or sufficient, use the Alignment Cropping feature of Screen Acquisition to correct for misalignment during acquisition. If correction at the time of acquisition is not possible, it is possible to preprocess your images in a journal to bring them into alignment.

Changes in cell morphology or loss of compartment integrity

A change in the cell shape might cause the regions for measurement to extend beyond the edge of the cell. As you continue, the measurements will have an increased fraction of background pixels. Likewise, changes in cell state might lead to changes in the integrity of the compartment. When the compartment looses its integrity, it will appear as though the second probe has translocated into the compartment.

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