

MetaXpress® Software Guide

Configuring Transwell plates for image acquisition



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Chapter Purpose

The purpose of this chapter is to explain the process of configuring and optimizing a Transwell plate for high-content imaging with the ImageXpress system and MetaXpress software.





Transwell assay



Transwell plates have inserts with a membrane at the bottom added to each well. Also known as Boyden chambers, these are used for measuring chemotaxis or cell migration.





Transwell schematic



- 1. Insert added to well with or without chemoattractant
- 2. Cells added to insert

- 3. If chemoattractant present, cells migrate through porous membrane
- 4. Read fluorescent signal from below

Tip: Phenol Red in the media increases background fluorescence in the FITC channel. Use Phenol Red-free media if this interferes with measurements

Transwell plates and Laser Autofocus

In a Transwell plate, the distance between the plate/well bottom and the insert may be inconsistent, causing focus issues.

Also, it can be difficult to adjust a very large Z offset from the normal plate/well bottom.

The insert introduces another reflective surface which can be read by the Laser Autofocus. The following procedure explains how to configure this.

Denment- AK-endo-Boyden-B1-12	Plate name:	Falcon-24 well-353	3504	-	Save Config	guration
Names and Description						
Objective and Camera- 10X Plan I						
Plate- Falcon-24 well-353504		Number of rouse		Number of oclumn		V fall share
Wells to Visit- 18 of 24				Re -	.	Casta
Sites to Visit- multi-site		1ª 🛄		19		I Cacle -
Timelapse- 1 time point(s)	$\overline{\Box}$	- Well	$\overline{\Omega}$	Column	(***********	Plate
Acquisition Loop	IQQ	15/90 -	190	19200 -	888888888888888888888888888888888888888	127.6 -
Autofocus	$\Box \ominus \Theta$	110400 -	100	113500 🖃	000000000000000000000000000000000000000	127.0 -
🦰 W1 Transmitted Light 20%	100		100			
W2 DAPI	60	Column	$\overline{\Omega}$	Row	000000000000000000000000000000000000000	Plate
W3 FITC	PU	0//sec (μ/m).	$ \Psi \psi $	spacing (µm):		Wath (mm)
W4 Cy3	OO	11.020 -	$ \bigcirc \bigcirc$	10000	000000000000000000000000000000000000000	00.0
Journals- 0 selected	100		100			
Display Settings	(\square)	- Row		Well		⁶ Plate beight (mm
Post Acquisition	100	13840 -		17980 -		20.2 -
Summary	()()					leare -
	100		U		U	
	Edit Plate Bo	ttom Settings	Laser Autofoc	us Wizard		
						0
						~

- 1. Go to **Screening > Plate Acquisition Setup**.
- 2. Select a long working distance objective, ideally the 10x Plan Fluor 0.3 NA. This will be used to measure the plate/insert dimensions.
- 3. Select or create a suitable plate file. Enter the plate dimensions as accurately as possible.
- 4. The Laser Autofocus Wizard will probably give incorrect results for a Transwell plate, and it is recommended to manually measure the plate bottom using this procedure, either after or instead of using the Wizard.

Names and Description	Exposure
Objective and Camera- 10X Plan I	Illumination setting: Transmitted Light 20%
Plate- Falcon-24 well-353504	Exposure (ms): 20 + Auto Expose Target max intensity: 33000 +
Wells to Visit- 18 of 24	
Sites to Visit- multi-site	Autofocus options
Timelapse- 1 time point(s)	Test Settings Post-laser
Acquisition Loop	offset (um)
Autofocus	Laser with z-offset 🔄 🛛
W1 Transmitted Light 20:	
W2 DAPI	
W3 FITC	Calculate Offset
W4 Cy3	
Journals- 0 selected	
Display Settings	
Post Acquisition	
Summary	
	Shading Correction: Off (C:\Shading Images\shading_10X Plan Fluor_Transmitted Light

- 5. Move to a well with an insert and make sure the site is towards the center, not towards the edge.
- 6. On the **W1** tab, select an appropriate illumination setting and wavelength. Set the Autofocus option to **Laser with z-offset** using a 0 offset.

