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Optimizing Laser Autofocus Parameters in MetaXpress[®] 5.x

Revision B

What is Laser Autofocus (LAF)?

- Laser : device emitting focused beam of light
- Autofocus: device that automatically adjusts the focus of a microscope
- The laser autofocus mechanism in the ImageXpress uses a 690 nm (far red) laser beam & dedicated camera
- The software and hardware (moving up and down in Z height) are working together to detect an in focus (brightest) reflection of that laser the bottom of a plate (or well)



Principles of Laser Autofocus



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What is the LAF Wizard Measuring?





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When Do I Need to Set Up or Optimize Laser Autofocus?

- When you set up a new plate (or slide) type on the ImageXpress Micro
- When you begin using a new objective with an existing plate type
- When you use environmental control with your assay
- Whenever you see issues with focus with a plate/slide that you have previously set up using the LAF Wizard





Advantages of Laser Autofocus (LAF) vs. Image Based Focusing

	Laser Autofocus	Image Autofocus
Type of light	Far red 690 nm	From 350-750, depending on filter cube selected
Exposure length	Short (us)	Long (ms)
Auto bleaching of sample	Nile to very low	Extremely likely
Damages/ kills cells	Very low	Yes
Speed	Fast	Slow
Frequency lack of signal	Low	High (If no cells/ signal, no focus)
Ability to follow signal that is moving in Z	No	Yes

In general LAF is preferred due to it speed and low photo bleaching effects. Assistance of image based focus is needed when the probe is moving to different compartments in the cells or when the plate is severely damaged



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Workflow for Initial Laser Autofocus Determination in MetaXpress





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Before Running the Laser Autofocus Wizard ...

- Plate dimensions (available through manufacturer)
- Notate the thickness of the plate as you will need to set the correction collar of any objectives in the system to this value before beginning
- Empty plate with no media or sample
- Plate with only media in all wells which are at least 50% full (recommended)

OR

Plate with cells and media in all wells (not recommended)

NOTE If the plate will be used with environmental control, it is recommended to allow the system to equilibrate for at least two hours. The plate(s) being test should also be equilibrated at the same temperature for at least 20 minutes





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Running Laser Autofocus Wizard

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Laser Autofocus Wizard is on the Plate Tab



- 1. Click on the **Plate** tab in the Plate Acquisition Setup wizard
- 2. To create a new plate, select a plate name that most resembles (i.e. plate type, manufacturer) your plate
- 3. Adjust the plate dimensions according to manufacturer specifications and click on the **Save Configuration...** button. It is recommended to save the plate as another name
- 4. Make sure that a 10X objective is in the system
- 5. Click on the Laser Autofocus Wizard ... button



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Laser Autofocus Wizard Instructions



- The Plate Acquire LAF Setup Wizard screen will appear
- Click Next >>



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Selecting Wells and Sites

Plate Acquire LAF Setup Wizard
Measure Bottom Variation
Well selection
All wells Selection pattern. Groups of wells Checkerboard with gaps, including A1
Group size (NxN): 2 😴 Gap between groups: 0 🛫
Selecting more wells takes longer to finish mesaurement but provides more robust autofocus settings, especially for plates with a large amount of variation.
Sites per well
Multiple sites 5 Performing measurements on multiple sites/well is slower but provides intra-well variation data, which may result in
C Single site faster site-to-site LAF.
1 2 3 4 5 6 7 8 9 10 11 12
IF ČČČČČČČČČČČČČ
G ČČČČČČČČČČČČČČ
Time estimate: 0:06:00
<< Back Next>> Cancel

- Well Selection
 - It is recommended to select all wells.
 Note that this can be quite time consuming but more accurate.
 - To complete a quicker LAF test, select
 Checkerboard with gaps, including A1.
 You can adjust the group size and gap
 between groups to adjust the number of wells test.
- Sites per well
 - It is recommended to select multiple sites. 5 sites is best, 3 should be the minimum



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Finding Physical Properties of the Plate



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- Measure Bottom Variation screen
 - It is recommended to have the 10X Plan Fluor objective in the system.
 - This screen will tell you which objective will be used to calculate plate bottom variation.
 - Click Next >>
- Load a Plate into the system
 - Click on the **Open door/clamp** button to open the stage door and load an empty plate (no media or cells)
 - Click on Close door
 - Make sure the radio button next to Calculated Values is selected
 - Only check the Allow Extreme Variation box if this is a thin bottom plastic plate ($<100 \ \mu m$)
 - Click Start >>



Laser Autofocus Wizard Status

Plate Acquire LAF Setup	Wizard			×
Measure Bottom Varia	tion Summary	1		
- Plate bottom thickness -				
Typical thickness (um)	: 0.0	Variation	n (+/-): 0.0	
Plate bottom variation —	Main	Mariana	A	Ord Days
Adjacent wells (um):		Maximum	Average 0.0	Std. Dev.
Intra-well (um):	0.0	0.0	0.0	0.0
Plate maximum variation	(um): 0.0			
- 11 C				
8			-	
	1.000	a - 2	-	
1				
	All LAF sev	arches succeed	di	
	<< Back	Next>>	1	Cancel
			1	

