Focus Strategies with ZEN2

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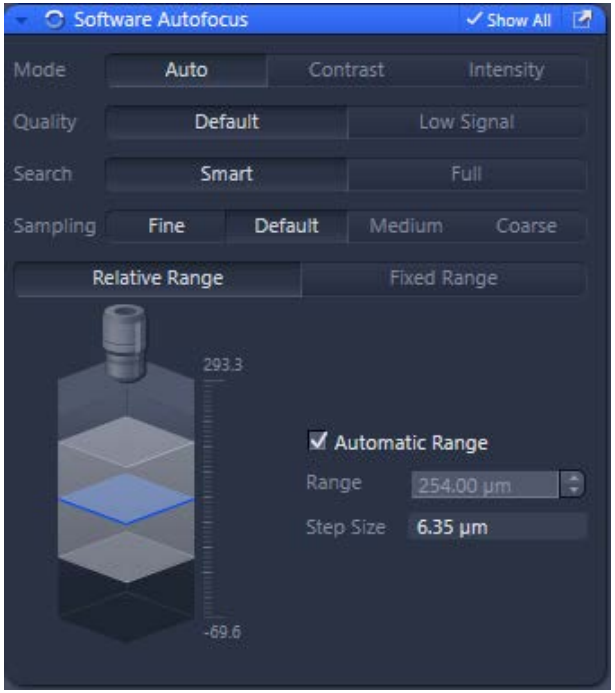
ZEN
Focus Strategies

- Focus Maintenance
  - Software Autofocus (SWAF)
  - Hardware: Definite focus (DF)
- Focus Strategies
  - Focus Strategy window
  - Only Autofocus
  - Only Definite Focus
  - A combination of both
- When to choose which strategy?
  - Different options with examples
  - Local versus Global Support Points

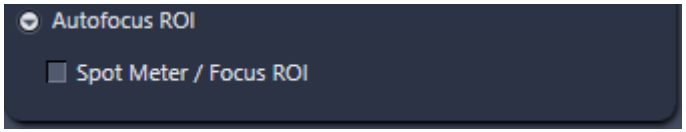
Focus Maintenance
Software Autofocus

- Find Focus runs Autofocus
- Based on parameters set in Software Autofocus window

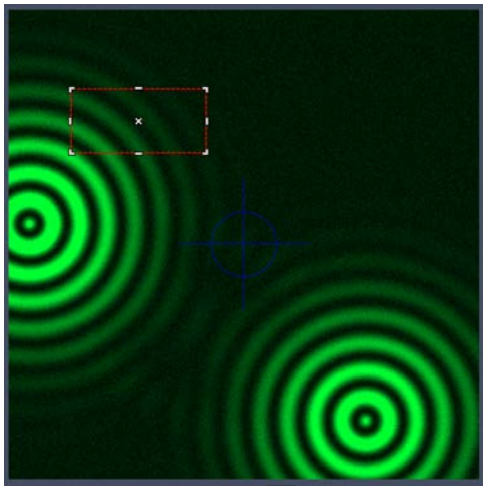


- **Mode**: Contrast for Widefield, Intensity for Confocal system
  (Auto will define best option based on system configuration)
- **Quality**: a more optimal algorithm for weaker signals is used
  (Choose Low Signal for sparse and/or weak signal)
- **Search**: Smart will stop z-movement on the first occurrence of a sharp object, Full will use the complete search range and then return
- **Sampling**: step-size, based on the depth of field of the objective. Typically Default is ok, fine will require 2x more steps (=potentially 2x more bleaching)
- **Range**: Relative Range is Default, if Automatic is switched off, one can define a larger depth to search. With Fixed Range, a specific minimum & maximum has to be defined (similar to z-stack First/Last).

