IN Cell Analyzer 6000

Customer Training

imagination at work

Name _____

Date _____

Index

General Safety Precautions

For detailed precautions please refer to the IN Cell Analyzer 6000 User Manual

WARNING! The operator of the IN Cell Analyzer 6000 is assumed to be trained in the correct operation of the instrument and the safety issues.

WARNING! Using controls, making adjustments, or performing procedures other than those specified in the IN Cell Analyzer 6000 documentation can result in hazardous exposure to high voltage or moving parts. Exposure to these hazards can cause severe personal injury.

CAUTION! Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure.

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Customer Instrument Specifics

Serial number	
Objectives	
Polychroics	
Camera	
Additional modules	
Acquisition Software	
Computer password	
Software build	

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Start up and Shut down Procedures

Start up procedure:

(1) Turn on computer and log on to Windows.

(2) Turn on instrument using momentary depress of main power switch*. Release switch after a click is heard, then wait for ~3 minutes as 2 of the 3 lights on the front of the system go out (orange, red) leaving one green light. Sample door should now be open.

(3) Connect to instrument by double clicking the IN Cell Analyzer 6000 icon on the PC desktop. 

Shut down procedure:

1) Exit/close the instrument software.

2) Depress system power switch* for one full second only. After a 20-30 second delay, the system should power down.

3) Repeat step 2 if system does not shut down after two minutes.

4) Switch off the workstation PC and monitor, if desired.

*** Main Power Switch is a rocker type, located on the right hand side of the IN Cell 6000 instrument, toward the rear, under rubber flap, immediately below communication cable.*

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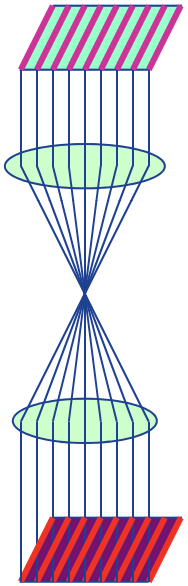
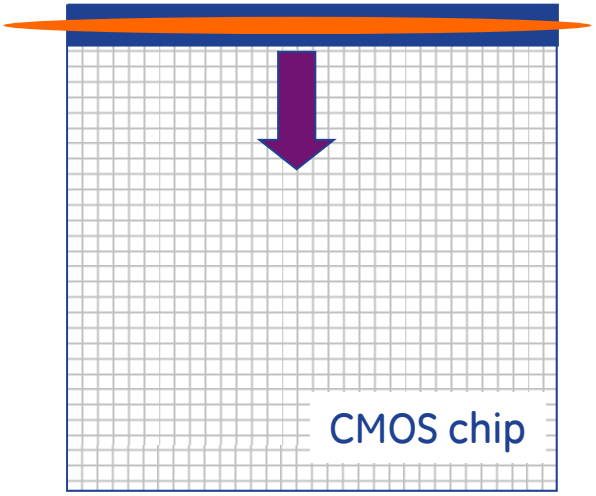
IN Cell Analyzer 6000 Principle

