MetaXpress® 6 Guide

Autofocus Options

April 2021 revB

Overview

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Module Purpose

The purpose of this module is to familiarize the user with the different focus options including the Laser Autofocus (LAF) and Image-based Autofocus (IAF) on the ImageXpress instruments with MetaXpress software

What is Autofocus?

Autofocus is the process by which the instrument automatically adjusts the objective so the sample is in focus.

In the MetaXpress software there are different options to achieve focus:

1. Laser Autofocus (LAF) – also known as hardware autofocus

2. Image-based Autofocus (IAF) – also known as software autofocus

3. Combinations of the above.

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What is Laser Autofocus (LAF)?
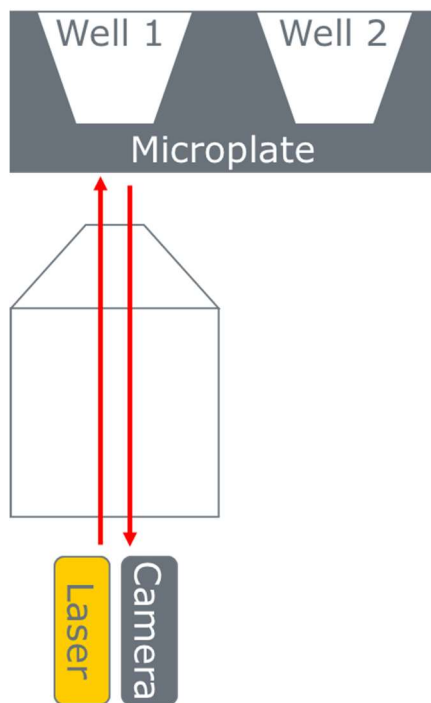
- A dedicated focus laser detects reflections at different surfaces

- The objective moves to the focus position by locating the reflection from the plate bottom and/or well bottom

- The laser autofocus mechanism uses a 690nm laser and dedicated focus camera

- Laser autofocus is independent of the cells and the staining

What is Image Autofocus (IAF)?
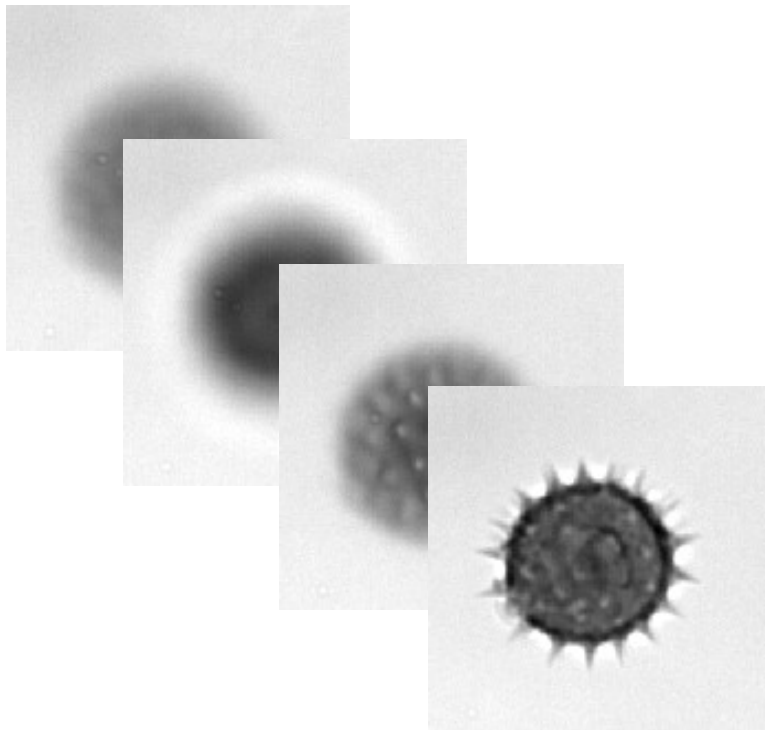
- Image-based Autofocus (IAF) uses the following routine:

1. The sample is illuminated at the chosen wavelength.

2. The selected objective moves up and down with a large step size to capture images of the sample at a range of focus (Z) heights.

3. The software evaluates the captured images for contrast.

4. The software iteratively narrows the search range and reduces the step size, minimizing the number of steps/images required.