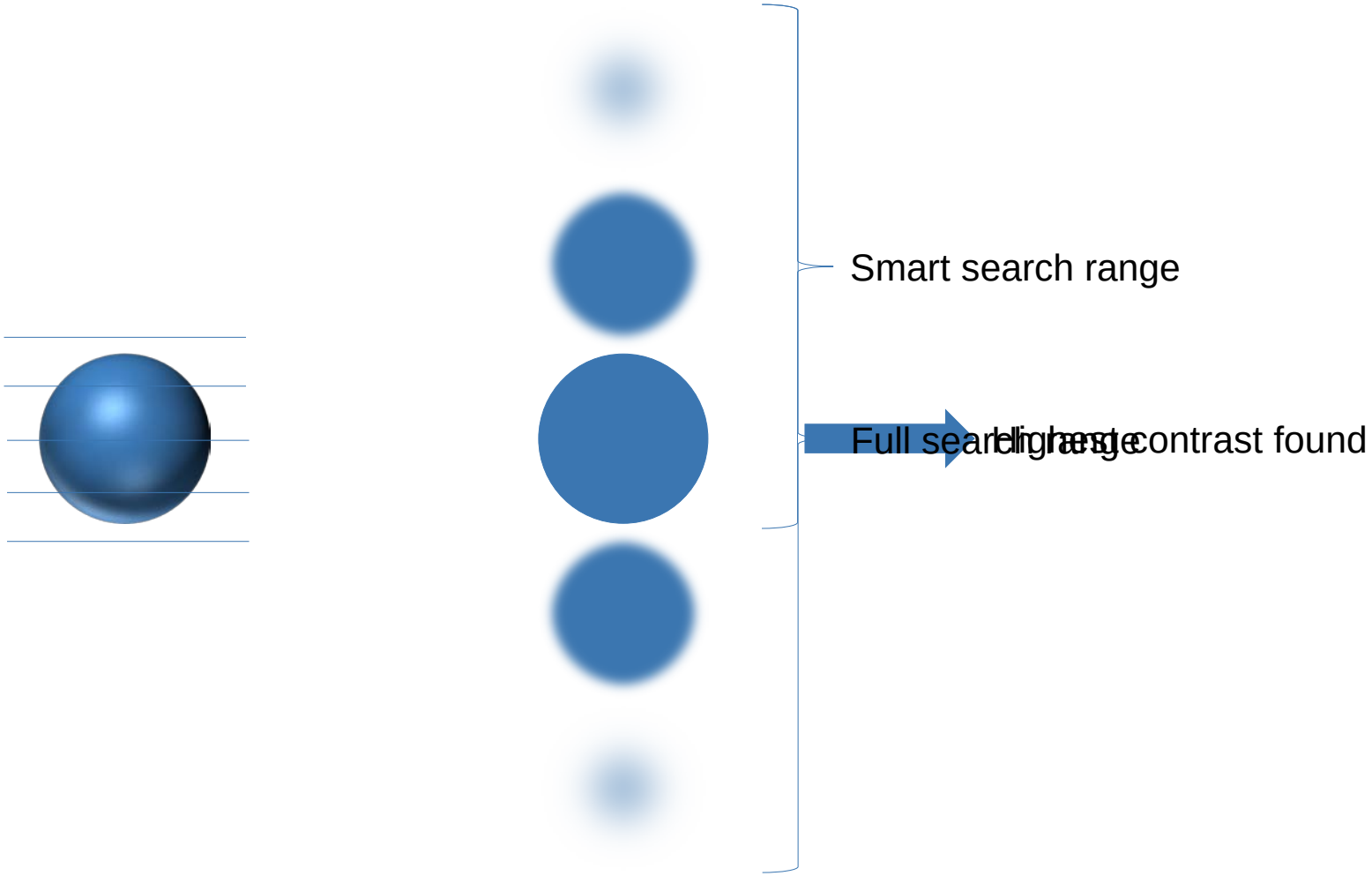
Focus Maintenance
Software Autofocus



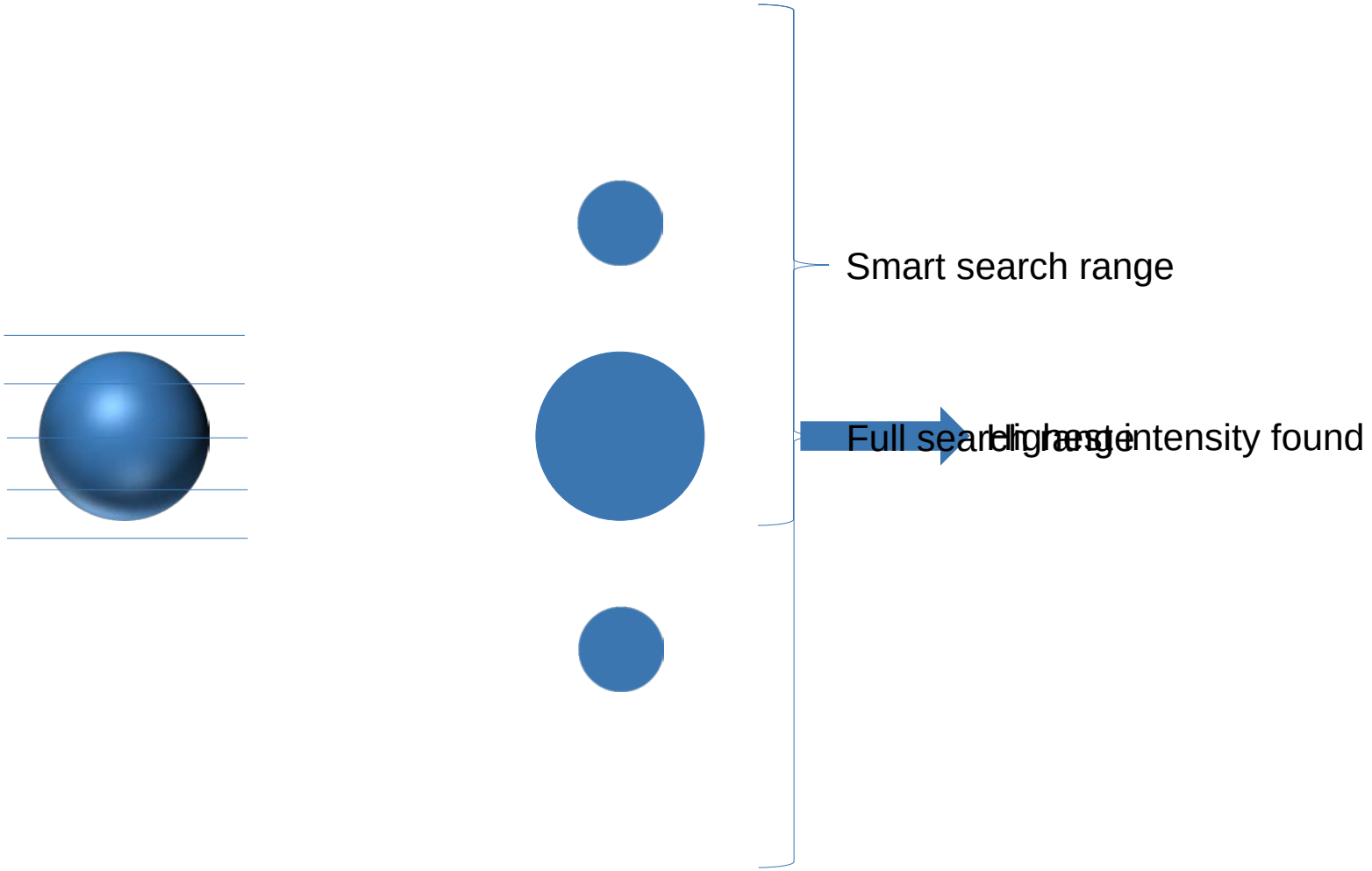
- Spot Meter / Focus ROI:
  - Mark small region of interest on image for focus maintenance
  - Particularly useful with fiducials (e.g. fluorescent beads)
  - When enabled, a red square will appear in the Live window which can be resized & repositioned



Focus Maintenance
Software Autofocus – Widefield

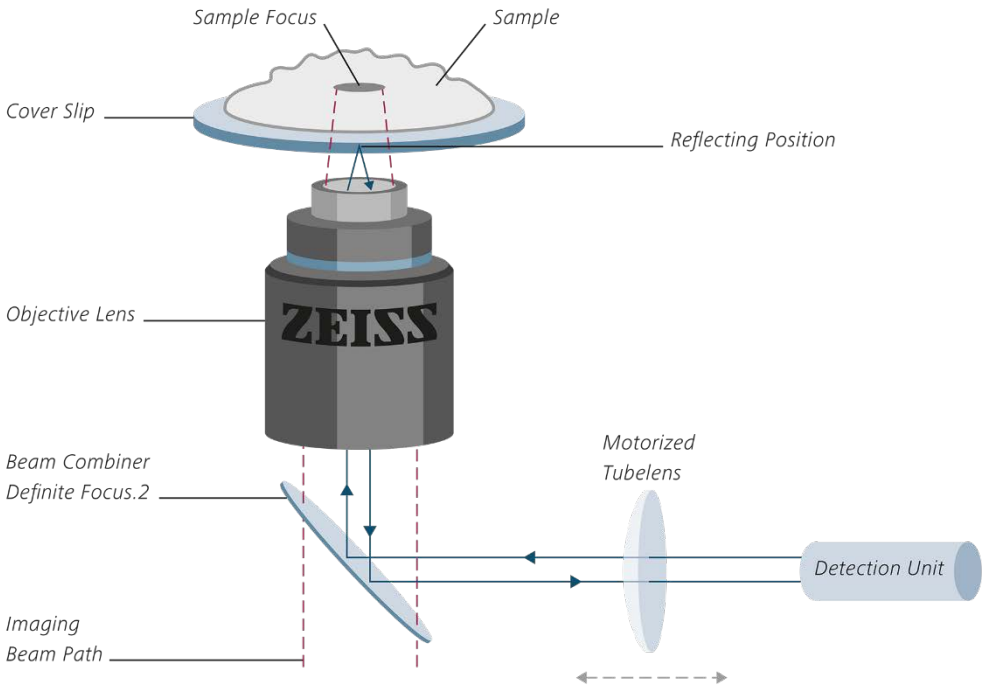


Focus Maintenance
Software Autofocus – Confocal



Focus Maintenance
Hardware Autofocus – Definite Focus 2

- Many new options introduced with DF.2 and ZEN2.3 or higher
- Searches for reflection of IR laser on coverslip → no interference with sample, thus no bleaching or phototoxicity