Sample scanned line by line



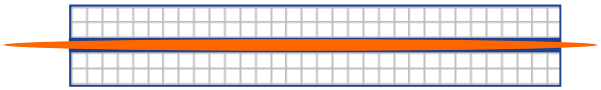
CMOS camera builds image line by line

Laser line and Confocal Slit arrangement



Wide Confocal Slit

Narrow Confocal Slit



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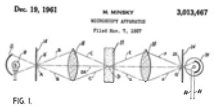
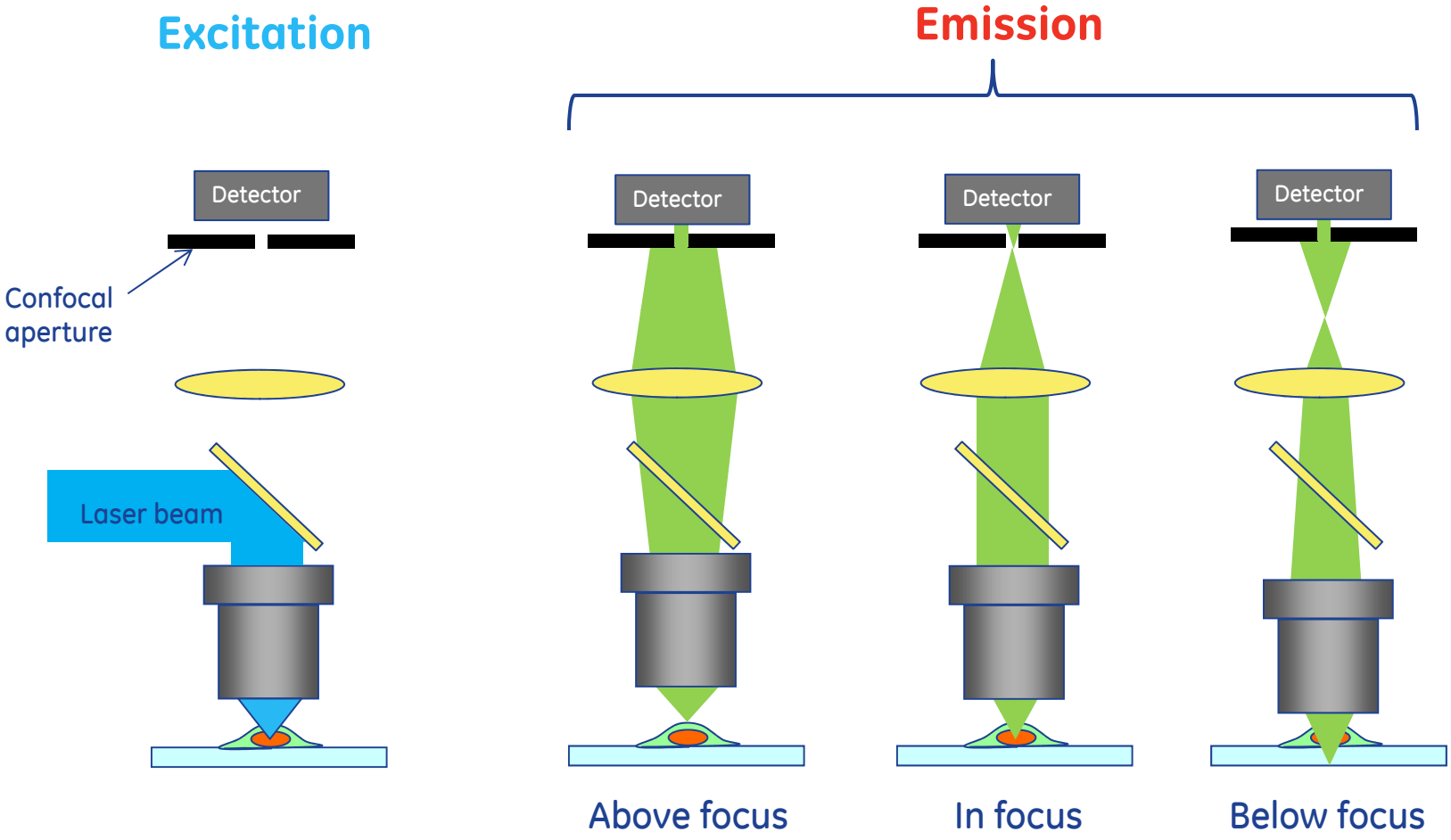
Concept of confocality:
Rejection of out-of-focus light





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- Confocal aperture blocks out-of-focus light
- Amount of light transmitted to detector depends on aperture size

Optical Path Schematic

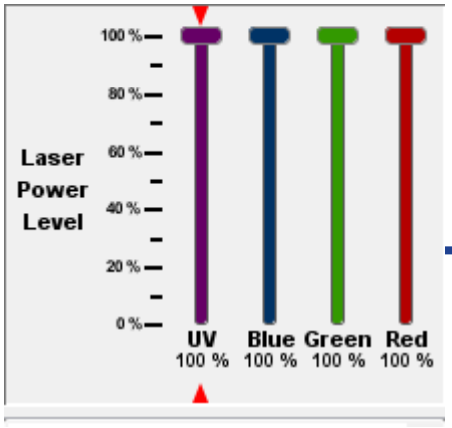
Transmitted light

B

SBS plate compatible

HWAF
SWAF

C



Laser Illumination

A

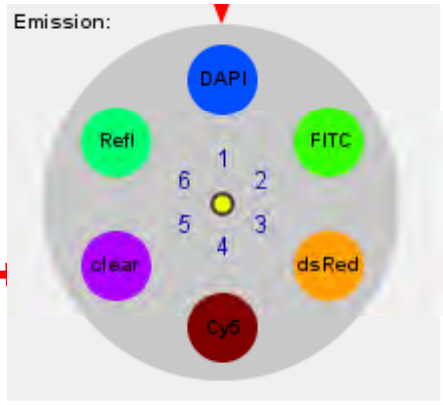
Objectives

D

Scan Head

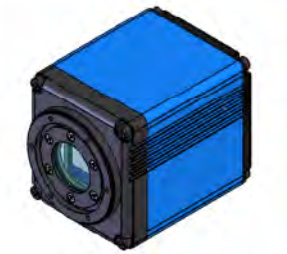
Polychroic Mirror

E



Emission Filters

F



G

Camera

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Laser Illumination

A

Laser Wavelengths

405	Diode	UV	DAPI, Hoechst, Quantum Dots
488	Diode	Blue	eGFP, Fluorescein, eYFP
561	DPSS	Green	Cy3, DsRed, Rhodamine, TxRed
642	Diode	Red	Cy5

Auto Calibration feature

- High operational stability
- Unsusceptible to extensive mechanical disturbances
- Unsusceptible to thermal fluctuations between 15 and 40 °C
- Pushbutton installation: Laser in spec after push-button command
- Combined fiber coupling values in excess of 50% can be restored by a single push-button command