Tip: Taping the plate lid onto the plate may reduce movement of the inserts within the wells.

eriment- AK-endo-Boyden-B1-12	Laser-based Focusing
Vames and Description	Configure Laser Settings
)bjective and Camera- 10X Plan I	
/late- Falcon-24 well-353504	Well to well autofocus Focus on plate and well bottom
Wells to Visit- 18 of 24	Image-based Focusing
Sites to Visit- multi-site	Algorithm Standard Binning 2 - K Custom autors us times
imelapse- 1 time point(s)	
Acquisition Loop	Allow image-based focusing for recovery from laser-based well bottom failures
Autofocus	
W1 Transmitted Light 20%	
W2 DAPI	Initial well for finding sample First well acquired 🗾 🖌 🚽 🛛 🕂
W3 FITC	Number of wells to attempt initial find sample 3
W4 Cy3	
	Charles and Second Se
Journals- 0 selected	Site Autorocus All sites

- 7. Go to the **Autofocus** tab and set well-to-well autofocus to "Focus on plate and well bottom".
- 8. Click **Configure Laser Settings**. This opens the Configure Laser Autofocus Settings dialog.

Plate Navigation ——		Acquisition Control	
X.Y	Z	Load Settings	Summary
	Go To Origin	Save Settings	Setup
	▼	Experiment base name:	
Walk P01 Site: 20	7.7000.00	AK-endo-Boyden-B1-12	132017
weil. DOT, Site. 20	2. 7000.00	Wavelength:	
Go To well: A1	Step size: 1000 🛨	W1 - Transmitted Light	20% 💌
Go To A1	Find Sample	Snap Current	Show Live
Eject Plate	Autofocus	Preview	Acquire Plate
?)			

- In addition to configuring the laser settings, you also will need to manually adjust the Z position. Go to Screening > Plate Acquisition and Control. This dialog will let you easily control the Z position.
- 10. Alternatively, you can use the **Focus** dialog from the **Devices** menu (**Control > Devices** in the simplified menu).

		💷 Configure Laser Autofocus Settings 🛛 🗖 🔍
		Autofocus Settings Plate name: Falcon-24 well-353504 Mag setting: 10X Plan Fluor Settings file: C:\MX5\plates\Falcon-24 well-353504.plt
		Save Settings Load Settings Laser Autofocus Wizard
		Plate Bottom Settings
		Bottom thickness 731 um Edit Plate Bottom Bottom thickness max variation 13um Adjacent well max variation 57 um Intra-well max variation 24 um Plate max variation 592 um
		Step Size
🖂 Plate Acquisition and Control		Coarse step (um) 5 🕂 Fine step (um) 1 🕂
Plate Navigation	Acquisition Control	Plate Bottom Exposure Well Bottom Exposure
-X, Y Z Go To Origin Well: B01, Site: 28 Z: 7000.00	Load Settings Summary Save Settings Setup Experiment base name: AK-endo-Boyden-B1-12132017	Attempt Exposure (us) 1 4.000 2 5.000 3 20.00
Go To well: A1 Step size: 1000 🛨	W1 · Transmitted Light 20%	Preview Pass
Go To A1 Find Sample	Snap Current Show Live	Note: Set the Preview Pass exposure by selecting an exposure time above or use "Override exposure" below
Eject Plate Autofocus	Preview Acquire Plate	Start: Current 🔽 Start from current position
?	Reset IX Close	Range: 7000 um Image: 7000 Image: Image: <thimage:< th=""> Image: <thimage:< th=""> Image: Image:</thimage:<></thimage:<>
		Exposure: 55.00us T Override exposure (us) 10 📑
		Find Sample Autofocus Focus Snap Close

- 11. Adjust the Z to a setting below the plate bottom (7000 typically works).
- 12. In the Configure Laser Autofocus Settings dialog, override the **Preview Pass Start** to "Start from current position" and override the **Preview Pass Range** to several thousand μm.
- 13. Select a high exposure time (usually 50-100 μ s will suffice).
- 14. Click **Preview Pass**.