- Measure Bottom Variation Status
 - This screen will tell you the status of the laser autofocus wizard
 - It will determine the optical thickness of the plate, variation within wells, and variation between wells.
 - The wells tested with be color coded. Only stop the wizard if after 4 wells all have been marked in red (click the ESC button the keyboard and refer to the rest of the document for troubleshooting guidelines)



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Plate Bottom Variation Summary

	mizaru			×
Measure Bottom Vari	ation Summary	,		
Plate bottom thickness				
Typical thickness (un	n): 0.0	Variation	n (+/-): 0.0	
Plate bottom variation – Adjacent wells (um): Intra-well (um): Plate maximum variation	Minimum 0.0 0.0 (um): 0.0	Maximum 0.0 0.0	Average 0.0 0.0	Std. Dev. 0.0 0.0
		1. 	۰. 	
	•			
	- ⁻		۰.	
	ALLAF se	auches succeede	id.	12
	All LAF se	arches succeede	be	
	All LAF se	arches succeede	ed!	
	All LAF se	arches succeed	d	
	All LAF se	arches succeeds	ed	
	All LAF se	arches succeeds	ed	

- Measure Bottom Variation Summary
 - This screen will tell you the typical thickness of the plate
 - If there are objectives with correction collars in the system, you may need to exit out of the wizard to change them if you cannot access the objectives easily from the sides. Use this number * refractive index to set the correction collar on any objectives
 - If the [Optical Thickness * Refractive Index (generally 1.5)] is more than 20% different than the suggested thickness from the manufacturer, you will need to adjust the plate bottom thickness before moving on.

Note This can be done in the Plate Acquisition Setup window under the Plate tab. You will need to exit out of the wizard by clicking on **Cancel**.



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Plate Bottom Variation Summary

Plate Acquire LAF Setup Wi	zard			×
Measure Bottom Variation	on Summary	,		
─ Plate bottom thickness ──				
Typical thickness (um):	0.0	Variation	n (+/-): 0.0	
Plate bottom variation	Minimum	Maximum	Average	Std. Dev.
Adjacent weils (um).	0.0	0.0	0.0	0.0
Plate maximum undation for		0.0	0.0	0.0
riate maximum variation (ur	ių. 0.0			
	:		•	
	All LAF ce	arches succeed	bel	
	<< Back	k Next>>		Cancel

- The software will tell you if the number of failures exceeds the ability to determine LAF successfully. If you receive this error, click **Cancel** and refer to the troubleshooting section
- Otherwise, click Next >>



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Calculating Laser Exposure Times

te Acquire LAF Setup Wi	izard	8
Calculate Exposure Par	ameters	
Select the medium that w magnification setting to u	ill be used when running this plate type. The se for calculating exposure times.	en select the
Medium: Aqueous m	nedia 🚽 Refractive index: 1.33	
Magnificati	on setting for calculating exposures:	
20x Super	Plan Fluor ELWD 🗨	
Select LAF step sizes (um)	
Calculated values (based on objectiv	(fine step = 0.0, coarse step = 0.0 (fine step = 0.6, coarse step = 4.9 ve N.A.)	
Press Next>>to measur	e new exposure settings.	

- Calculate Exposure Parameters
 - The next step will be to calculate laser autofocus exposure times based on media in the plate
 - Select the type of media from the drop down list. If you are unsure of what to use, select unknown
 - Select the 10x objective from the drop down menu (you will need to repeat this process for every other objective in the system)
 - Select the radio button next to Calculated Values
 - Click Next >>



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Well Selection and LAF Settings



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land a WET provide the late	diami.	
MOTE: Deare proceeds of Array or actual sequences	inted and particular datasets	e with a sec
LAF search type	Rose (clamp) Class door	underland the
 Place and well bottom Place bottom only 	infractive today of the plate and r man's districut to a granuplate of paged medium?	andur ye di gyondi

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- Well Selection
 - It is recommended to select all wells. Note that this can be time consuming but more accurate
 - To complete a quicker LAF test, select **Checkerboard with gaps, including A1**. You can adjust the group size and gap between groups to adjust the number of wells tested.
 - It is recommended to select multiple sites per well . 5 sites is best, 3 should be the minimum
 - Click Next >>
- Calculate Exposure Parameters
 - Click Open door/clamp and replace the dry plate with the plate containing media in all wells
 - Click on the Close door button
 - Select Plate and well bottom under LAF search type (only select plate bottom only if working with low magnification objectives (1x, 2x, 4x) or samples with glycerol and mounting media
 - Click Next >>



