

## Determining settings for Screen Acquisition

### Overview:

This document describes how to determine the optimal parameters for automated multi-well plate acquisition using Screen Acquisition. The settings might differ from plate type to plate type (manufacturer, lot, and format), and depend on the objective and fluorescent probes used. This document describes in general terms which parameters will have an effect on the quality of images and how to establish the parameters for auto focus. These parameters should be optimized for each system and assay. Once the settings are optimized, they can be saved in a State File, which can be recalled and used at a later time.

**Note: This document does not discuss the use of any laser auto focus devices.**

This document describes the following procedures:

- Determining the optimum exposure for your sample
- Determining the correct set up for auto focus
- Saving the parameters in a state file for future use

This document assumes the following:

- The sample is fluorescently-stained and the correct filter settings have been determined
- There is a signal in all the selected wells on which to focus

### Initial focus on the sample:

From the Apps menu, choose, Screen Acquisition to open the dialog box. This dialog is divided into two major areas: a "sidebar area" that contains settings and controls that apply to the entire dialog, and a "tabbed" area that provides from five to eight tabs (depending on your settings) that are used to set the acquisition parameters for wavelength, focus, and well and site selection. The dialog box is opened to the Main tab by default.

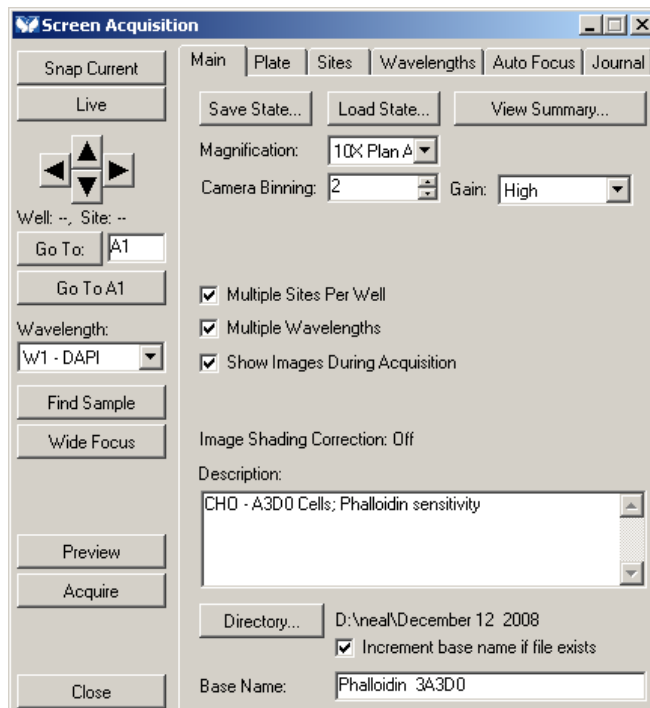
When trying to find a sample for the first time, use a low-power magnification. For cells, begin with the 4 – 10 X magnification. Select the appropriate magnification in the *Magnification* box (Main tab). Make sure that the correct wavelength settings are selected for your sample. Click on the Wavelength tab, then in the Illumination box select the correct filter combination.

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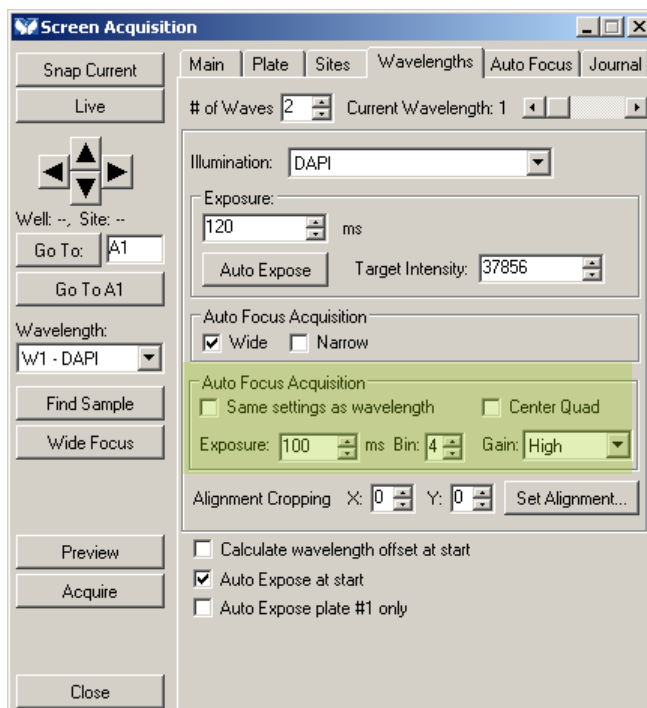


## Setup the Auto Focus Acquisition parameters

The auto focus acquisition parameters are also the parameters used to show a live image.