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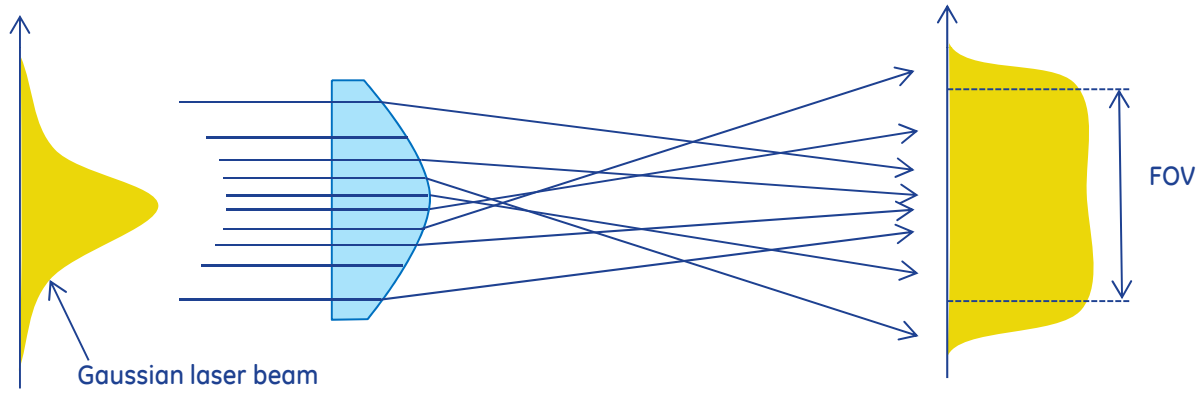
Laser Illumination

Powell lens serves two functions:

- It creates a laser line by focusing the collimated laser beam in one direction
- It distributes a laser intensity angularly to make laser line uniform along its length



- Typical line intensity uniformity is better than 10% for visible lasers
- UV line (405 nm laser) may have less uniform line

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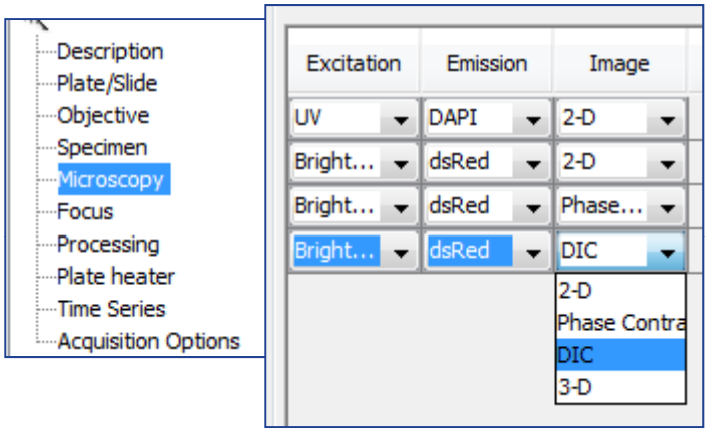
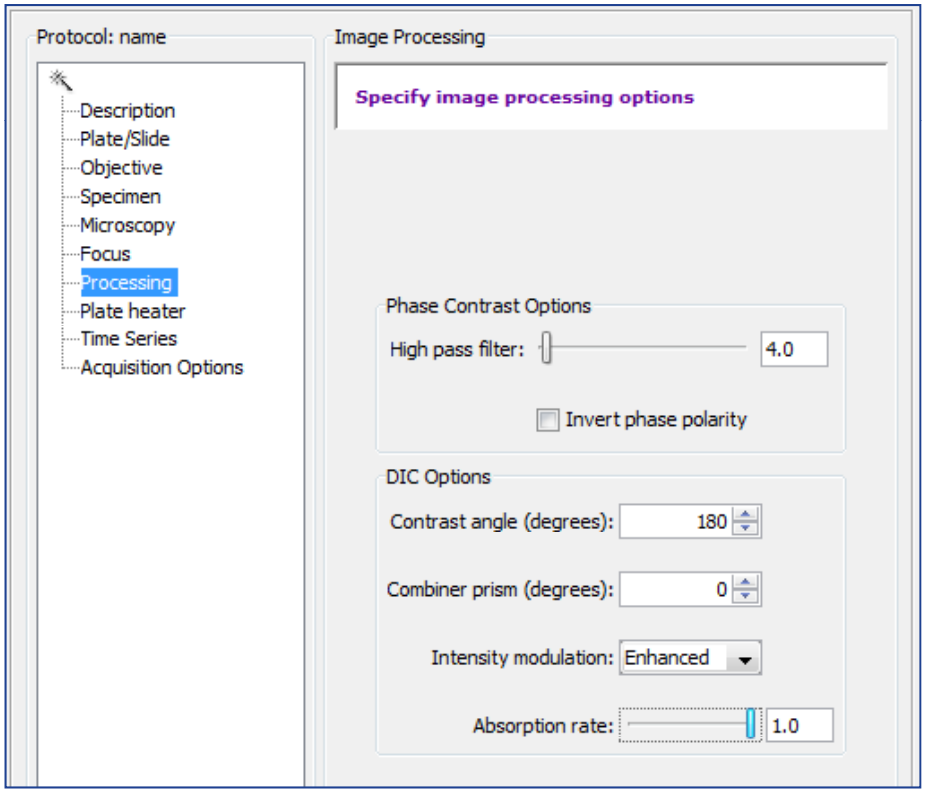
Transmitted Light