LAF Exposure Summary

	r Setup wi	zard			
Exposure Mea	asurement	Summary			
Exposure varia	tion —	Junnary			
		Minimum	Maximum	Average	Std. Dev.
Plate bottom ex	(posure (us):	1.0	1.0	1.0	0.0
Well bottom exp	posure (us):	1.0	1.0	1.0	0.0
LAF search ste	ep sizes (um)				
Fine step =	= 0.6	Coa	rse step = 4.9		
- Plate bottom ex	xposures (us	;)		m exposures (us	3)
1st pas	s- 1.0		1st	t pass- 1	0
2nd na	ss- 2.0		20	d pass- 2	0
3rd page	5.0		20	10366 5	0
		All LAF sea	rches succeeded		
	Save se Discard s	All LAF sea Save settin ttings and me ettings and m	rches succeeded	d ter objective	

- Exposure Measurement Summary
 - When LAF wizard is complete, a summary will appear with the exposure times
 - The software will automatically determine if the failures exceed the ability to determine exposure times successfully. If there are too many failures, click on Discard Settings and measure with another objective or Cancel to exit the wizard. Refer to the rest of the document for troubleshooting guidelines
 - If the wizard was successful, select one of the Save settings options
 - You will need to repeat this process for all objectives in the system



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Check LAF Wizard Settings



- 1. Put a plate with sample in the system and open the **Plate Acquisition Setup** wizard
- 2. Select the appropriate plate type from the drop down menu on the **Plate** tab
- 3. On the **Wells to Visit** tab, right click on a well that has sample to move the stage
- 4. On the **Sites to Visit** tab, select single site
- 5. On the **Acquisition Loop** tab, only select *Enable laser-based focusing*
- 6. On the **Autofocus** tab, select the appropriate *Well to well autofocus* routine for your objective and plate (Refer to the *Ensure Proper Focus Method is Selected* section in this document for details)



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Check LAF Wizard Settings

Names and Description	Expension setting [DAP]
Objective and Camera- Wx Super	
Piece: 90 Wells (0x52)	Exposure (ms) 200 🖞 Auto Expose Target max intensity (3000 🖞
Sites to Visit- 90 or 90	A second second
Timelapse-1 time point(s)	Tet Care Barding
Acquisition Loop	test settings offset (un)
Autofocus	Laser with z-offset • 0 4
WI DAPI	
W2 FITC	
Journals- 0 selected	Range (um) Step (um)
Display Settings	Cacolan one 1 1 1 one c mar 1 commissinge (17) 1 2 (24)
Post Acquisition	1
Summary	
	Digital Confocal ☐ Englise
	Shading Correction: Off

- On the W1 tab, select an appropriate wavelength and exposure time for your sample
- 8. Select Laser with z-offset in the Autofocus options section
- 9. Make sure the **Post-laser offset** is set to 0
- 10. Click on the **Test Settings** button





Check LAF Wizard Settings

Names and Description	Spoare
Objective and Camera- 20x Super	
Plate- 96 Wells (8x52)	Exposure (ms): 200 (1) Auto Expose Target max Intensity (2000 (1)
Wells to Visit- 96 of 96	
Sites to Visit- multi-well	Autofocua optione
Timelapse-1 tinve point(s)	Test Settings Post-laser
Acquisition Loop	Offset (un)
Autofocus	Laser with z offset
WI DAPI	
W2 FITC	
Journals- 0 selected	Coloulete Officet c V Use Zistack Custom Rance
Display Settings	
Post Acquisition	
Summary	
	Digital Confocal
	Shading Correction: Off

- An image should appear that is relatively in focus (this may not be true if your sample is in a completely different plane of focus or if you imaging through extracellular matrix)
- 12. Use the **Calculate offset** button to determine the true offset for your sample
- 13. Acquire the plate
- 14. During acquisition, take note if laser autofocus is taking a long time between wells or if the images are out of focus. If so, refer to the rest of the document for troubleshooting guidelines



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Running Laser Autofocus Wizard for Additional Objectives





- To run Laser Autofocus wizard again for a particular objective or for a new objective
 - Click on the Laser Autofocus Wizard... button on the Plate tab in Plate Acquisition Setup wizard
 - You can bypass testing plate variation settings by selecting Calculated Values on the Measure Bottom Variation screen and clicking Next >>
 - This will bring you back to Calculate Exposure Parameters
 - Follow the guidelines in previous slides



Troubleshooting Laser Autofocus

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Double Check if Laser Autofocus Settings Exist