To set up these parameters complete the following steps:

1. On the Wavelength tab, in the Auto Focus Acquisition area, uncheck *Same settings as wavelength*.
2. If the signal strength is unknown, set the exposure to 100ms (this will allow a fast enough refresh rate; decrease this rate if the image becomes saturated).
3. Set binning to 4.



How to determine the optimum acquisition exposure for the sample will be discussed later.

Select the live button on the left side of the dialog and manually focus up and down to find the sample. For most fluorescent samples the camera will become saturated as focus is approached (seen as gray values greater than 4094 and/or the screen becoming white or black), when this occurs decrease the Auto Focus exposure time.

**Caution: Some objectives have short working distances and may not be able to focus on outside wells before hitting the plate skirt. Also, some samples may be too thick to successfully focus on the sample. Use caution to avoid damaging the objective or displacing the sample from the stage holder. Discontinue focusing immediately if an objective contacts the plate or the stage.**

Once the sample is in focus, use the Magnification pull down option on the Main tab to switch to the desired magnification and verify the focus accuracy. Choose the auto focus tab of Screen Acquisition and click *Set Z Origin* so the program will store the focus position.

***To determine autofocus exposure time:***

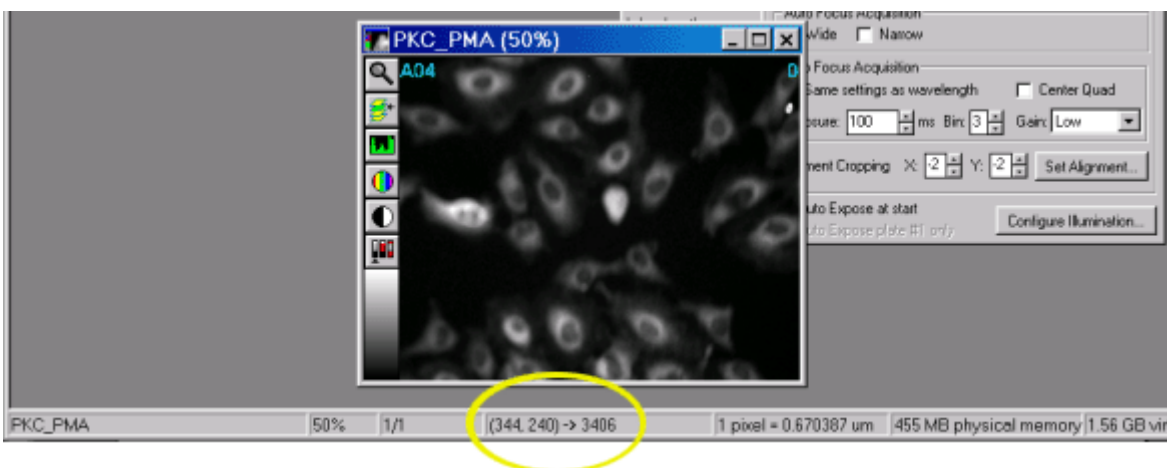
Step	Action
1	From the Apps menu, click Screen Acquisition.
2	On the Main tab, choose a low magnification.
3	On the Wavelengths tab, choose the correct wavelength.
4	Uncheck <i>Same settings as wavelength</i> , then set Auto focus exposure to 100 ms, and set Binning to 4.
5	Click <i>Live</i> to see the result.
6	Manually focus the sample.
7	If the maximum grey value in the image is within $\frac{3}{4}$ of the saturation grey value, reduce the Auto Focus exposure time.
8	On the Main tab, change the magnification setting to the desired magnification.
9	Repeat steps 5 - 7.
10	On the Auto Focus tab, click <i>Set Z Origin</i> .