B

Iatia Phase Contrast and Differential Interference Contrast (DIC) Imaging Modes





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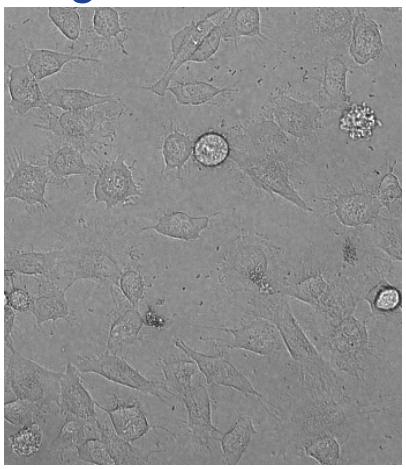
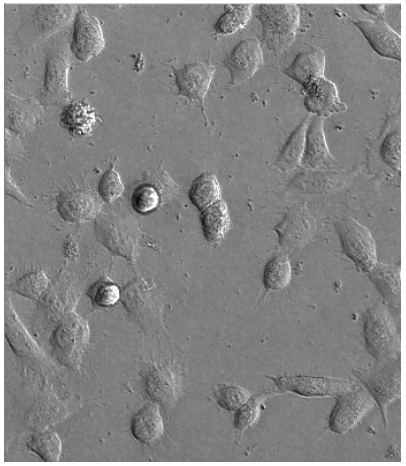
Transmitted Light

B

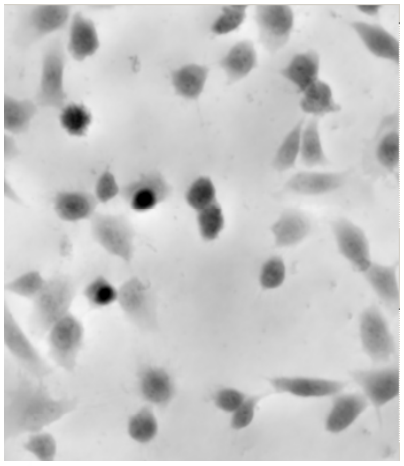
Brightfield



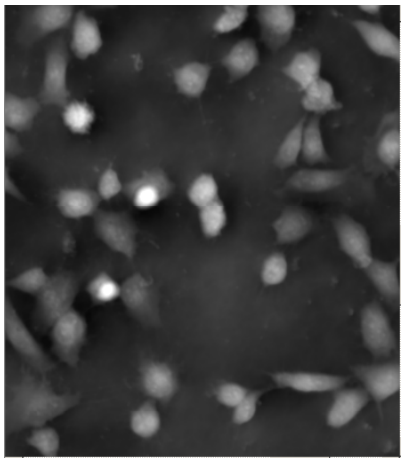
DIC



Phase Contrast



Phase Contrast (Inverted)



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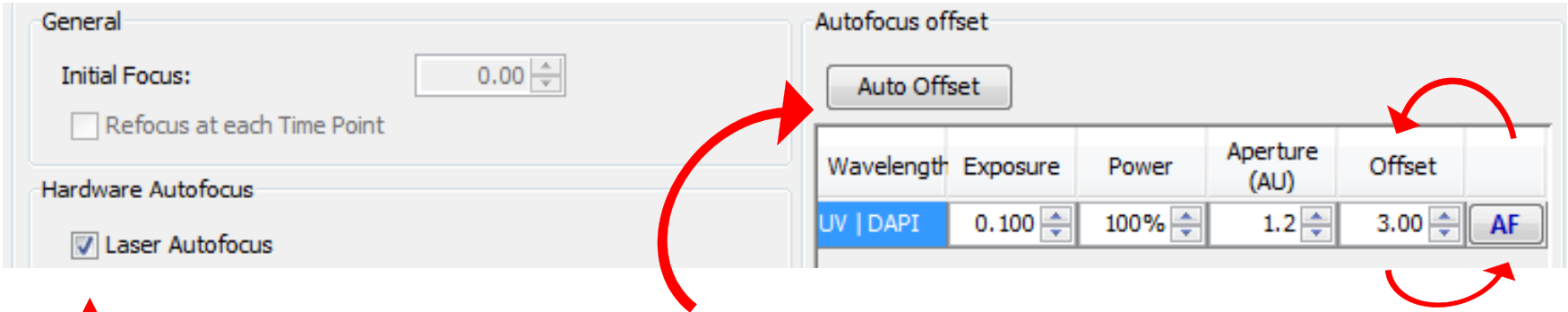
Autofocus: Laser-based (HardWareAutoFocus)

HWAF A 785nm laser is directed through the objective and focused on to the target whilst z-axis is moved. A sensor monitors the reflected beam to determine the exact position of the target.



Step 1

Coarse focus to locate the well bottom (glass or plastic to liquid interface)

Step 2

Fine focus for each wavelength using a software image contrast algorithm. (Only needs to be run once for each plate or group of plates prepared the same day.)