- 15. You should see a graph window similar to the one above, with 3-4 distinct peaks.
- 16. If you do not see any peaks or you only see 1-2 peaks, try increasing the exposure time and/or modifying the start Z and range. Also, try moving to other wells/sites.

Tip: To confirm that the 3rd peak is the membrane, adjust the Z to that position and acquire an image. When you are done, return the Z to the previous setting.

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17. To measure the peaks and the difference between them, click on the icon in the lower left corner of the graph and select **Point Statistics**.

- 18. Click on the first and the third peaks to measure their positions and the difference between them. Usually the green peaks (focus score) are easier to measure than the red peaks (brightest pixel).
- 19. Note the plate bottom Z position (8463 in the example above), the membrane position (9974), and the difference (1511 μ m).

- 20. Repeat for several wells so that you can determine the variability of the Z and the thickness (difference).
- 21. You may sometimes see a 4th peak. This is the top of the liquid and generally can be ignored. If it is too close to the 3rd peak, add more liquid to the well.

itofocus Settings	Plate name: Falcon-24 well-353504	
late name: Falcon-24 well-353504	Settings file: C:\MX5\plates\Falcon-24	well-353504.plt
ag setting: 10X Plan Fluor	Save Settings Load Settings	
ettings file: C:\MX5\plates\Falcon-24 well-353504.plt	Plate Bottom Settings	
Save Settings Load Settings Laser Autofocus Wizard	Note: All values are optical measurem	ents
Plate Bottom Settings	Bottom thickness (um)	1480 🕂
Bottom thickness 731 um Edit Plate Bottom	Bottom thickness max variation (um)	100 🔶
Bottom thickness max variation 13um	Adjacent well max variation (um)	80 🔅
Adjacent well max variation 57 um	Intra-well max variation (um)	24 🔅
Intra-well max variation 24 um	Plate max variation (um)	592 -

- 22. Once you have measured sufficient wells, click Edit Plate Bottom.
- 23. Enter the "Bottom thickness" (difference between plate bottom and membrane Z positions) and variation parameters that you estimated from your measurements.
- 24. Click Save Settings.

Tip: If the measured bottom thickness varies significantly, use a number towards the lower end and make sure the Bottom thickness max variation setting is sufficient to cover the thickness range that you measured.

Autofocus					
W1 Transmitted Light 20%					
W2 DAPI	Initial well for finding sample	First well acquired	- A	<u>v</u> 1	
W3 FITC	Number of wells to attempt in	nitial find sample 3			
W4 Cy3	C2. A 1.4	- 1 [40.5			
Journals- 0 selected	Site Autorocus	All sites	-		
Display Settings					
Post Acquisition					
Summary				1.1	
			1.2.4		
			View	v Focusing De	tails
-	and the second s				13
	Save Settings Su	mmary	Previous	► <u>N</u> ext	Close

- 25. Changing the bottom thickness and/or the plate max variation will affect the start Z position.
- 26. Go to Autofocus tab > View Focusing Details to view the Plate Find Start Z Position and Plate Find Range.

Display of underlying focus parameters f	or diagnosit	ic purposes
Parameter	Value	Description
Plate Bottom Reference	7450	Reference Z position of flat sheet in plate holder.
Reference Objective	3	Objective position used for setting reference point.
Parfocality Offset	0	Offset distance between current objective and reference objective.
Working Distance	16000	Distance from front of lens to plate bottom when focused on well.
Working Distance Tolerance	0	Tolerance to subtract from working distance.
Plate Height	20200	Height defined for current plate.
Well Depth	17980	Depth of well for current plate.
Bottom Thickness	731	Median thickness for current plate.
Bottom Thickness Max Variation	13	Max variation for thickness across plate.
Plate Max Variation	592	Max variation between expected and measured bottom position across
Adjacent Well Max Variation	57	Max variation in well focus position for any adjacent wells.
Intra-well Max Variation	24	Max variation in well focus position for sites within any well.
User Z Offset	1135.3	Offset to be applied after well finding.
Laser Course Step	5	Course step size for laser autofocus (um).
Laser Fine Step	1	Fine step size for laser autofocus [um].
Safe WD Focus Depth	16724.5	Max safe distance from plate bottom to highest focus position for object
Acquisition Best Case:		Best case focus ranges for normal acquisition sequence.
Validation	0K	Basic validation of autofocus parameters.
Plate Find: Start Z Position	8325.5	Starting position for plate bottom LAF search [um].
Plate Find: Range	1227	Depth of LAF plate bottom search range [um].
Plate Find: Safe WD	OK	Is plate range safe for current working distance.
Adjacent Well: Plate Find: Offset Delta	-57	Offset from adjacent well plate bottom for plate bottom LAF search (um
Adjacent Wellt Plate Find: Range	114	Depth of LAF plate bottom search range [um].
Adjacent Well: Well Find: Offset Delta	631	Offset from plate bottom for LAF well search [um].
Adjacent Well: Well Find: Range	200	Depth of LAF well search range (um).
	(cont	Mit at the state to the AP will search from?