**How to determine the optimum exposure time**

The exposure time is a critical parameter in achieving accurate acquisition and auto focus. If the exposure time is too long, there is a potential for over saturation of the camera (going beyond the brightness range of the camera), photo damage of the sample (photo-bleaching of the fluorophore and/or photo-toxicity in living specimens), or bleed through from another wavelength. In addition, acquisition or auto focusing will take longer. If the exposure time is too short, the signal-to-noise ratio might not be very high. All of these factors can prevent accurate auto focusing or proper analysis of the images.

So, what is a correct exposure time? The optimal exposure time is short, but with an adequate signal to background ratio. This will help reduce artifacts and increase acquisition speed (reducing collection time). When determining the exposure time, it is a good practice to start with the auto expose button on the wavelength tab, using a target intensity setting of  $\sim \frac{3}{4}$  of the maximum grey value for the camera. Once the image is acquired, move the mouse pointer over

the image. Check the intensity of the cells/objects. A number for the intensity is shown in the status area at the bottom of the screen (X, Y -> intensity/gray value). Then move the cursor to a location in the image that has no cells/objects (background) and check the value. There should be a noticeable difference between the background and your cells/objects. The general rule is to attain a threefold or better signal over background. Thus, if the cell signal is 3000 and the background is 150, the exposure time could safely be decreased. If the signal is only 300 and the background is 150, consider increasing the exposure time, increasing binning (see next section), or improving the staining protocol.



### *To determine the optimum exposure time*

Step	Action
1	On the sidebar of the Screen Acquisition dialog box, click <i>Live</i> . A continuously updating image of the sample opens.
2	Manually focus the sample.
3	When the sample is in focus, press F2 or click <i>F2:Stop</i> .
4	On the Wavelength tab, choose Auto expose with a target intensity of ~3/4 of the dynamic range of the camera.
5	Move the mouse pointer over the brightest cell object to determine its intensity or gray value. This value is reported on the status bar at bottom of screen (x,y -> gray value/intensity).
6	Move the mouse pointer over the background area of the image and determine the intensities or gray values.

7	Calculate the ratio of the brightest cell pixel value to the background pixel values.
8	If the ratio is less than 3:1, increase the exposure time, binning and/or improve the sample staining.

### Binning

One way to increase the signal over the background is to combine the signal from several pixels together so that 4, 9, 16 or 64 pixels are all combined into one (Binning of 2x2, 3x3, 4x4 and 8x8). Binning enables the use of much shorter exposures to get the same signal over background. Binned images are also much smaller because they have fewer pixels (1/4, 1/9, 1/16 or 1/64), leading to faster processing and smaller storage size. On the negative side, binning diminishes the image X,Y resolution. For focusing, this is usually not a problem, so a higher level of binning (4x4 or 3x3) is recommended. For the actual result images, the resolution (binning) needed for analysis will have to be determined.

### Auto focus

Auto focus is one of the most essential parts to collecting images adequate for analysis. Due to the nature of multi-well plates and assay conditions, these settings should be optimized for a particular setup. There are a number of parameters in the software that can affect auto focusing:

- Exposure times for auto focusing
- Auto focus algorithm
- Maximum Single Step size
- Range and accuracy for Find sample, Wide Focus and Narrow Focus

### Setting up Auto Focus Acquisition

The selection of binning during auto focus is found on the *Wavelengths* tab. In the first section of this document *Initial focus on the sample*, the "Same settings as wavelength" box was deselected with the exposure set to 100ms and a binning of 4 or 3. To optimize the settings for the auto focus decrease the exposure until the live image does not saturate and retains a decent S/N. To check for saturation, select the "live" mode, and move the mouse over the brightest spots/cells and check the intensity. If the value at the bottom of the screen indicates a value at or close to the maximum grey value for the camera then decrease the exposure time under auto focus, until the maximum grey value is not near the maximum for the camera.. If it is still near saturation with an exposure time of 1ms, then decrease the binning.

Now switch to the Auto Focus tab: There are a number of parameters that can be configured here.

**Algorithms**

The Screen Acquisition dialog Auto Focus tab includes auto focus algorithms that provide two different levels of sensitivity: Low Signal and Standard. Use the Low Signal algorithm when using higher magnification objectives or there are bright artifacts or debris in a different focal plane than the sample. Otherwise, use the Standard algorithm.