xperiment- Experiment1	Laser-based Focusing
Names and Description	Configure Laser Settions
Objective and Camera- 4X PA	
Plate- 96 Wells (8x12)	- Well to well autofocus Focus on well bottom
Wells to Visit-1 of 96	mage-based Focusing
Sites to Visit- single site	Alexandread and Research 1 and 10 Contractioners
Timelapse-1 time point(s)	
Acquisition Loop	Allow image-based focusing for recovery from laser-based well bottom failures
Autofocus	
W1 FITC	
Journals-1 selected	Initial well for finding sample Skip Find Sample beliect if sample is 💌 A 🚽 1 😳
Display Settings	Number at wells to attempt relial field sample 1
Post Acquisition	
Summary	
	Wew Focusing Details
	View Focusing Details
	Vew Focusing Details
Configure Laser Autofocus Settings Adfocus Settings Ide name: 96 Wells (&k12) Iag setting: 10X S Plan Fluor FLWD ettings file: C.XMX 5.1 vplates 36 Wells (&k1	View Focusing Details
Configure Laser Autofocus Settings utofocus Settings Yate name: 96 Wells (&12) Mag setting: 10X S Plan Puor ELWD Save Settings (C. C.MX 5.1 vplates 36 Wells (&1 Save Settings) Load Settings Lase	View Focusing Details
Configure Laser Autofocus Settings Lutofocus Settings Tate name: 96 Wells (kr.12) Mag setting: 10X S Plan Ruor ELWD Save Settings Load Settings Lase Plate Bottom Settings	View Focusing Details
Configure Laser Autofocus Settings utofocus Settings Plate name: 96 Wells (&r.12) Hag settings file: C:VMS 5 Tylates 95 Wells (&r.1 Save Settings Lase Plate Bottom Settings Lase Plate Bottom Settings 360um Bottom thickness may validion 10um	View Focusing Details
Configure Laser Autofocus Settings utofocus Settings Plate name: 96 Wells (&:12) Mag settings file: C:VMX 5 Tiplates 96 Wells (&:1 Save Settings Plate Bottom Settings Bottom thickness 360um Bottom thickness 10um Bottom thickness 060um	Vew Focusing Details
Configure Laser Autofocus Settings Lidiocus Settings Vate name: 96 Wells (&x12) lag settings: 10X S Plan Fluor ELWD lettings file: C:VMX 5. 1'vplates '96 Wells (&x1 Save Settings Load Settings Lase Plate Bottom Settings Bottom thickness 360um Bottom thickness 360um Bottom thickness 10um Adjacent well max variation 60um Intra-well max variation 5um	Vew Focusing Details
Configure Laser Autofocus Settings Udfocus Settings Yate name: 96 Wells (&x12) Jag settings: 10X S Plan Hour ELWD Save Settings: Load Settings Lase Plate Bottom Settings Bottom thickness 360um Bottom thickness max variation 10um Intra-well max variation 60um Intra-well max variation 5um	Vew Focusing Details
Configure Laser Autofocus Settings tutofocus Settings Yate name: 96 Wells (&r.12) Ag settings file: C.V.MS 5.1yalaes 95 Wells (&r.1 Save Settings Load Settings Lase Plate Bottom Settings Bottom thickness 360um Bottom thickness 360um Rottom thickness 360um Intra-well max variation 50um Plate max variation 60um Step Size Coarse step (um) 11 © Fine st	Vew Focusing Details
Configure Laser Autofocus Settings tutofocus Settings Plate name: 96 Wells (&r.12) Mag settings 10: XS Plan Flour ELWD Settings file: C: XWA 5. Tylates 95 Wells (&r.1 Save Settings Load Settings Lase Plate Bottom Settings Bottom thickness 360um Bottom thickness 360um Intra-well max variation 60um Intra-well max variation 5um Plate max variation 60um Step Size Coarea step (um) 11 💼 Fine st	Vew Focusing Details
Configure Laser Autofocus Settings Lutofocus Settings Tate name: 96 Wells (8x12) dag setting: 10.5 Pion Fluor ELWD Settings file: C-VMX.5 Tiplates '96 Wells (8x1 Save Settings Laad Settings Lase Plate Bottom Hickness max variation Bottom thickness max variation 10 um Adjacent well max variation 60 um Plate max variation 60 um Plate max variation 60 um Plate max variation 60 um Plate bottom Exposure Plate Bottom Exposure (us) Attempt	Vew Focusing Details
Configure Laser Autofocus Settings utofocus Settings Iate name: 96 Wells (8x12) dag setting: 10.05 Plan Fluor ELWD Save Settings Load Settings Lase Plate Bottom Settings Load Settings Lase Bottom Hickness 360um 80um Bottom Hickness max variation 10um Adjacent well max variation 60um Plate Bottom Exposure Coarse step (um) 11 ⊕ Fine st Plate Bottom Exposure Well Bottom Attempt Exposure (us) Attempt Exposure (us) 1 1 1	New Focusing Details Save Settings Save Settings Previous Next Qost Qost Lippoure pt Expoure (x)

Preview Pass

Start

Range

Step size:

Exposure:

Find Sample

Preview Pass

Note: Set the Preview Pass exposure by selecting an exposure time above or use "Override exposure" below

Autofocus

Override range (um)

Focus Snap

11.00um Coveride step (um)

400.0us Override exposure (us)

610 0um

 On the Plate Acquisition Setup Wizard, click on the Objective and Camera tab

- 2. Select the objective you plan to image with
 NOTE In MX 5.1.0.46 and above, the Autofocus tab
 will be highlighted if laser autofocus settings do not exist
- 3. Click on **Plate** tab and select your plate from the drop-down menu
- Click on the Autofocus tab and then click on the Configure Laser Settings button
- 5. The Configure Laser Autofocus Settings dialog box will appear
- 6. The fields beneath Step Size and Plate Bottom / Well Bottom Exposure settings should be populated
- If not, go back to the Plate tab and run the Laser Autofocus Wizard for Plates or click on Setup Slide as Plate on the taskbar for Slides