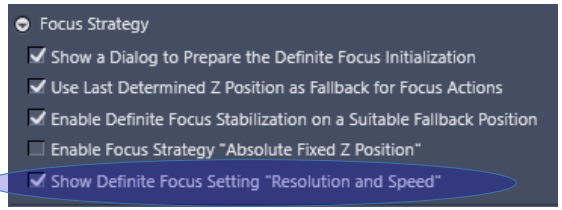


Focus Maintenance
Hardware Autofocus – Definite Focus 2

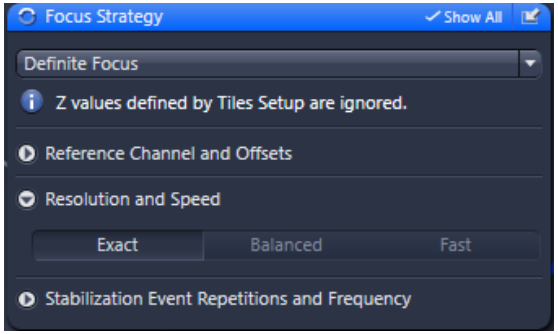
- **Find Surface**: will detect coverslip based on reflection of IR light
- **Store Focus**: after finding coverslip and focusing (manually or with SWAF), stores the offset (distance) between coverslip and sample
- **Lock Focus**: will keep DF.2 AND offset active while screening over sample (=continuously maintain focus)
- **Recall Focus**: with Lock Focus inactive, or during an experiment, the previously defined offset is applied once.
- **Period**: DF.2 can be used continuously, set here the interval. 0s is continuously, often 30s or even 1min is sufficient

Focus Maintenance
Hardware Autofocus – Definite Focus 2

- In menu: Tools | Options | Acquisition | Focus Strategy



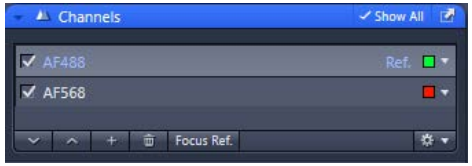
- Enabling ‘Show Definite Focus Setting “Resolution and Speed”’ shows the DF accuracy parameter in the Focus Strategy window when using DF

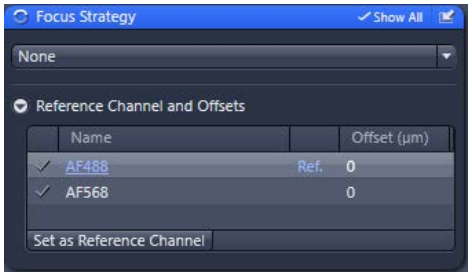


  - Exact: very accurate, but slower
  - Balanced: good accuracy and speed
  - Fast: faster focusing, but possibly less accurate

Focus Strategies
Focus Strategy window

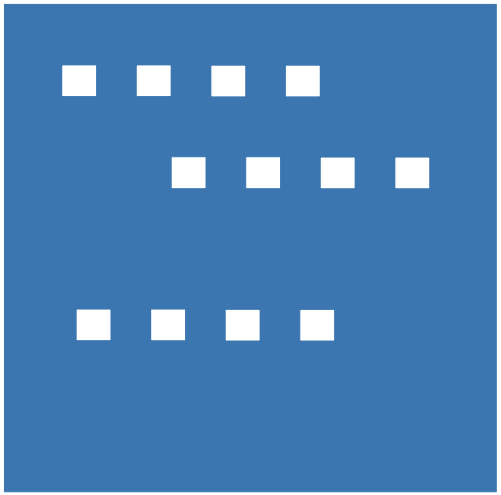
- For SWAF, a Reference channel is required
  - Select a channel in Channels or Focus Strategy window & click on Set as Reference





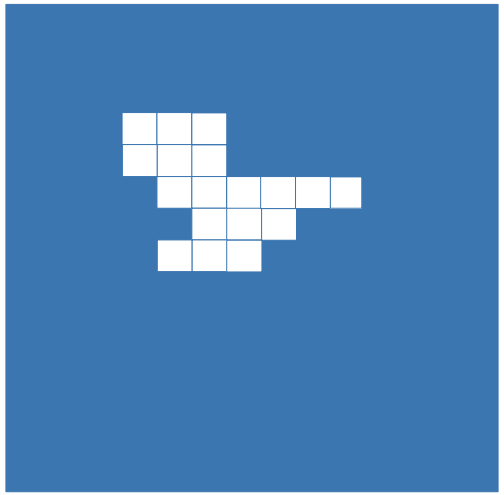
  - Choose a channel that can handle some bleaching, or if possible, a transmitted light image
  - Beware of dirt on coverslip or sample, it may influence the focus result as it is generally very bright
  - Consider the use of fluorescent beads in a channel not used for the experiment (e.g., when imaging BFP, GFP and DsRed, one could use Far-red stained beads for SWAF)

Tiles, Regions & Positions



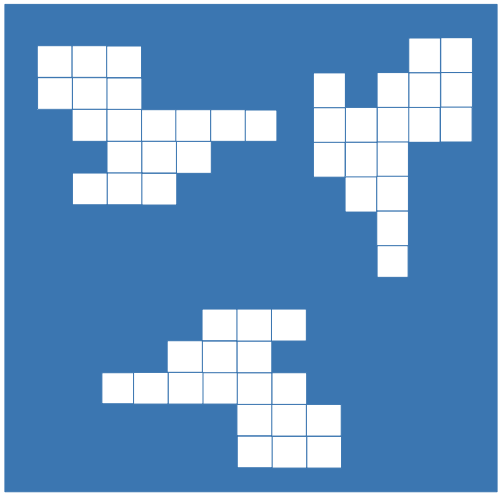
Positions
=
collection of single Tiles

Example: screening of cell culture



Region
=
collection of multiple Tiles, linked and often overlapping to be able to stitch & fuse into one large image
(=extending field of view)

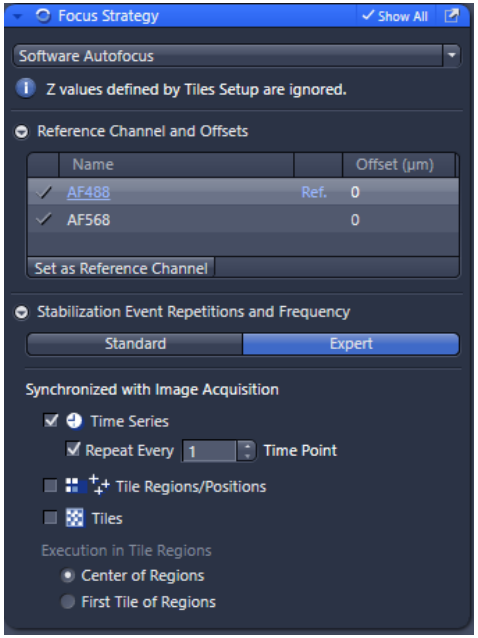
Example: imaging of 1 zebrafish, complete at high magnification