5. The instrument captures the final image at the focus height giving the greatest contrast.

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Combination of Laser and Image Autofocus
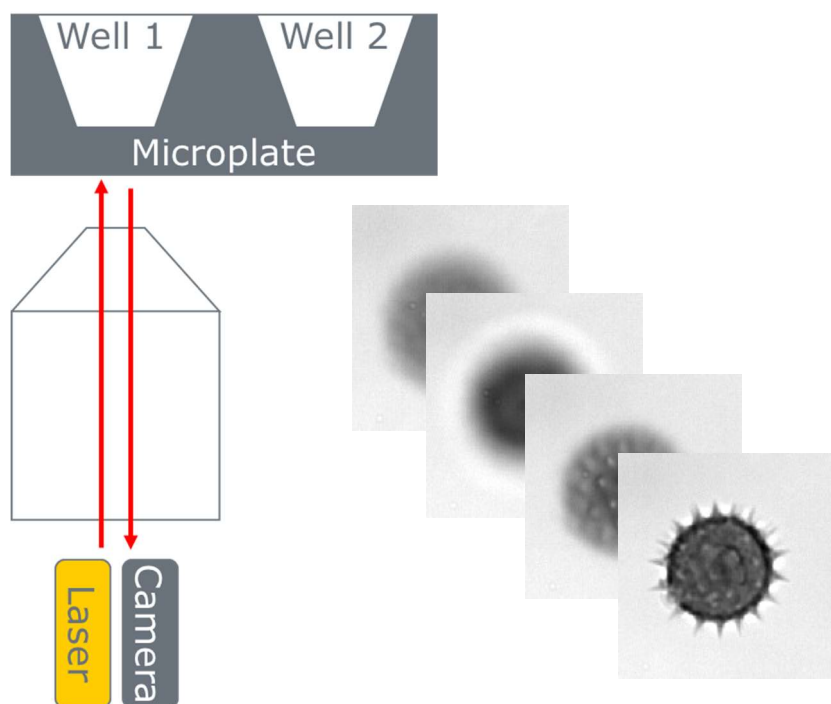
When a combination of LAF and IAF is selected:

1. The instrument first uses the LAF routine to find focus for the plate and/or well bottom.
2. The instrument then uses the IAF to optimize focus based on the specific sample.
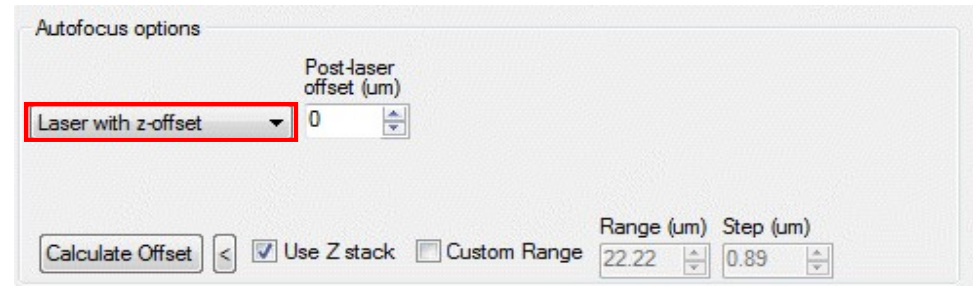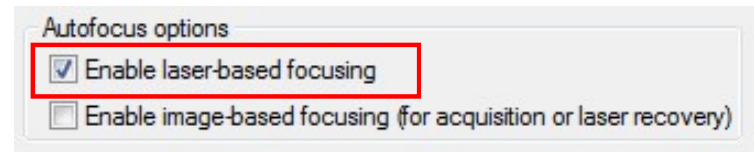
When is a combination of LAF & IAF useful?

- Multiple cell types in the same sample plate
- Stains that move between cellular compartments with different focal planes
- Sample in mounting media, which can interfere with LAF
- Samples in a thick matrix such as Matrigel
- Suspension cells
- Whole organisms

Autofocus Settings in MetaXpress 6

- Enabling Laser-based Autofocus (LAF) alone

- Enabling Image-based Autofocus (IAF) alone

- Enabling All Autofocus options (Recommended)

- Well-to-Well Laser Autofocus options

- Find Sample options

- Site Autofocus options

- Timelapse Autofocus options

- Autofocus Routine Selection Guide

- When Do I Need to Optimize Laser Autofocus Settings?