Selecting Well for Focus

Recommendations for Initial Well for Finding Sample

- Plate Acquisition Setup Autofocus Experiment-Experiment1 Laser-based Focuring Names and Description Configure Laser Settings Objective and Camera- 10X S Pla Record and set of Jell Focus on well bottom Plate-36 Wells (Bd2) cus on well bottom Wells to Visit-1 of 95 Image-based Focusing. Focus on plate bottom, then offset by bottom tricking Sites to Visit-single site Aportion: Standard Timelapse-1 time point(s) Afore mage-based focusing for recovery from laser-based well bottom failures Acquisition Loop Autofocus W1 FITC Initial well for finding sample. First well acquired A | A lournals-1 selected **Display Settings** Amber of wells to attempt initial find sample. **Pest Acquisition** Summary Sove Settings. Synnay ... < Perox
- Default settings are select First well acquired
- If only using laser autofocus, select *First well* acquired. If you find that focus is not found due to the objective / plate combination, you can select to focus on another well.
- If using image based focus (with or without laser autofocus), you may chose to select a specific well where you sample is located. Image autofocus searches a larger range for the first well acquired. Therefore, if your first well does not contain an appropriate sample this can result in out of focus images.
- Change Number of wells to attempt to initial find sample to 3.



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Ensure Proper Focus Method Is Selected



Check that the proper focus method is selected for your plate/slide, objective, and sample type under the **Well to well autofocus** drop-down menu

Focus on well bottom

• Fastest, best for 10x, 20x, and 40x objectives

Focus on plate bottom, then offset by bottom thickness

- Always use for Slides
- Always use for 1x and 2x, best for 4x with thin bottom plates
- Plates with agar or other thick matrix

Focus on plate and well bottom

 Best for thin, variable plates or weak reflections on well bottom



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Ensure Proper Focus Method Is Selected



Recommendations for Initial Well for Finding Sample

- Default settings are select *First well acquired*
- If only using laser autofocus, select *First well acquired*. If you find that focus is not found due to the objective / plate combination, you can select to focus on another well.
- If using image based focus (with or without laser autofocus), you may chose to select a specific well where you sample is located.
 Image autofocus searches a larger range for the first well acquired. Therefore, if your first well does not contain an appropriate sample this can result in out of focus images.
- Change Number of wells to attempt to initial find sample to 3.



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Checking if Laser Autofocus Settings Need to be Optimized

Nement 1530 Wek 4X Sample	Number of wavelengths	
Objective and Cemera-Aultager Plate: 1536 Well Plate Walks to Vise: 9 of 3072 Strey to Vise: multi-well	Adobcus options 19 Toutie lase based focusing 19 Endle magebased focusing for scquattor or lase recovery)	
Timelapse: 1 time point()) Acquidition Loop Autofocus WL DAPI W2 FITC	Febtos inalog constant Service 1 - sette tabates interdes (2011)	
Inumate: 8 selected Display Settings Post Acquisition		
Summary		

aperiment- 2590 We8 4X Sample	Laser Loand Focusing		
Names and Description	Carlgare Lawr Settings.		
Objective and Certera-4x Super	Well to well autotocks Focus on plate botton, then reflect by bottom their ress		
Pune-15/0 mel Pune			
With to Find: 9 of 30/2	high based Focueing		
adds to the mark-well	Acathei Santat V Broom A C Outre encourings		
timespise- 2 time pow(d)	22 Allow processing the second way have based well before failures		
- Acquaiment coop			
Sat Dabl			
W2 FTC	initial well for finding sample (Feet well accurred +) [A] [-+]		
Incomerce & option that			
Diselas Settings	Number of welle to abende initial foot sample: [1]		
Fort Accusition			
Summary			
and the second s			
	1 P		
	Very rocuting Details		

- Click on the Acquisition Loop tab in the Plate Acquisition Setup Wizard and make sure there are check marks in the boxes next to Enable laser-based focusing and Enable image-based focusing
- 2. Click on the Autofocus tab
 - Select the appropriate method for Well to well autofocus
 - Make sure there is a check mark next to the box for Allow image-based focusing for recovery from laserbased well bottom failures
- Click on the Display Settings tab and make sure there are check marks in the box next to Display images during autofocus