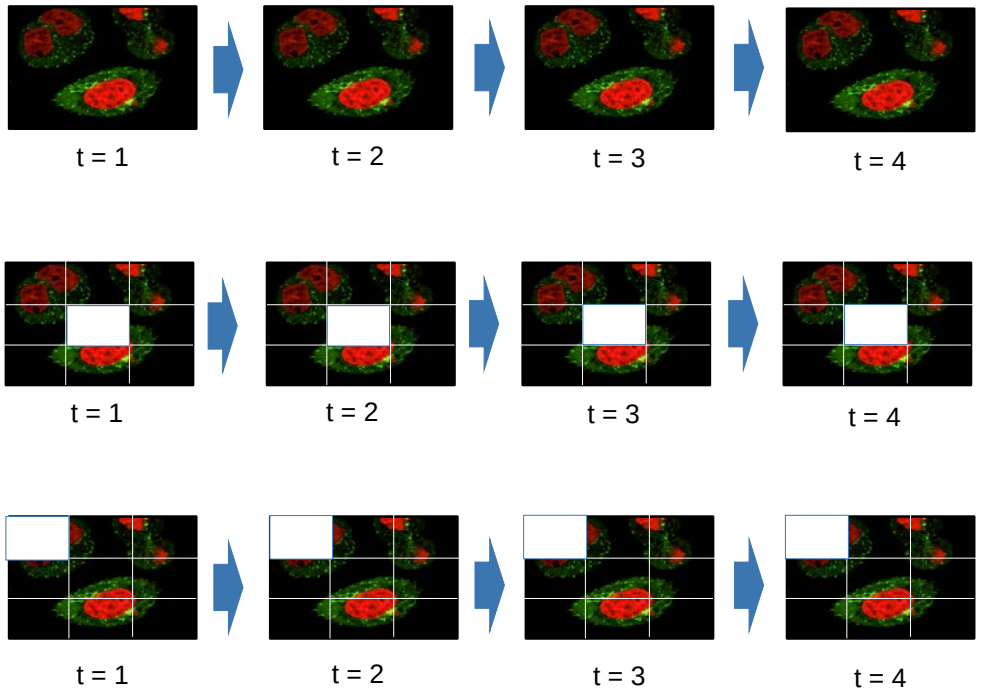


Regions
=
Combination of Positions & Region: multiple regions, with each a different position.

Example: imaging of multiple zebrafish embryos, complete at high magnification

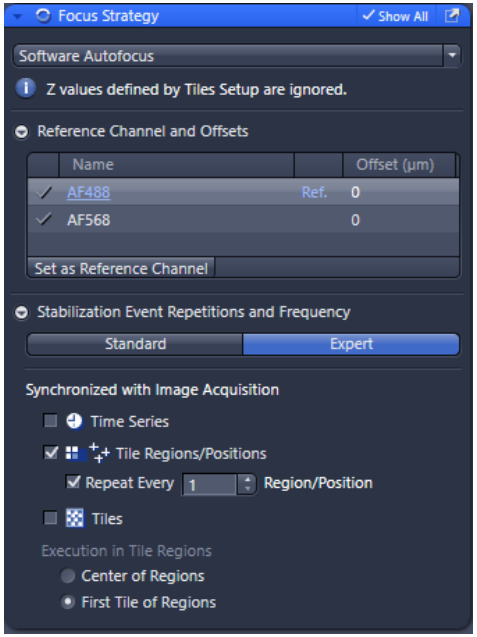
Focus Strategies
Software Autofocus only



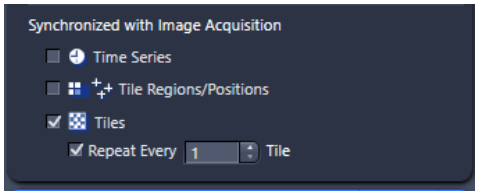


- Without Tiles enabled, Time lapse SWAF runs before every nth acquisition
- With Tile enabled, two options:
  - Before every time point in center of region
  - Before every time point on first tile of region

Focus Strategies
Software Autofocus only

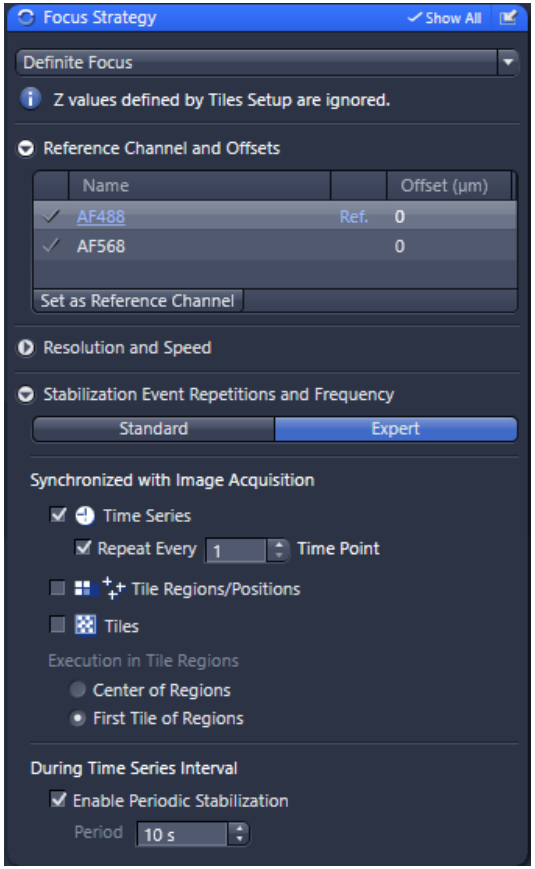


- When using multiple Positions or Regions, time-lapse runs SWAF on every Region.
- To speed up acquisition, select Region/Position and choose to skip positions or regions.
- Setting it to “Repeat Every 1 R/P” is similar to selecting “Check SWAF every time-point”



- For large Region(s), one can benefit from checking SWAF every n^{th} Tile to cover for sample unevenness

Focus Strategies
Definite Focus only



- Definite Focus has similar options as SWAF
- One extra option: Enable Periodic Stabilization
  - For time-lapse with long interval (>5min), drift may be too high resulting in DF not finding the reflection anymore when only checking every time-point or position
  - With Periodic Stabilization enabled, DF will maintain focus also during the interval, e.g. every 10s

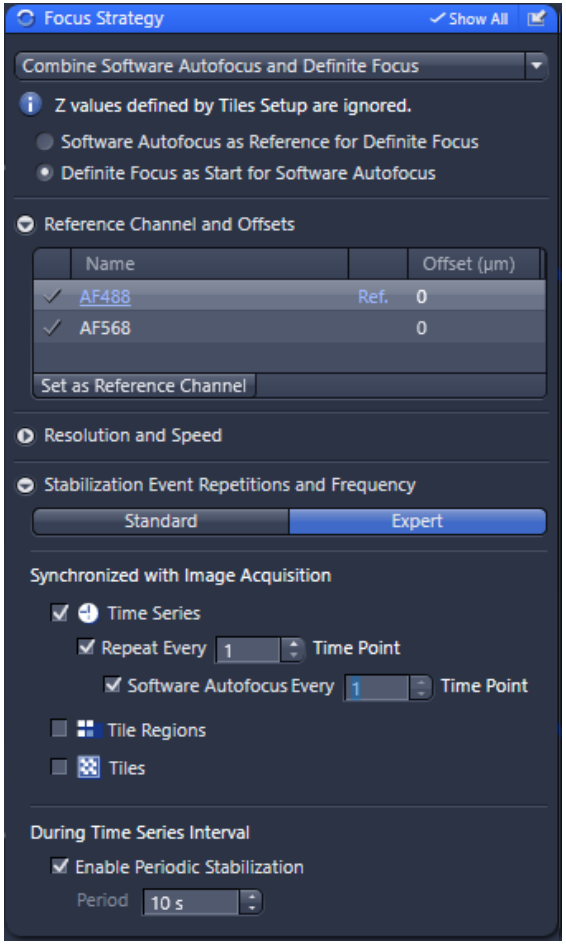
Focus Strategies
Combined SWAF and DF

- A combination offers more options and often higher accuracy & speed
- **SWAF as reference for DF**: useful e.g. when SWAF is required but at the same time to be avoided due to bleaching.