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Enabling Laser-based Autofocus (LAF) Alone

- **Plate Acquisition Setup > Acquisition** tab
  - Select **Enable laser-based focusing**
  - Deselect **Enable image-based focusing**

- **W1** autofocus options:
  - Laser with z-offset

- **W2 – W8** autofocus options:
  - Z-offset from W1

- LAF alone is the default setting:
  - Fastest performance
  - Minimizes photobleaching
  - Independent of sample/stain quality

Autofocus options

☑ Enable laser-based focusing

☐ Enable image-based focusing (for acquisition or laser recovery)

Autofocus options

Post-laser offset (um)

Laser with z-offset ▼ 0

Calculate Offset < ☑ Use Z stack ☐ Custom Range Range (um) 22.22 Step (um) 0.89

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Enabling Image-based Autofocus (IAF) Alone

- **Plate Acquisition Setup > Acquisition** tab
  - Deselect **Enable laser-based focusing**
  - Select **Enable image-based focusing**

- **W1** Autofocus options:
  - Image-based

- **W2 – W8** Autofocus options:
  - Image-based
  - Z-offset from W1

- Using IAF alone is generally not recommended, but can occasionally be useful when:
  - LAF has not been configured for your chosen plate/objective
  - LAF fails due to plate imperfections, low volume, or the presence of mounting media

Autofocus options

☐ Enable laser-based focusing

☑ Enable image-based focusing (for acquisition or laser recovery)

Autofocus options

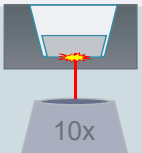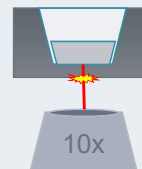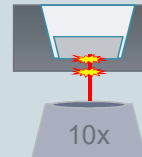|	Image-based range +/-(um):	Max. step (um):
Image-based ▼	200	5

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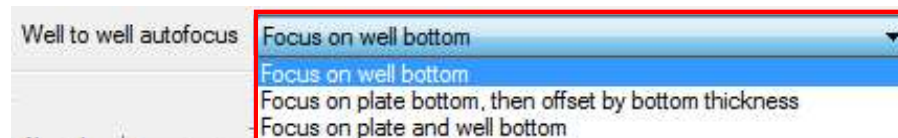# Enabling All Autofocus Options (Recommended)

- **Plate Acquisition Setup > Acquisition** tab
  - Select **Enable laser-based focusing**
  - Select **Enable image-based focusing**

- **W1** autofocus options
  - Laser with z-offset
  - Laser And Image
  - Laser with Image Recovery

- **W2 - W8** autofocus options
  - Z-offset from W1
  - Image-based

- **Plate Acquisition Setup > Autofocus** tab
  - Enable **Allow image-based focusing for recovery from laser-based well bottom failures**

Well to well Laser Autofocus Options

Well-to-well option	When to use
Focus on well bottom	• Thicker plates with 10x and above • Very flat thin plates with 10x and above • For fastest performance

Focus on plate bottom, then offset by bottom thickness	• Most plates with 4x and 2x objectives
	• Slide/coverslips with all objectives
	• Together with IAF for all objectives with:
	    • Samples in thick matrices (Matrigel)
	    • Ultra-thin plates

Focus on plate and well bottom	• Most thin plates with 10x and above • When Focus on well bottom gives inconsistent results

Well to well autofocus | Focus on well bottom ▼

Focus on well bottom
Focus on plate bottom, then offset by bottom thickness
Focus on plate and well bottom

- Thicker plates: Physical bottom thickness ≥ 0.35 mm
  Optical thickness ≥ 220 um
- Thin plates: 0.15 mm < Physical bottom thickness < 0.35 mm
  100 um < Optical thickness < 220 um
- Ultra-thin plates: Physical bottom thickness ≤ 0.15 mm
  Optical thickness ≤ 100 um

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Find Sample Options

Initial well for finding sample	When to use
First well acquired	• Most of the time
Specific well	• When First well acquired gives errors • When using IAF only and the first well to be acquired doesn't have a bright sample
Skip Find Sample	• When you have already manually focused on the sample, and First well acquired gives inaccurate focus
Number of wells to attempt initial find sample	**When to use**
3 or higher	• When using automated plate loading

Site Autofocus Options

Site Autofocus Option		When to use
First site only		• For fastest performance
Center of well only		• Most plates with 4x and 2x objectives • Round-bottom plates
All sites		• Most of the time with 10x and above