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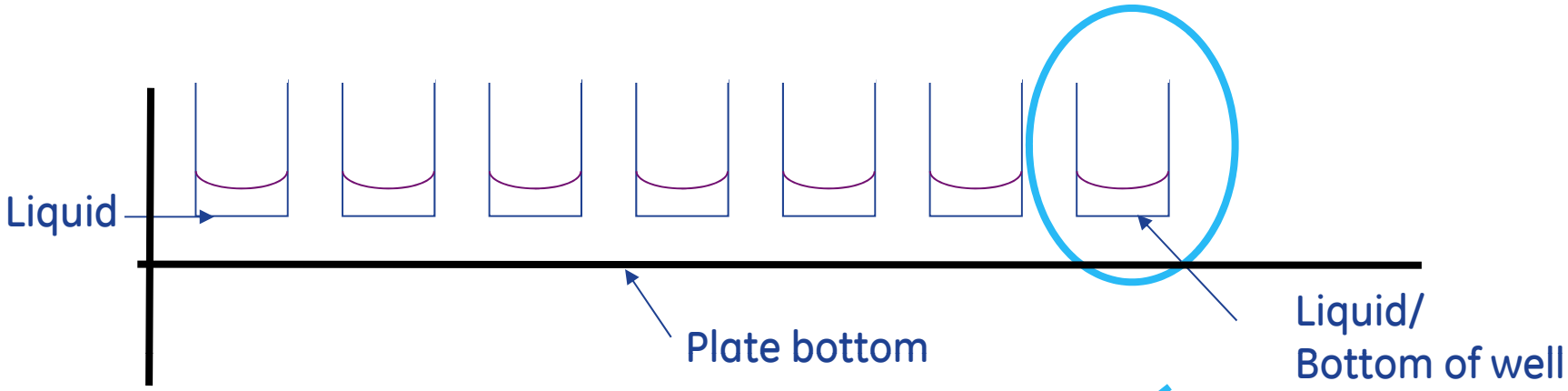
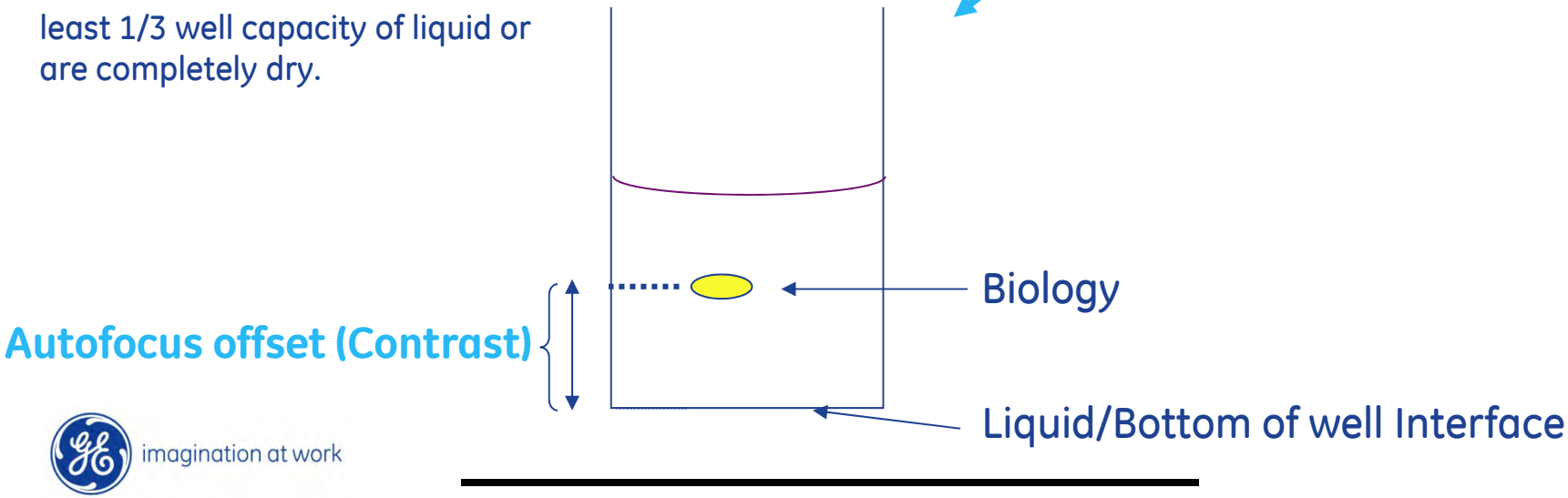
HWAF and Autofocus Offset



Hint: Make sure wells contain at least 1/3 well capacity of liquid or are completely dry.



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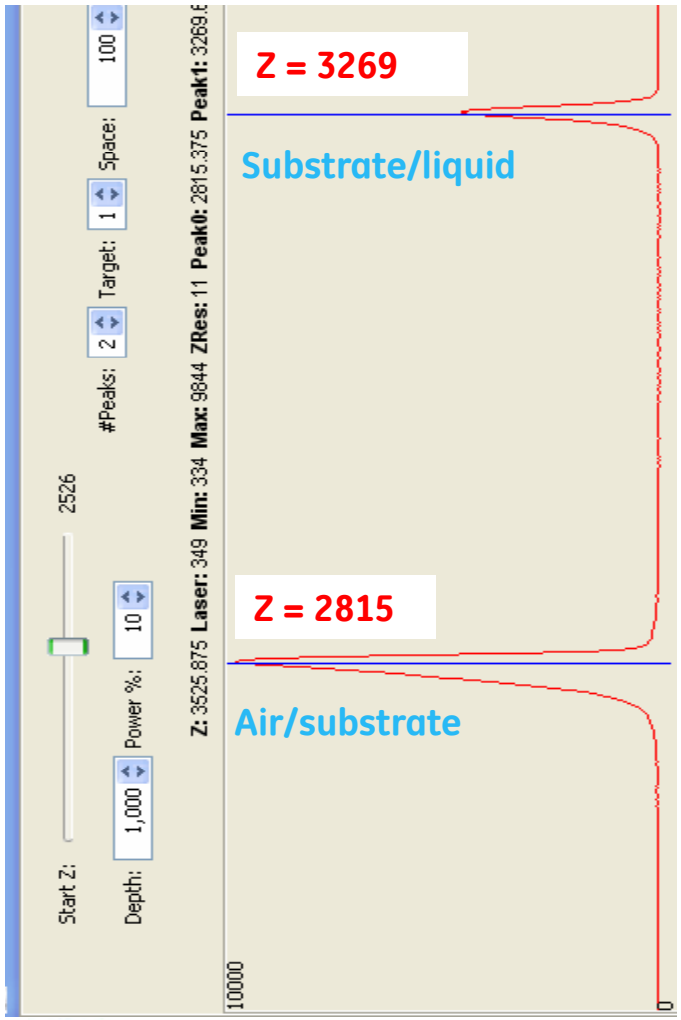
Typical Plate HWAF Trace