  Example: cells on a gel. DF will focus on coverslip, SWAF on cells. Choosing SWAF every 5 t-points, DF every t, limits phototoxicity.

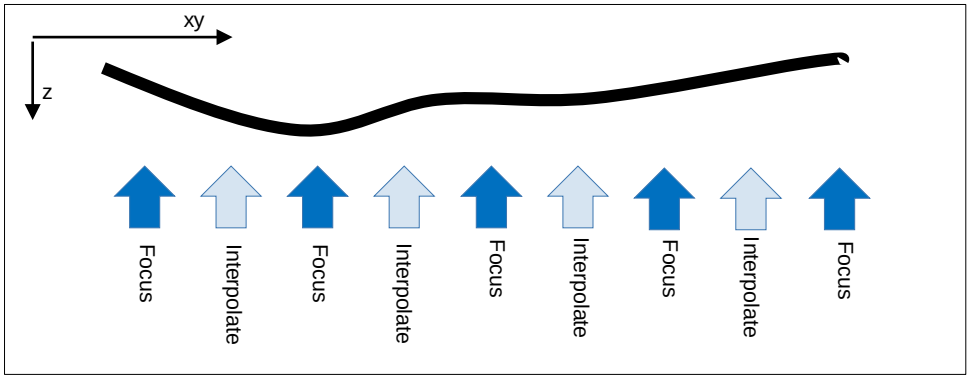
- **DF as reference for SWAF**: speed up SWAF

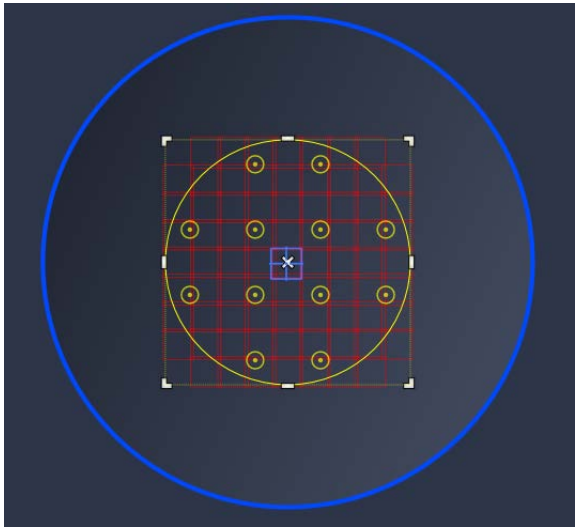
  Example: DF finds coverslip, distance to focus on cells is now minimal and can be performed much quicker by SWAF

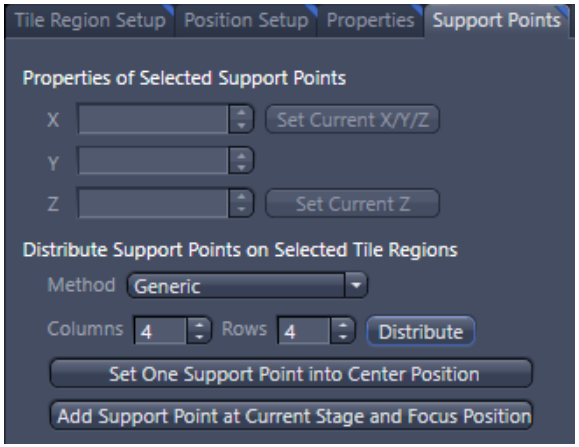


Focus Strategies
Support Points

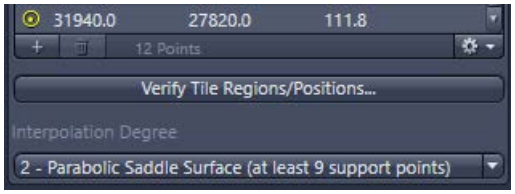
- Create a Tile Region in Tiles window & select the tab Support Points to create positions that will be used as focus point to cover for sample tilting/tortuosity



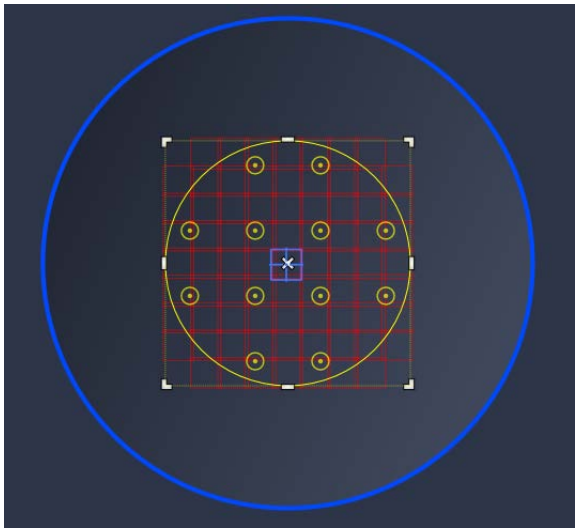




- In the Tiles window, under Support Points, one can then additionally define an optimized interpolation



Focus Strategies
Support Points

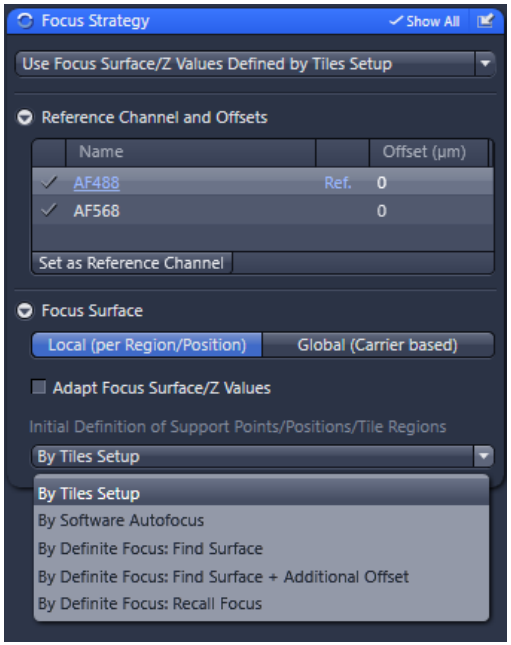


- Why not SWAF Only, every nth Tile?