- 27. Compare the **Plate Find Start Z Position** and **Plate Find Range** to your measurements of the plate bottom.
- 28. The range must cover all of the possible plate bottom Z positions, but does not necessarily need to reach the membrane Z position.

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Experiment- AK-endo-Boyden-B1-12 Plate name	Falcon-24 well-353504	• Sa	en Configuration
Names and Description	1. 1001 24 1101 000004		re coningaration
Objective and Camera- 10X Plan I			
Plate- Falcon-24 well-353504			
Auto Wells to Visit- 18 of 24	Number of rows:	Number of columns:	Well shape:
Display Sites to Visit- multi-site	14 🖆	6 코	Circle 💌
Timelapse-1 time point(s)		Column	sssss Plate
Plate B Acquisition Loop		spacing (µm): 0000000	2000000 [127.6 -1
Referer Autofocus		13300 - 000000	000000 127.D I
Parfoc: W1 Transmitted Light 20%			
Workin W2 DAPI		Row	Plate
Workin W3 FITC		spacing (µm):	width (mm);
		19300 🖻 👬	000000 85.5 🛨
Nell Di Iournalse Il selected	100		
Bottom Display Settings	Bow	= Well	Plate
Plate M Bost Acquisition) offset (µm):	depth (um)	height (mm):
Adjace			
Intra-wa	/ [—	I	
UserZ Edit Plate F	lottom Settings	ous Wizard	
Laser C		ous mizad	
Laser F			(?)
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A	n I Commun I		New Classe
Validati	s Symmaty	Elevious F	
Plate Find: Start Z Position 7533 Starting position f	for plate bottom LAF search [um].	ia .	
Plate Find: Range 1314 Depth of LAF pla	te bottom search range [um].		996 0
Plate Find: Safe WD OK Is plate range sal	fe for current working distance.	10	
Adjacent Well: Plate Find: Offset Delta -80 Offset from adjac	ent well plate bottom for plate bottom l	"AF search (um).	
Adjacent Well: Plate Find: Range 160 Depth of LAF pla	te bottom search range [um].	T.S.	N
Adjacent Well: Well Find: Offset Delta 1335 Offset from plate	bottom for LAF well search [um].	1.00	Par
Adjacent Well: Well Find: Range 290 Depth of LAF we	Il search range [um].		Custo
Intermetries with the difference of the dif		· · · · · · · · · · · · · · · · · · ·	Name:
Com.		Church	Email: m
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29. If the start Z position is inappropriate, adjust the **Well Depth**.

- a) Increase the well depth for a lower start Z position.
- b) Decrease the well depth for a higher start Z position.
- 30. If the range is inappropriate, adjust the **Plate Max Variation**.
- 31. Click **Save Configuration** after modifying plate dimensions.