Observed reflectance peaks



This peak used for focusing with 4x lens (for plates with base >500µm thick) 10x, 20x and higher power lenses

(Thickness calculated from Z values = 3269-2815 = 454*1.55 = 703µm)

This peak used for focusing with 2x lens and 4x lens (for plates with base <500µm thick)

Correct Plate thickness and skirt height values are important for effective HWAF

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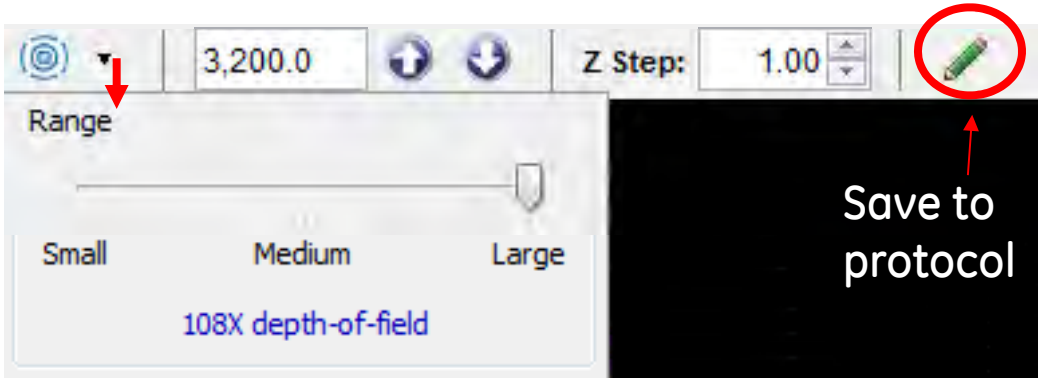
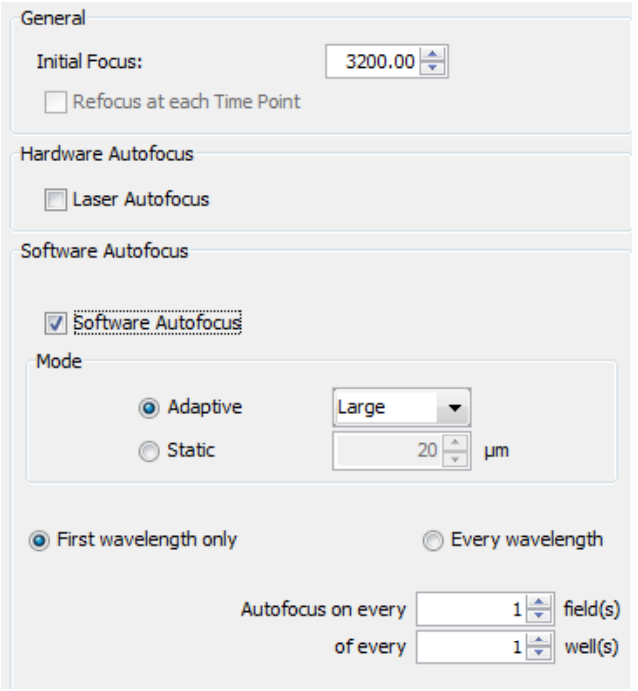
Software-based Autofocusing (SWAF)

C





Adaptive Mode – For each Z-step, autofocus starts from the previous in-focus point. Search range from the previously discovered in-focus point; **Large** specifies a ±200µm search range, **Medium** specifies a ±100µm search range, and **Small** specifies a ±50µm search range.

Static Mode – For each Z-step, autofocus uses the Z position in the Initial Focus field as a starting point, and then uses the number indicated in the field next to the **Static** radio button as a search range. Static Mode is usually the fastest software autofocus mode and works well for a relatively flat target, such as a slide.

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Capturing Images

	**Laser Autofocus** **Drop down to laser power**	Takes you to an interface e.g. plastic/liquid interface -
	**Software Autofocus Drop down to scan ranges**	Select from the dropdown list of available scan ranges will start from the position defined by the Z position window For software autofocus to be effective you need to be within range of the sample biology
	**Z Position**	View current position in the Z-stack, -1500 is the default position at rest.
	**Digitize**	Takes an image at the current position defined by the Z position window. Depending on its position within the GUI takes images using different parameters.
	**Digitize with Autofocus**	When using HWAF takes an image at the Interface found using Laser Autofocus +HWAF Offset value. When using SWAF takes an image applying a small SWAF search.
	**Initial Focus**	When using SWAF this is the Z position that the SWAF search will start from when you run the protocol