SWAF Only: every 3rd Tile

Support Points: interpolation between points

Focus Strategies
Focus Surface Support Points



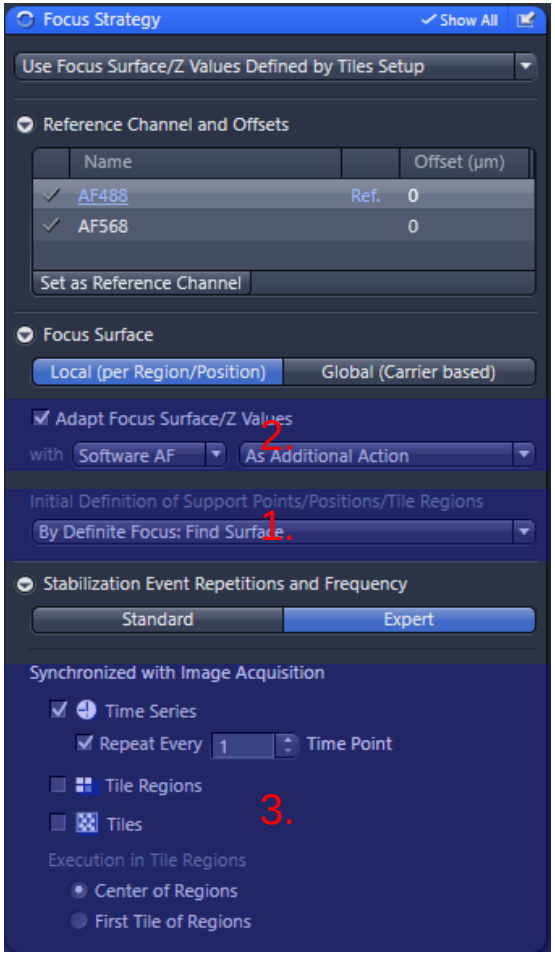
- Uses Support Points (see earlier slide)
- '**By Tiles Setup**' does not run DF or SWAF, uses z-position of Positions or Regions as defined
- '**By SWAF**' runs SWAF to define Support Points
- '**By DF: Find Surface**' define position of coverslip, no further adjustment (works well on objectives with lower resolution due to long depth of focus)
- **'By DF: Find Surface + Additional Offset'** applies an user-defined focus offset after DF finds the surface – similar to:
- **'By DF: Recall Focus'** uses the focus offset as defined by clicking Store Focus in the Definite Focus window

Focus Strategies
Focus Surface Support Points with SWAF and DF combined

1. All Support Points, or Positions or Tile Regions (!) are first defined by DF

2. In a following step, SWAF will be performed based on settings in (3), as an **‘Additional Action’**

3. Similar settings to “Only SWAF or DF” (slides 12-14)

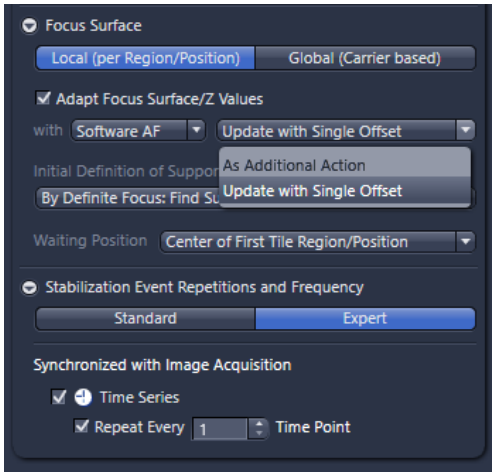


Focus Strategies
Focus Surface Support Points with SWAF and DF combined

‘Adapt Focus Surface/Z Values’ has a few options:

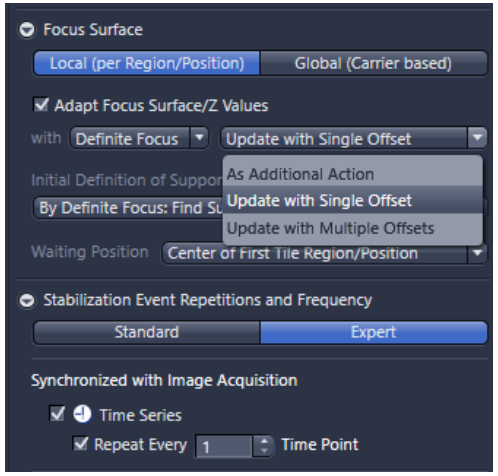
For SWAF:

1. ‘**As Additional Action**’: see previous slide
2. ‘**Update with Single Offset**’: only available with Time-lapse acquisition and will define a single offset on one position and apply this to all support points (faster, assuming cells are all on same distance from coverslip)



For DF:

1. ‘**As Additional Action**’: similar to SWAF
2. ‘**Update with Single Offset**’: similar to SWAF
3. ‘**Update with Multiple Offsets**’: each position has a different offset. Covers situations where cells are moving combined with coverslip deformation due to e.g. heating stage. Distance between coverslip & sample is continuously variable.



When to choose which strategy?!

Choice of strategy

Software Autofocus:

- Preferably avoided for live cell imaging due to photo-bleaching/-toxicity, ok for tissue
- Requires some time to find focus
- Focus may be affected by dirt, bright spots

Definite Focus:

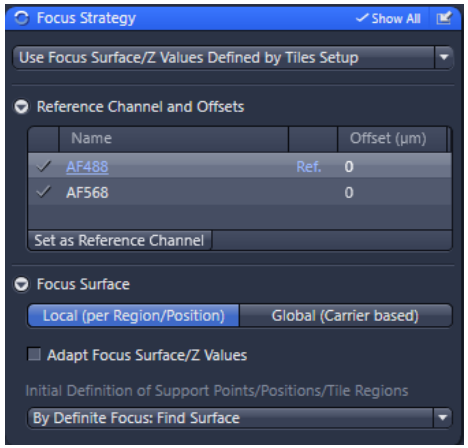
- Requires a refractive index mismatch between coverslip & medium to generate reflection
- Fast & accurate

Tiles Setup (fixed positions):

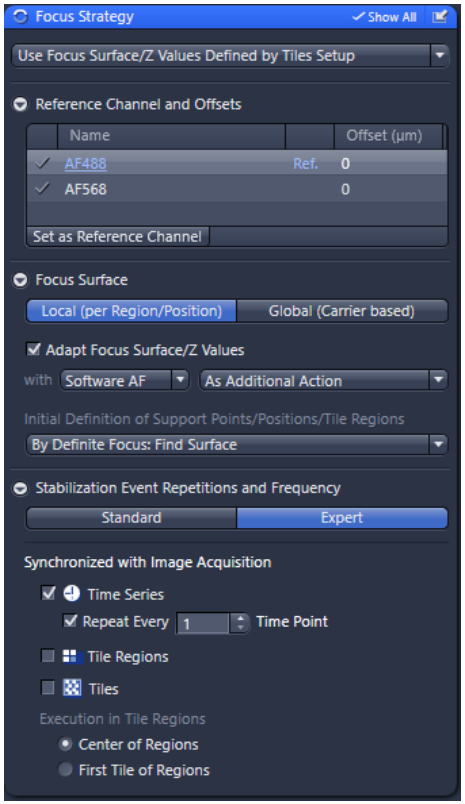
- Requires user input to define each single position
- More time-consuming