Only available when acquiring multiple sites per well

Timelapse Autofocus Options

Timelapse Autofocus Option	When to use
First timepoint only	• Most fast kinetic assays
All timepoints	• Most longer timelapse assays
Every Nth timepoint	• Fast kinetic assays when the focus may occasionally drift (for example after pipetting in compound)
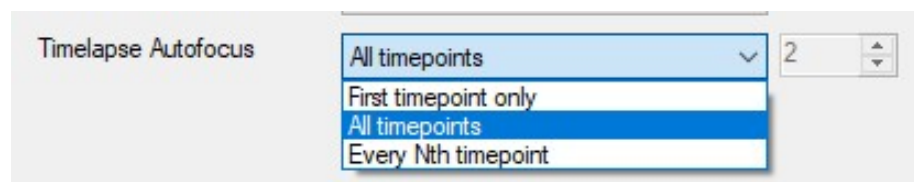
Only available when acquiring multiple time points

Autofocus Routine Selection Guide

Objective	Plate/Slide Type	Well-to-well autofocus option	Site autofocus option
1x	• Any plate • Any slide	None (Use fixed focus across the plate)	None (Use fixed focus across the plate)
2x, 4x	• Any plate • Any slide	Focus on plate bottom then offset by bottom thickness	Center of well only
10x and above	• Thin plate	Focus on plate and well bottom	All sites for flat-bottom plate Center of well only for round-bottom plate
10x and above	• Slide with coverslip • Ultra-thin plate • Plate with Matrigel	Focus on plate bottom then offset by bottom thickness IAF may also be needed	All sites for flat-bottom plate Center of well only for round-bottom plate
10x, 20x, 40x ELWD, 60x ELWD	• Thicker plate • Chamber slide with wells	Focus on well bottom	All sites for flat-bottom plate Center of well only for round-bottom plate
40x Plan Apo or other high-mag short working distance objective	• Thin plate (avoid edge wells) • (Do not use thick plates)	Focus on plate and well bottom	All sites for flat-bottom plate Center of well only for round-bottom plate

- Thicker plates: Physical bottom thickness ≥ 0.35 mm
  Optical thickness ≥ 220 um
- Thin plates: 0.15 mm < Physical bottom thickness < 0.35 mm
  100 um < Optical thickness < 220 um
- Ultra-thin plates: Physical bottom thickness ≤ 0.15 mm
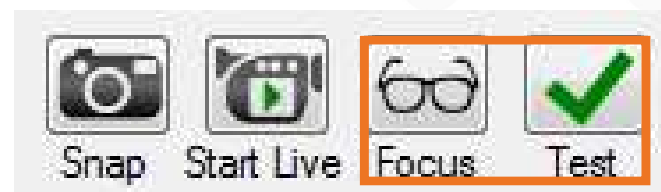 Optical thickness ≤ 100 um

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Additional notes on Autofocus Routines

1. 2x and 4x objectives: For these low-magnification objectives, the plate and well bottom laser reflections cannot reliably be distinguished because of the wide depth of field.

2. Thin-bottom plates: For thin-bottom plates with high variation, the large search range for the well bottom may accidentally detect the plate bottom. The **Focus on plate and well bottom** option forces the system to search for both surfaces.

3. Short working distance objectives: These objectives may bump the skirt of the plate when focusing on edge wells, pushing the plate up.

4. Matrigel-based samples: The LAF cannot detect the Matrigel or the cells. The LAF locates the plate bottom and a smaller image-based autofocus is used to find the cells.

5. Slides with coverslips: There is no equivalent well bottom to produce a reflection at the interface between the coverslip, mounting media, and slide. Generally if the sample is located on the bottom surface (e.g. **cells on the coverslip/coverslip down** or **tissue on the slide/coverslip up**) then **LAF alone** is sufficient. If the sample is on the top surface (e.g. **tissue on the slide / coverslip down**) then you may need **LAF + IAF**.

•	Thicker plates:	Physical bottom thickness ≥ 0.35 mm
		Optical thickness ≥ 220 um
•	Thin plates:	0.15 mm < Physical bottom thickness < 0.35 mm
		100 um < Optical thickness < 220 um
•	Ultra-thin plates:	Physical bottom thickness ≤ 0.15 mm
		Optical thickness ≤ 100 um

When Do I Need to Optimize Laser Autofocus Settings?

- When working with a new batch/lot of plates from the manufacturer

- If you are seeing many out of focus images

- When the software is very slow or unresponsive after clicking the **Focus** or **Test** buttons

- When plate acquisition time is taking longer than expected, or acquisition speed generally needs improvement

- If you see the error "The initial autofocus failed to find the plate"

- If the automated plate loading system is reporting error 14, and the plate was loaded correctly

Support Resources

- F1 / HELP within MetaXpress® Software
- Support and Knowledge Base: https://support.moleculardevices.com/
- Contact us: https://www.moleculardevices.com/contact