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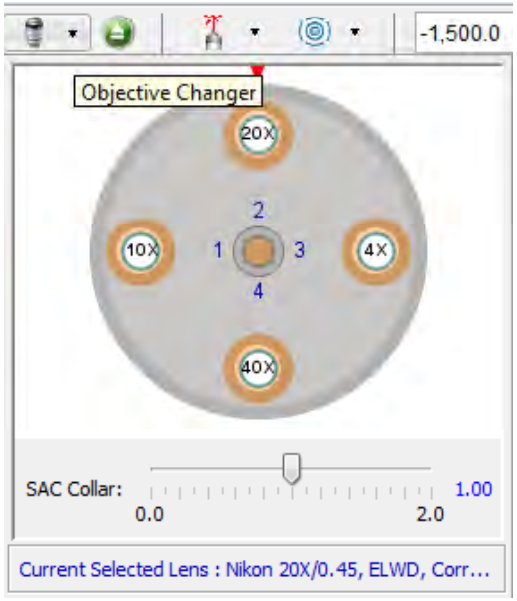
Objectives



- Automated - 4 position turret
- Automated Spherical Aberration Collars (ASAC) - 2 positions

Standard Objective Description			
Mag.	NA	ASAC (range mm)	Working Distance
10X	0.45	No	4 mm
20X	0.45	Yes (0-2)	7.5 mm
Optional Objective Description			
Mag.	NA	SAC	Working Distance
2X	0.1	No	8.5 mm
4X	0.2	No	20.0 mm
20X	0.75	No (0.17)	1 mm
40X	0.6	Yes (0-2)	3.2 mm
60X	0.7	Yes (0.1-1.3)	1.8 mm

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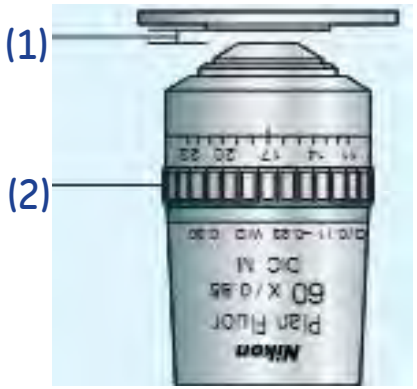
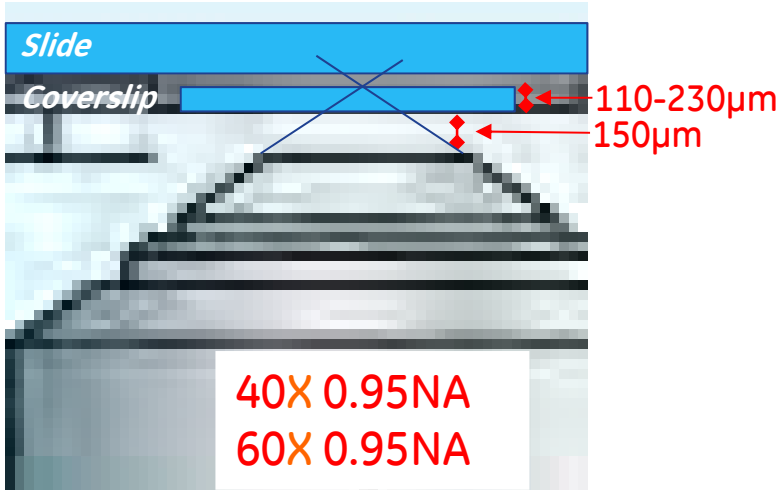
High NA Objectives

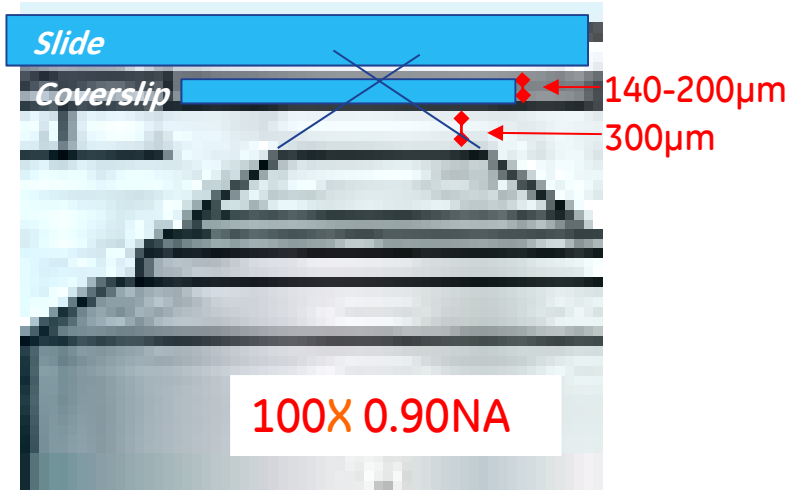


Optional Objective Description			
Mag.	NA	ASAC	Working Distance
40X	0.95	Yes (0.11-0.23)	0.15 mm
60X	0.95	Yes (0.11-0.23)	0.15 mm
100X	0.9	Yes (0.14-0.20)	0.3 mm

(1) Working distance defines the distance from the top lens of the objective and the surface of the coverslip