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Checking if Laser Autofocus Settings Need to be Optimized

ennent-1536 Well 4X Sample	/ booke
Harris and Celophen	Burgration setting: DAPI
Objective and Catters- 4s Super	
Plate-15/6 Weichate	Expense test 200 1 Atta Dapawe Target was viewely 2000 1
Wate to Visiti 9 of 3072	
Sites to Visit-multi-well	Addisse gilen
Timelapse- I time point()	Test Settices Populater Inspectated Mar. Map
Acquisition Loop	Construction and a second seco
Autofocus	Loser with image Record and IP2 500521 (100 21 P 21
W1 DAP1	The second se
W2:FETC	leage-based autofocus maximum # of Z moves = 101
Inumate-0 selected	Renge (and Step (and The Association Party and Step (and
Display Settingi	Catter Oren (4) w Ore 2 mox 1 Cumm range (112.5 1) (12.5 1)
Post Acquisition	
Summary	
	DytelCeffood
	Stating Constitution Of
	F Bradle Une Dealing Connection CP

- 3. Click on the W1 tab
 - Select Laser with Image Recovery from the Autofocus options drown-down menu
 - Definte and offset and range appropriate for the chose objective
- 4. Acquire the plate and look for the following:
 - Rhythmic acquisition with no hangs-ups; this indicates that LAF settings are good
 - Number of times image based focusing occurs.. When LAF fails, you will see an image appear which will indicate image based focus is active/in progress. If this happens only once during the imaging routine, your LAF settings are probably okay. However, if you see more than one instance, proceed with further LAF Optimization.



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How to Adjust Laser Autofocus Parameters in MetaXpress

🗟 Configure Laser Autofocus Settin	gs 🗖 🖻 🕱
Autofocus Settings Plate name: 96 Wells (&x12) Mag setting: 10X S Plan Fluor ELWI Settings file: C:\MX 5.1\plates\96 W) /ells (8x12).plt
Save Settings Load Settings	Laser Autofocus Wizard
Plate Bottom Settings	
Bottom thickness	360um Edit Plate Bottom
Bottom thickness max variation	10um
Adjacent well max variation	60um
Intra-well max variation	5um C0um
Fiate max variation	muuo
Step Size	
Coarse step (um) 11 🌲	Fine step (um) 1.4 🌲
Plate Bottom Exposure	Well Bottom Exposure
Attempt Exposure (us)	Attempt Exposure (us)
1 10.00	1 100.0
2 20.00	2 200.0
3 40.00	3 400.0
Preview Pass	
Note: Set the Preview Pass exposure exposure time above or use "Override	by selecting an exposure" below
Start: 1892um 🔲 Start fro	om current position
Range: 610.0um 🔲 Overrid	e range (um) 🛛 🖯
Step size: 11.00um 🔲 Overrid	e step (um) 10 🚊
Exposure: 400.0us 🔲 Overrid	e exposure (us) 10 🚊
Preview Pass	
Find Sample Autofocus	Focus Snap Close

- Open the Configure laser Autofocus Settings dialog box (click on the Autofocus tab and click on the Configure Laser Settings button)
- 2. Double-check that LAF settings exist under the Step Size and Plate Bottom / Well Bottom Exposure sections
- 3. There should not be any check marks in the boxes under the **Preview Pass** Section



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How to Adjust Laser Autofocus Parameters in MetaXpress

Configure Laser Autofocus Settings	
Autofocus Settings Plate name: 96 Wells (&x12) Mag setting: 10X S Plan Fluor ELWD Settings file: C:\MX 5.1\plates\96 Wells (&x12).plt	4.
Save Settings Load Settings Laser Autofocus Wizard	
Plate Bottom Settings	
Bottom thickness 360um Edit Plate Bottom	
Bottom thickness max variation 10um	
Adjacent well max variation 60um	E
Intra-well max variation 5um	э.
Plate max variation 60um	
Step Size	
Coarse step (um) 11 🚔 Fine step (um) 1.4	
Plate Bottom Exposure Well Bottom Exposure	6.
Attempt Exposure (us) Attempt Exposure (us)	
1 10.00 1 100.0	
2 20.00 2 200.0	
<u>3</u> 40.00 <u>3</u> 400.0	
Preview Pass	
Note: Set the Preview Pass exposure by selecting an exposure time above or use "Override exposure" below	
Start: 1892um Start from current position	
Range: 610.0um Override range (um) 1000	
Step size: 11.00um 🔲 Override step (um) 10 🛓	
Exposure: 400.0us Override exposure (us)	
Preview Pass	
Find Sample Autofocus Focus Snap Close	

- The exposure times under the Plate Bottom and Well Bottom Exposure fields are the autofocus laser exposure times calculated by the system tools (LAF Wizard or Set Up Slide As A Plate)
- Click on the lowest exposure time in the Well Bottom Exposure field, then click on the **Preview Pass** button
- The Preview Pass graph window will appear



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Preview Pass Graph Window

What is a Preview Pass?

- A search along the z-axis to find the plate bottom and well bottom positions
- Allows you to see the intensity peaks of the focus laser reflection from the plate and/or well bottom (red peaks)
- Used to measure plate bottom thickness
- Used to measure plate bottom variation
- Preview Pass is not LAF!!!





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What Does an Ideal Preview Pass Look Like?