**Maximum Single Step size**

The Maximum Single Step is the largest Z move the objective will make while scanning to find focus. The value entered on the Auto Focus tab will depend on two things: the objective being used, and the signal to background noise ratio (S/N) of the sample.

A higher magnification requires a smaller step size. This is due to the smaller depth of field and narrower point spread function of the higher numerical aperture objective. Numbers for the typical accuracy and maximum single step size for different objectives are listed in the Table 1 below. In general the Maximum single step size is 2-5 times the accuracy.

The S/N of the sample also affects the maximum single step size that can be used. An extremely bright sample exhibiting a high S/N can be detected from further away in comparison to a sample with low S/N. Thus a larger step size can be used with the higher S/N sample and a smaller step size with a smaller S/N.

So, if the sample is relatively weak and a higher numerical aperture objective is used then the Maximum Single Step size should be decreased.

**Setting the Parameters for Find Sample, Wide Focus, Narrow Focus**

The Auto Focus tab includes three types of autofocus operations that can be run at different times in the experiment. The user can specify the range and the accuracy that are used for each operation. If there are multiple focus devices on a system the user can specify which focus device is used for which operation based upon the range and speed of the devices. The three operations and their general uses are shown below.

**Find sample:**

The find sample operation is always run at the very start of reading a plate. The find sample operation is run at the start of an acquisition to reset the focus origin. The range used for the find sample operation depends upon how the system is being used. If plates are being loaded and focus is determined (either manually or using automation) the range for the find sample operation can be small or even 0. If plates are being loading and no auto focusing is done before hitting acquire and there is a lot of variation in the plates or in the seating of the plates, a larger range of travel may be needed. Suggested accuracy here is usually the same as is given for the objective under typical accuracy for objective.

It is also important to pick a good position for finding the first sample. This position is set on the Plate tab (*First Well for Finding Samples*). If the outer wells are always lower and the middle of the plate is always the highest choose a good location that is somewhere in between to be able to minimize the Wide Focus Range. For example C4 in a 96 well plate- not the outside edge and not the middle could be used.

### Wide focus:

The wide focus operation can be run at every well, every wavelength or every site within a well. The range used for wide focus depends on the plate being analyzed and the way the operation is used. The most common use is to focus at the start of each well. To use the wide focus in this way, the maximum distance (in Z) from the Z origin needed to carry out focusing at any position on the plate needs to be known. Since larger ranges require more exposures to light and movements in Z there is an advantage in trying to minimize the focus range. To manage this, pick a position in the middle of the Z range, so that the distance to the top and bottom points from that position are approximately equal.

### *To determine a range and a starting position for focusing in the image*

Step	Action
1	Use Screen Acquisition to move to position A1
2	Bring the sample in the well into focus. Record the Z position
3	Move to positions .A6, A12, D1, D6, D12, H1, H6, and H12 and repeat step 2 for each position.
4	Determine the maximum variation in Z across the plate.
5	Estimate a position on the plate that might be equidistant in Z from the highest and lowest position. Check the position to see if its focal position is close to expectation, otherwise pick a different location on the plate.

Figure 1.

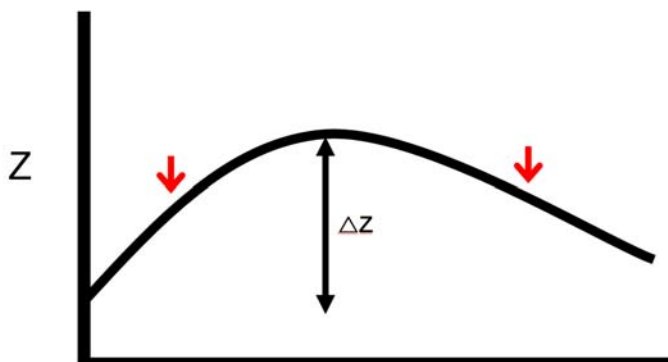


Figure 1: In the graph above the Y axis is focal height (Z) and the X axis is distance across the plate (any X,Y dimension). The graph shows a bowing of the plate such that the center is higher than the outside edges. The maximum variation in Z across the plate (step 4) is represented by  $\Delta Z$ . Two estimates for positions on the plate that might be equidistant in Z are shown by the red arrows.