Choice of strategy
Cell culture: multiple position(s)/region(s), time-lapse

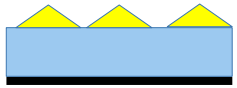
- Cells adhering to bottom



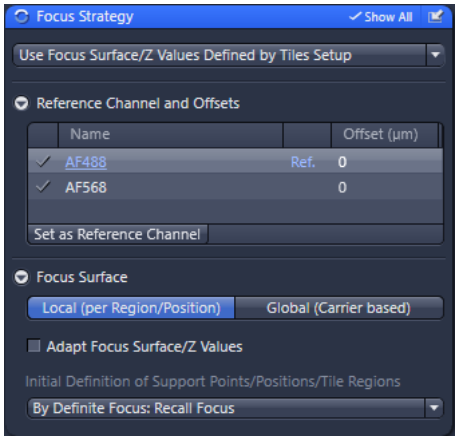
- Cells not adhering to bottom
- Free floating in solution

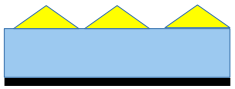


Choice of strategy
Cell culture: multiple position(s)/region(s), time-lapse

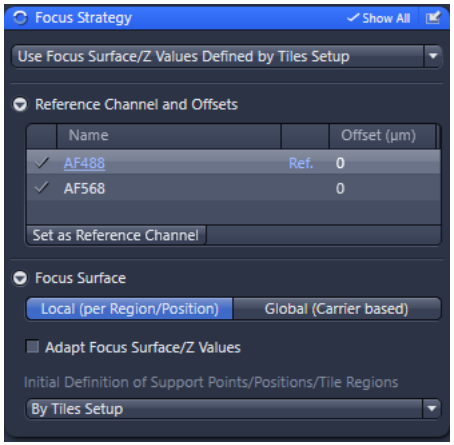


- Cells leveled, fixed, at homogeneous thickness above coverslip
- DF detects reflection





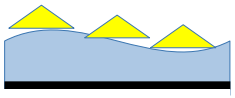
- Cells leveled, fixed, at homogeneous or heterogeneous thickness above coverslip
- No DF due to RI matching



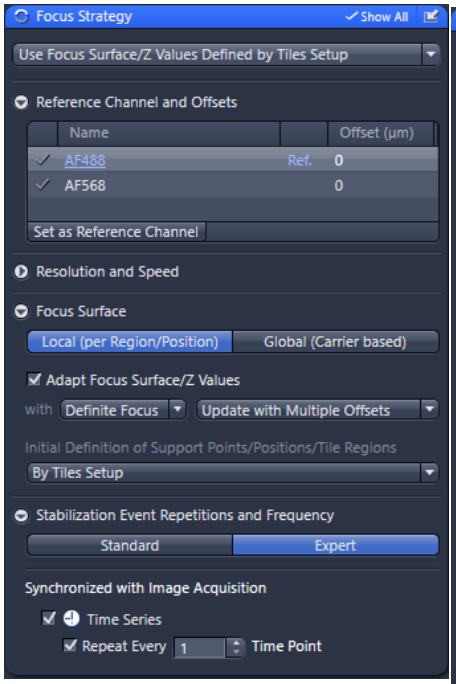
Disadvantage of ‘By Tiles Setup’ is that all z-positions have to be pre-defined

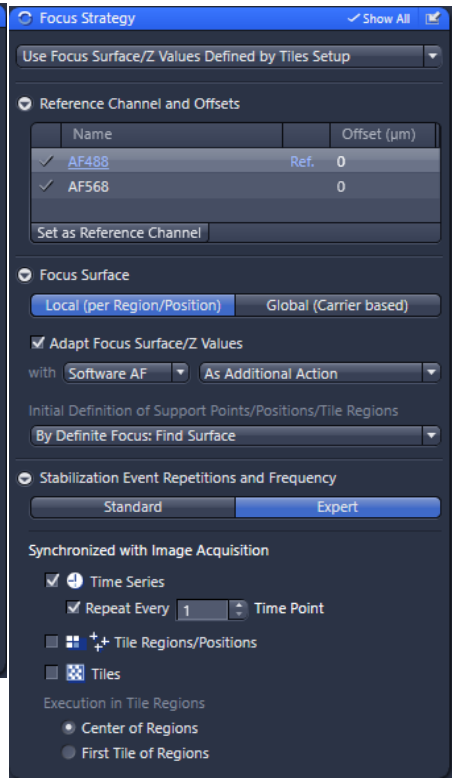
Choice of strategy
Cell culture: multiple position(s)/region(s), time-lapse

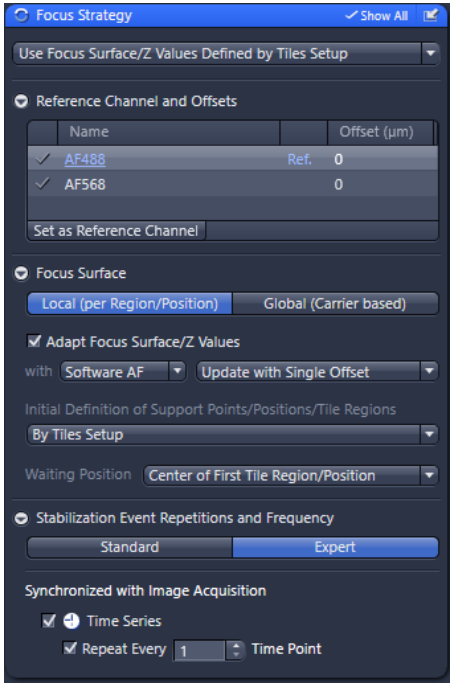
- Cells leveled, fixed, at heterogeneous thickness above coverslip
- DF finds coverslip



- Cells leveled, fixed, at heterogeneous thickness above coverslip
- DF fails due to RI matching





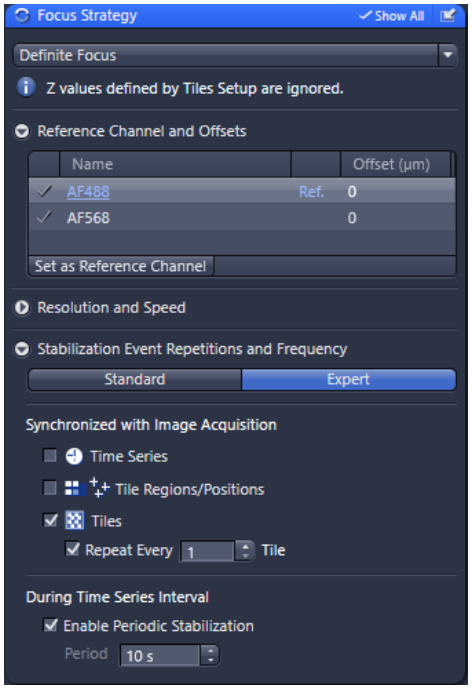


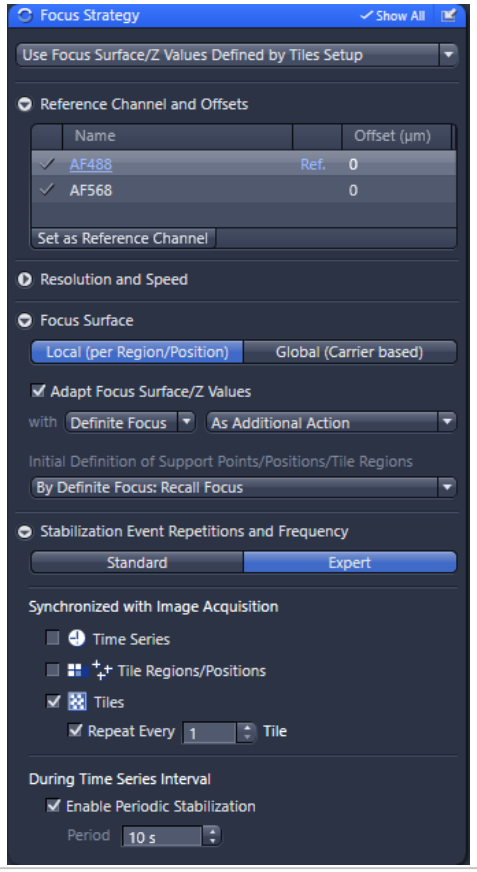
Choice of strategy
Cell culture: time-lapse, long interval