Experiment- AK-endo-Boyden-B1-12 Names and Description Plate name: Falcon-24 well-353504 Save Configuration Diplay Plate - Falcon-24 well-353504 Image: Save Configuration Save Configuration Image: Autor Wells to Visit- 18 of 24 Sites to Visit- multi-site Number of rows: Number of columns: Wells Image: Plate B Referer Acquisition Loop Vell Image: Save Configuration Plate W1 Transmitted Light 20% W1 Transmitted Light 20% Column Save Configuration Plate Workin W3 FITC Save Configuration Save Configuration Plate	
Names and Description Objective and Camera-10X Plan I Plate- Falcon-24 well-353504 Number of rows: Wells to Visit- 18 of 24 I Display Sites to Visit- multi-site Timelapse-1 time point(s) Acquisition Loop Acquisition Loop Itage Pater Autofocus W1 Transmitted Light 20% W2 DAPI Workir W3 FTTC Plate Golumn Image: Column offset (µm); Image: Column offset (µm);	hape:
Image: Construction of the system of the	hape:
Plate- Falcon-24 well-353504 Number of rows: Number of columns: Wells Display Wells to Visit- 18 of 24 Id Id<	hape:
Image: Autor Display Wells to Visit- 18 of 24 Number of rows: Number of columns: Wells Display Sites to Visit- multi-site Image: Autor of rows: Number of rows: Image: Autor of rows: <td< td=""><td>hape:</td></td<>	hape:
Display Sites to Visit- multi-site Image: Parfore Vell Column Column Plate Binger Acquisition Loop Acquisition Loop Image: Parfore Multification Loop Image: Parfore Image: Parfor	(mm):
Plate B Acquisition Loop Well Column spacing (µm): Image: Colum	(mm):
Plate B Acquisition Loop anameter (µm) spacing (µm) spacing (µm) interest	(mm):
Refere Autofocus Image: Second secon	3
Parfocs W1 Transmitted Light 20% Workin W2 DAPI Workin W3 FITC Plate H W3 FITC	
Workin W2 DAPI Column Spacing (µm): <	
Workin W3 FITC offset (µm); spacing (µm); social spacin	The second s
Plate H (1/520 - 1/520	(mm):
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Well D lournale 0 calacted	
Bottom Diverse Selected Row Well Plate	
Plate M Display Security - height height	(mm):
	1
Intrawy summary	
User Z Edit Plate Bottom Settinge Laser Autologue Wizard	
Laser 0	-
Laser F	(?)
Safe V.	~
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Acquis 2ave seturgs Sginnay Elevious Mexic	Fiose
Plate Find: Start Z Position 8033 Starting position for plate bottom LAF search (um).	
Plate Find: Range 1314 Depth of LAF plate bottom search range [um]	
Plate Find: Safe WD DK Is plate range safe for current working distance.	
Adjacent Well: Plate Find: Offset Delta -80 Offset from adjacent well plate bottom for plate bottom LAF search [um].	
Adjacent Well: Plate Find: Range 160 Depth of LAF plate bottom search range [um].	
Adjacent Well: Well Find: Offset Delta 1335 Offset from plate bottom for LAF well search [um].	 Par
Adjacent Well: Well Find: Range 290 Depth of LAF well search range [um].	Cust
Internal Park Mell Fack Offices Date: 1200 Offices from also bettern fact [F well course from]	
	Name
Lopy Close o second	Name: Email: r

29. If the start Z position is inappropriate, adjust the **Well Depth**.

- a) Increase the well depth for a lower start Z position.
- b) Decrease the well depth for a higher start Z position.
- 30. If the range is inappropriate, adjust the **Plate Max Variation**.
- 31. Click **Save Configuration** after modifying plate dimensions.

Configure Laser Autofocus Settings [Modified]	Preview Pass	
Autofocus Settings Plate name: Falcon-24 well-353504 Mag setting: 10X Plan Fluor Settings file: C:VMX5\vplates\Falcon-24 well-353504.plt Save Settings* Load Settings Laser Autofocus Wizard Plate Bottom Settings Bottom thickness 1480um Addigeent well max variation 100um Adjocent well max variation 80um Intra-well max variation 24 um Plate max variation 592 um	60 -	 Ørightest pixel Focus Score- root mean square
Step Size Coarse step (um) 5 Plate Bottom Exposure Attempt Exposure (us) 1 2 5.000 3 20.00 3 20.00 3 20.00	Locus Score	
Note: Set the Preview Pass exposure by selecting an exposure time above or use "Override exposure" below Start: 8033um Range: 2894um Override range (um) 7000 Step size: 5.000um Exposure: 3.000us Override exposure (us) 10 Preview Pass 10	20 -	
Find Sample Autofocus Focus Snap Close"	8033.0 8757.1 9461.3 Z Position	10205.4 10929.6

- 32. You can now disable the overrides in **Configure Laser Autofocus Settings** for the **Preview Pass Start** and **Range**.
- 33. Test and adjust the exposure times for the plate bottom. Exposure 1 should give a red peak between 50-150 for most wells. Exposures 2 and 3 should be higher as they will be used for wells with dim reflections. It is fine to only see one peak during this test.