#### Narrow focus:

The Narrow focus operation has been used to adjust the focus between sites in a well or between wavelengths at a site. There might be enough of a focal difference between sites that a single Z position will not work for the entire well. Likewise it is possible that two wavelengths focus on different Z positions. The range used for Narrow Focus depends upon these different operations. The focal position across a well can vary greatly, especially for plates with smaller numbers of wells. Because the range of the narrow focus operation is less than the wide focus operation a high speed short range focus device is often used.

**Table 1. Suggested settings for plastic plates:**

Magnification	Max. Single Step	Find Sample		Wide Focus		Narrow Focus	
		Range	Accuracy	Range	Accuracy	Range	Accuracy
2x	160-400	750	60	250	40	10-20	40
4x	50-150	750	15	250	10	10-20	10
10x	30-75	750	10	250	7.5	10-20	7.5

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20x	10-40	750	4	250	3	10-20	3
40x	5-20	750	0.25	250	0.1	10-20	0.1
60x	1-5	750	0.1	250	0.05	10-20	0.05

### Multiple Wavelengths

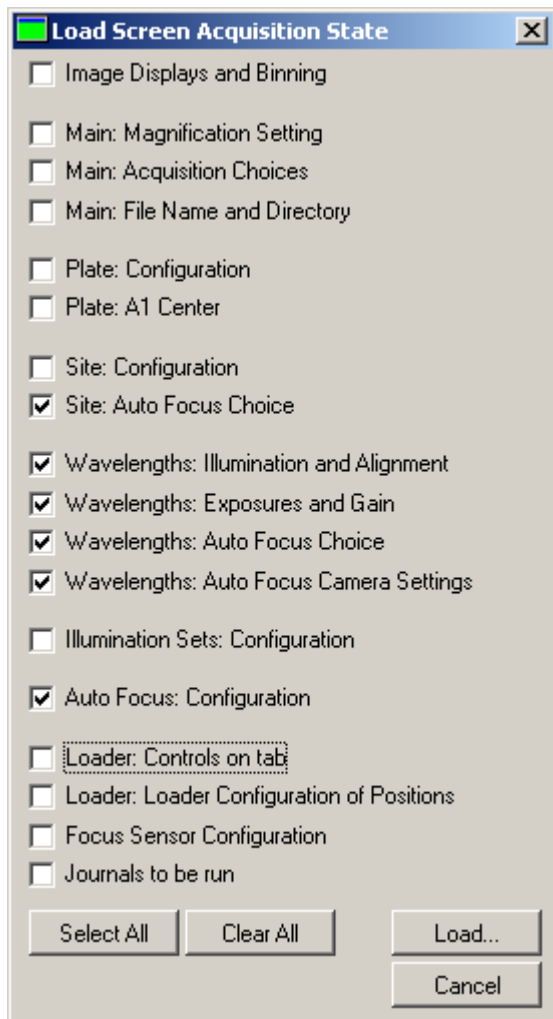
When multiple wavelengths are collected, the first wavelength is used to find the sample during auto focusing. The first wavelength should:

- Be present in all wells of your sample.
- Have the best signal to noise at shorter exposures.
- Be the most photo-stable (less likely to photo-damage).

The first wavelength often uses transmitted light since no probes are required and there is usually a high signal to noise at low illumination intensities. Other typical first wavelengths are nuclear probes since most cells have nuclei and many nuclear probes have high signal to noise and are fairly photostable.

### Save and Retrieve Saved Parameters

Once you have configured the appropriate screen acquisition settings that work for the specific samples, save the parameters in a state file by choosing Save State on the Main tab. To recall parameters, select Load State on the Main tab. From the Load Screen Acquisition State dialog choose the options to load. Most often these settings are: *Site: Auto Focus Choice, Wavelength: Illumination and Alignment, Wavelengths: Exposures and Gain, Wavelengths: Auto Focus Choice, Wavelengths: Auto Focus Camera Settings, and Auto Focus Configuration.*



For further assistance please contact Meta Imaging Series<sup>®</sup> technical support at 800-635-5577 option 3-2-2 or email [support.dtn@moldev.com](mailto:support.dtn@moldev.com).