- Cells adhering to bottom
- Single position

- Cells adhering to bottom
- Multiple positions

- Cells growing on top of a gel or alike
- DF detects reflection
- Single/Multiple positions





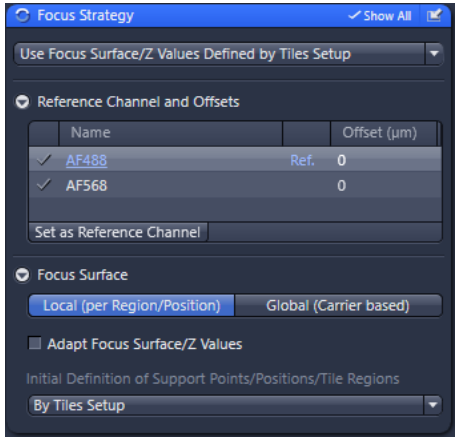
Alternatively, when adhering to coverslip, choose 'By Definite Focus: Find Surface'

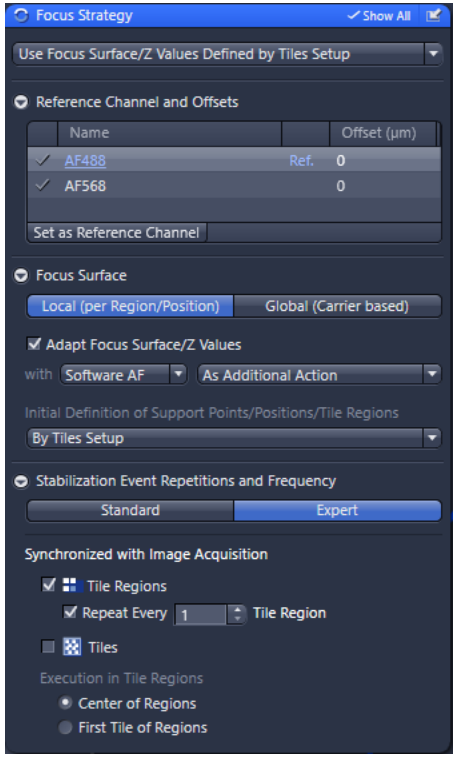
Choice of strategy
Tissue section: region(s), no time-lapse

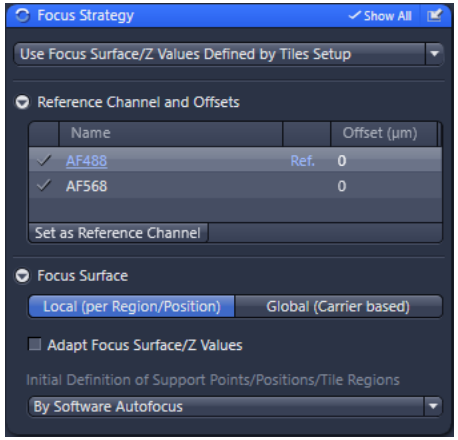
- Small region
- About 3x3 tiles
- No support points

- Small, multiple regions
- About 3x3 tiles
- No support points

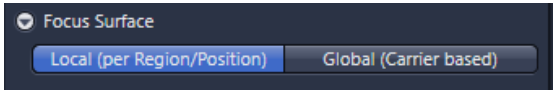
- Large region(s)
- More than 3x3 tiles
- >9 support points





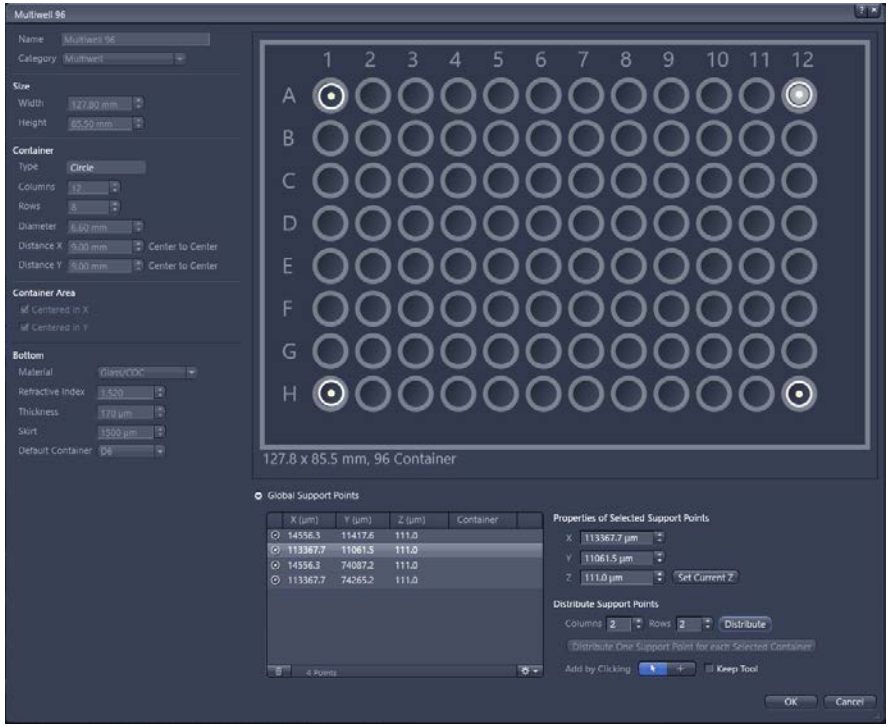


Choice of strategy
Local versus Global Support Points



- Instead of ‘Local’ Focus Support Points, ‘Global’ can be chosen. Global Support Points have to be defined in Sample Carrier Template
- Local Focus Points are always per position, or region
- Global Focus Points can compensate tilting of a large carrier at once, regardless of regions in each well

- Useful for:
  - Well-plate where cells are adhering to bottom (same fixed offset)
  - Outer wells can be filled with beads and used for SWAF without affecting sample in other wells



Focus Strategies with ZEN
Summary

- A solution for every condition, it's a matter of finding the best option.
  Questions to ask:
  - Is the sample adhering? → **include DF**
  - Is the sample staying on a fixed distance of the bottom? → **DF with Recall Focus**
  - Is DF not finding a reflection? → **include By Tiles Setup (or SWAF)**
  - Floating/tortuous? → **include SWAF**
  - Time-lapse with long interval? → **remember to Enable Periodic Stabilization**
- Be creative:
  - If SWAF is required, consider using far-red beads if possible → no effect on other channels
  - Consider using Global Support Points where some wells only contain beads
  - Try SWAF on brightfield channel
- **But above all: make sure the system's temperature is stabilized. A system that is warming up will show focus maintenance instability**.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