- 2 focus peaks should appear when the **Attempt 1 Well Bottom** exposure time is highlighted
- Both peaks are at the center of the Z Position Range (X Axis on the Preview Pass Graph)
- Brightest pixel (red peak) on the Well Bottom reflection is NOT saturated i.e. focus score is less than a value of 255 on the Y axis of the Preview Pass Graph
 - The first attempt exposure time should result in a red peak that is around 60
- There should be at least 4 points that compromise the peaks. If there are less than 4, than the course step is too big
- There should be no noise in the green line between the plate bottom and well bottom peaks. Noise usually indicates that the exposure time is too high.



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Adjusting Exposure Times

Whether **Plate Bottom** or **Well Bottom** exposure times are used will depend on the Well to well autofocus method you chose in the Plate Acquisition Setup wizard

- 1. You will need to adjust these exposure times to optimize Laser Autofocus Settings
- 2. In the Plate Acquisition Setup
 - Select the appropriate objective from the Objective and Camera tab
 - Select the appropriate plate from the drown down menu under the Plate tab
 - Move to a well preferably in the middle of the plate on the Wells to Visit tab

	Experiment- Experiment1	Later-based Focusing
	Names and Description	Configure Laser Settings
	Objective and Camera- 4X PA	Holite and a state of the second state of the
	Plate- 96 Wells (8x12)	Well to well subtrocue Focue on well bottom
	Wells to Visit-1 of 96	Image-based Focusing
	Sites to Visit- single site	Acother Standard Bonno: 1 1 Diston emosure times
	Timelapse-1 time point(s)	
	Acquisition Loop	Allow image-based focusing for recovery from laser-based well bottom failures
	Autofocus	
	W1 FITC	
	Journals- 1 selected	Initial well for finding sample Skip Rind Sample (select if sample is 💌 A 🔍 1
	Display Settings	Number of wells to attempt ential field sample: 1 (2)
	Post Acquisition	
	Summary	
		(9)
and the second		Wew Focusing Details

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Adjusting Exposure Times for Plate Bottom

Coarse step	(um) 11	Fine step (um) 1.4	-
Attempt 1 2 3	Exposure Exposure 10.00 20.00 40.00	e (us) Attempt Exposure (1 100.0 2 200.0 3 400.0	us)
Preview Pass Note: Set the exposure time	Preview P above or	iss exposure by selecting an se "Override exposure" below	
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Exposure: Preview Pa	10.00u iss	□ Override exposure (us) 10	A F

- 3. Highlight the first exposure time under **Plate Bottom Exposure** and click on the **Preview Pass** button.
 - You should see at least one peak relatively in the middle of the graph
 - The Red peak should not be saturated and reach at least 60 on the Yaxis
 - Increase the exposure time if the red peak is less than 60 or decrease the exposure time if the red peak is saturated at 255
 - The lowest exposure time is 1 μs
 - Increment in 1 μs for exposures between 1-10 μs and 10 μs for anything above
- 4. Adjust exposure times for Attempt 2 so that the red peak reaches between 100 -200 on the Y-Axis
- 5. Adjust exposure times for Attempt 2 so that the red peak reaches above 225 on the Y-Axis
- 6. It is recommended to test the above procedure on several wells spanning the plate

The exposure time used by preview pass is the one highlighted in blue. Select values with cursor to highlight.

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Adjusting Exposure Times for Well Bottom



- 3. Highlight the first exposure time under **Well Bottom Exposure** and click on the **Preview Pass** button.
 - You should see two peaks relatively in the middle of the graph
 - The **Second Red** peak should not be saturated and reach at least 60 on the Y-axis, you can ignore the first red peak
 - Increase the exposure time if the red peak is less than 60 or decrease the exposure time if the red peak is saturated at 255
 - The lowest exposure time is 1 μs
 - Increment in 1 μs for exposures between 1-10 μs and 10 μs for anything above
 - Typically well bottom exposures are longer than plate bottom
- 4. Adjust exposure times for Attempt 2 so that the red peak reaches between 100 -200 on the Y-Axis
- 5. Adjust exposure times for Attempt 2 so that the red peak reaches above 225 on the Y-Axis
- 6. It is recommended to test the above procedure on several wells spanning the plate

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When Your Peaks Are Not Centered in Preview Pass

If your peak(s) are present but not centered on the Z-position axis (X-axis) of the graph, this is most likely due to the well-depth of the plate being incorrect in the plate definition

To adjust well depth

- 1. On the Plate Acquisition Setup dialog, click on the **Objective and Camera** tab
- 2. Select the 10x Objective (It is suggested to change plate definition settings first using the 10x objective)
- 3. Click on the **Plate** tab
- 4. Adjust the value in the Well Depth (μm) field
- 5. Click on the **Save Configuration** button and return to the Configure Laser Autofocus Settings dialog window to check your settings.
- 6. Repeat this process until the peaks are centered along the X-axis of the Preview Pass Graph
- If you were initially adjusting settings for another objective, return to the Objective and Camera tab to select that objective and check that your peaks are in the center
- 8. If the peaks are not centered, this usually indicates that the parfocality (objective z offset) settings need to be adjusted
 - You can use the MetaXpress taskbar to determine objective parfocality OR
 - Create multiple plate definitions for each objective to adjust the well depth