34. Test and adjust the exposure times for the "well bottom" (actually the membrane surface). Exposure 1 should give a red peak between 50-150 for most wells. Exposures 2 and 3 should be higher as they will be used for wells with dim reflections. It is fine for the other peaks to be saturating during this test.

Plate name: Mag setting Settings file:	Falco 10X1 C:\M	on-24 well-353504 Plan Fluor X5\plates\Falcon	-24 well-35	3504.plt	
Save Setti	ngs*	Load Settings	Laser	Autofoci	us Wiz
Plate Botto Bottom thi Bottom thi Adjacent i Intra-well i Plate max	m Settir ckness ckness well max max vari variatio	ngs max variation « variation iation n	1480um 100um 80um 24um 592um	Edit Pla	te Bott
Step Size Coarse ste	ep (um)	5 🔶	Fine ste	p (um)	1
Plate Botto	m Expo	sure	Well Botte	om Expo	sure
Attempt	Expe	osure (us)	Attempt	Exp	osure (
1	3.000		1	25.00	i.
2	6.000		2	55.00	
3	20.00		3	220.0	-
Preview Pas Note: Set th exposure tin Start:	s ne Previa ne abov 80	ew Pass exposure e or use "Override)33um ┌── Start fr	by selectin exposure* om current	ig an 'below position	
Range:	28	894um 🕅 Overrie	de range (u	m)	7000
Step size:	5.0	000um 🖵 Overrig	te step (um)	10
Exposure:	25	00us 🖵 Overrid	te exposur	e (us)	10

- 35. After adjusting settings, click **Save Settings** to update your plate file.
- 36. Click **Find Sample** to test the autofocus settings on the current well and site.

37. If the Find Sample focus succeeded, you will see a "Focus found!" message, along with a zoomed Preview Pass graph and a focused image

38. If the focus failed, adjust autofocus settings and retry. Also, try other wells.

39. Repeat the exposure time optimization and Find Sample test for other long working distance objectives that you want to image with. This type of plate is incompatible with short working distance objectives.

DEVICES

Names and Description	Exposure
Objective and Camera- 10X Plan I	Illumination setting: Transmitted Light 20%
Plate- Falcon-24 well-353504	Exposure (ms): 20 + Auto Expose Target max intensity: 33000 +
Wells to Visit- 18 of 24	
Sites to Visit- multi-site	Autofocus options
Timelapse- 1 time point(s)	Test Settings Offset (um)
Acquisition Loop	
Autofocus	Laser with z-offset 🗾 🛛 📩
W1 Transmitted Light 20:	
W2 DAPI	
W3 FITC	Calculate Offset
W4 Cy3	
Journals- 0 selected	
Display Settings	
Post Acquisition	
Summary	/
	Shading Correction: Off (C:\Shading Images\shading_10X Plan Fluor_Transmitted Light

- 40. After you have verified that the autofocus is working for your objective, go to the W1 tab and Calculate Offset.
- 41. Verify that the exposure time is appropriate.
- 42. Repeat for the other wavelengths.
- 43. Once the settings are optimized, run a test acquisition.

Transwell assay imaging

Tip: If Transmitted Light is used, expect to see both the membrane pores and cells. A custom module or custom journal analysis is required to distinguish these. Alternatively, use Fluorescence for more accurate cell counts.

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Support Resources

- F1 / HELP within MetaXpress® Software
- Support and Knowledge Base: <u>http://mdc.custhelp.com/</u>
- User Forum: http://metamorph.moleculardevices.com/forum/
- 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

MOLECULAR DEVICES

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