When Peaks are Not Present in Preview Pass

There are several possible reasons if you do not see peaks in Preview Pass (100 s exposure should be sufficient for to see plate bottom for a 10x objective)

- 1. Sample plate is not loaded or objective is not within we the well area
 - Open and Close the door, then move to a well
 - Try several wells before adjusting any parameters
- 2. Laser exposure time is not high enough (see **Plate and Well Bottom Exposure** times in Configure Laser Autofocus Settings dialog box)
- 3. Coarse Step size is too large
- 4. Incorrect Z Start position for objective lens
 - For 4x, 10x, 20x ELWD, and 40x ELWD objective lens, increase the max plate variation by 200
- 5. Plate may have been bumped by objective. Open the stage door and check if plate is sitting flat in the plate nest.
- 6. Short working distance objectives may not be able to image edge wells due to the plate height and skirt.

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Be Sure to Save Settings!

Autofocus Settings Plate name: 96 Wells (8x12) Mag setting: 10X S Plan Fluor ELWD Settings file: C:\MX 5.1\plates\96 Wells (8x12).plt Save Settings Load Settings Laser Autofocus Wizard	1
Save Settings Load Settings Laser Autofocus Wizard	
Plate Bottom Settings	
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Bottom thickness max variation 10um	
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Intra-well max variation 5um	
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2 20.00 2 200.0	
3 40.00 3 400.0	
Preview Pass	
Note: Set the Preview Pass exposure by selecting an exposure time above or use "Override exposure" below	
Start: 1892um C Start from current position	
Range: 610.0um 🕅 Ovenide range (um) 1000	A.
Step size: 11.00um 🔲 Ovenide step (um) 10	di. V
Exposure: 400.0us Override exposure (us)	h. V
Preview Pass	
Find Sample Autofocus Focus Snap Close	

Once your peaks are optimal, be sure to click the **Save Settings** button in the Configure Laser Autofocus Settings dialog before closing.



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Double Checking Your Laser Autofocus Settings

🗟 Configure	Laser Autofocus Sett	ings 🗖 🗖 🖾	
Autofocus Settings Plate name: 96 Wells (8x12) Mag setting: 10X S Plan Fluor ELWD Settings file: C:\MX 5.1\plates\96 Wells (8x12).plt			
Save Setti	ngs Load Settings	Laser Autofocus Wizard	
- Plate Bottor	m Settings		
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Bottom this	kness max variation	10um	
Adjacent w	vell max variation	60um	
Intra-well m	nax variation	5um	
Plate max	variation	60um	
Step Size			
Coarse ste	p (um) 11 🌲	Fine step (um) 1.4	
Plate Bottor	m Exposure	Well Bottom Exposure	
Attempt	Exposure (us)	Attempt Exposure (us)	
1	10.00	1 100.0	
2	20.00	2 200.0	
3	40.00	3 400.0	

Preview Pass					
Note: Set the Preview Pass exposure by selecting an exposure time above or use "Override exposure" below					
Start:	1892um 🔲 Start from current position				
Range:	610.0um 🔲 Override range (um)	1000	×		
Step size:	11.00um 🔲 Override step (um)	10	×		
Exposure:	400.0us 🔲 Override exposure (us)	10	×		
Preview Pass					
Find Sample	Autofocus Focus Snap	Close			

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- 1. Make sure the correct objective is selected and you are on a well in the plate
- 2. Highlight the first exposure time
 - For low magnification objectives (under 10x), chose the first exposure time under Plate Bottom Exposure
 - For high magnification objectives (10x and above) or thin bottom plates, chose the first exposure time under Well Bottom Exposure
- 3. Click on Find Sample button
- 4. If you get a message that says Focus was found; LAF settings are now optimal
- If you get a message that says Focus was found but with overexposure, adjust the exposure time according and repeat steps 1-3



Contacting Technical Support for Help with LAF Optimization

• Before contacting Technical Support, please locate and obtain a copy of the following files:

From the temp folder: (C:\Users\ xxxxxx \AppData\Local\Temp) or enter in search %temp% MetaXpressAutofocusReport.txt MetaXpressAutofocusStatus.txt

- These files can be emailed along with your request for help to: <u>support@moldev.com</u>
- Please indicate in your message the nature of the issues you are encountering as well as the serial number of your instrument.
- Technical Support can also be reached by telephone: 1-800-635-5577, select options for Tech Support> Cellular Imaging Products > ImageXpress Instruments.



Support Resources

- F1 / HELP within MetaXpress[®] Software
- Support and Knowledge Base: <u>http://mdc.custhelp.com/app/home</u>
- Email <u>support@moldev.com</u>
- Technical Support can also be reached by telephone: 1-800-635-5577, select options for Tech Support → Cellular Imaging Products → ImageXpress Instruments.





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