(2) Correction Collar





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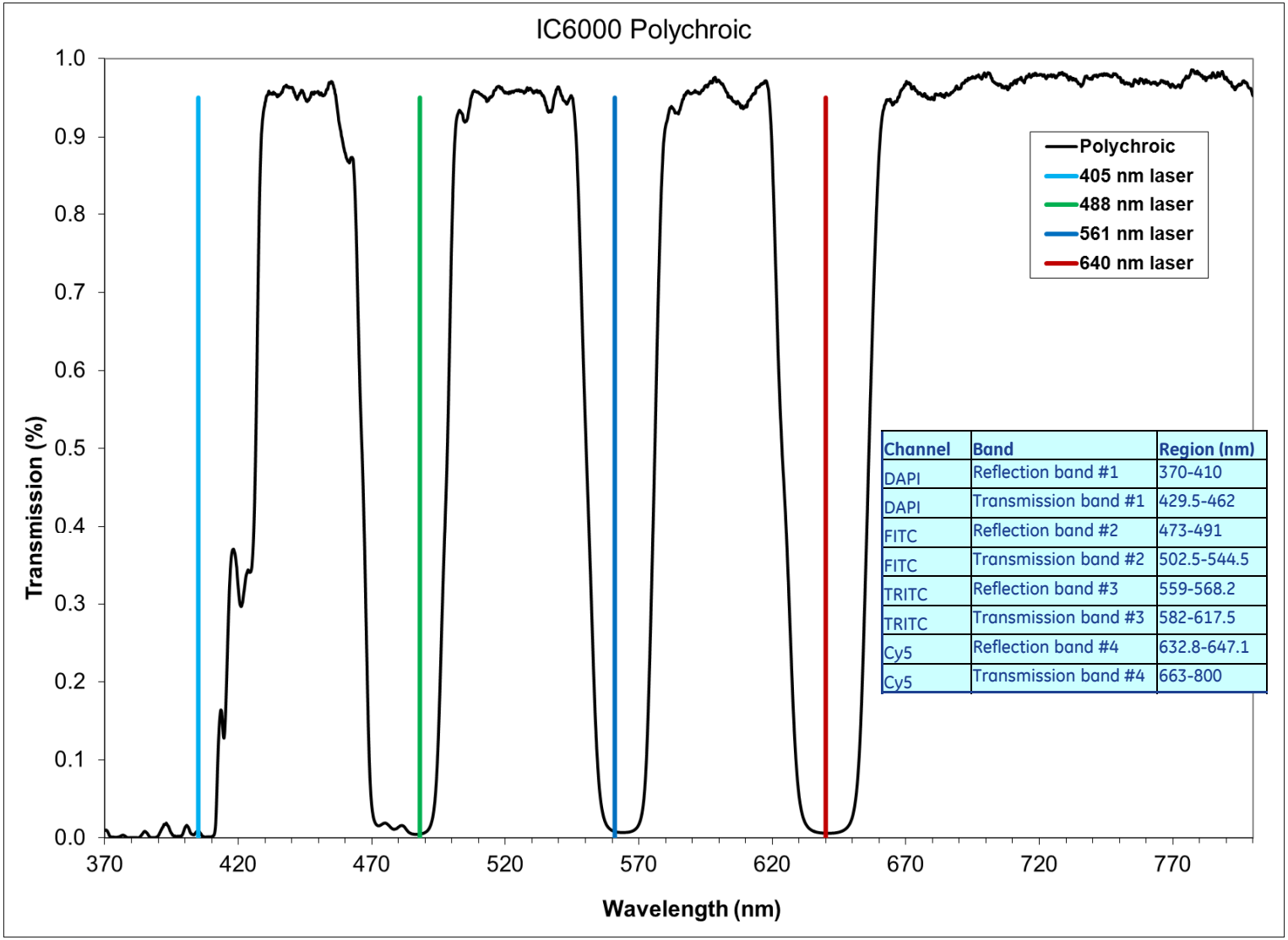
Considerations for plate imaging; plate thickness, skirt height and plate flatness

Nikon MICROSCOPYU THE SOURCE FOR MICROSCOPY EDUCATION

Objective performance summary

Magnification	NA	Airy Disc d=1.22*lambda/ NA, um	CMOS resolution 2*pixel size/mag, um	System imaging resolution, um	FOV area, mm2	FOV area, %	Depth of Field, um
2	0.1	6.10	6.50	6.50	44.302	100.00	115.0
4	0.2	3.05	3.25	3.25	11.076	25.00	28.8
10	0.45	1.36	1.30	1.36	1.772	4.00	5.4
20	0.45	1.36	0.65	1.36	0.443	1.00	3.9
20	0.75	0.81	0.65	0.81	0.443	1.00	1.8
40	0.6	1.02	0.33	1.02	0.111	0.25	1.9
40	0.95	0.64	0.33	0.64	0.111	0.25	0.9
60	0.7	0.87	0.22	0.87	0.049	0.11	1.3
60	0.95	0.64	0.22	0.64	0.049	0.11	0.8
100	0.9	0.68	0.13	0.68	0.018	0.04	0.8

Polychroic mirror and lasers spectral data E



Channel	Band	Region (nm)
DAPI	Reflection band #1	370-410
DAPI	Transmission band #1	429.5-462
FITC	Reflection band #2	473-491
FITC	Transmission band #2	502.5-544.5
TRITC	Reflection band #3	559-568.2
TRITC	Transmission band #3	582-617.5
Cy5	Reflection band #4	632.8-647.1
Cy5	Transmission band #4	663-800

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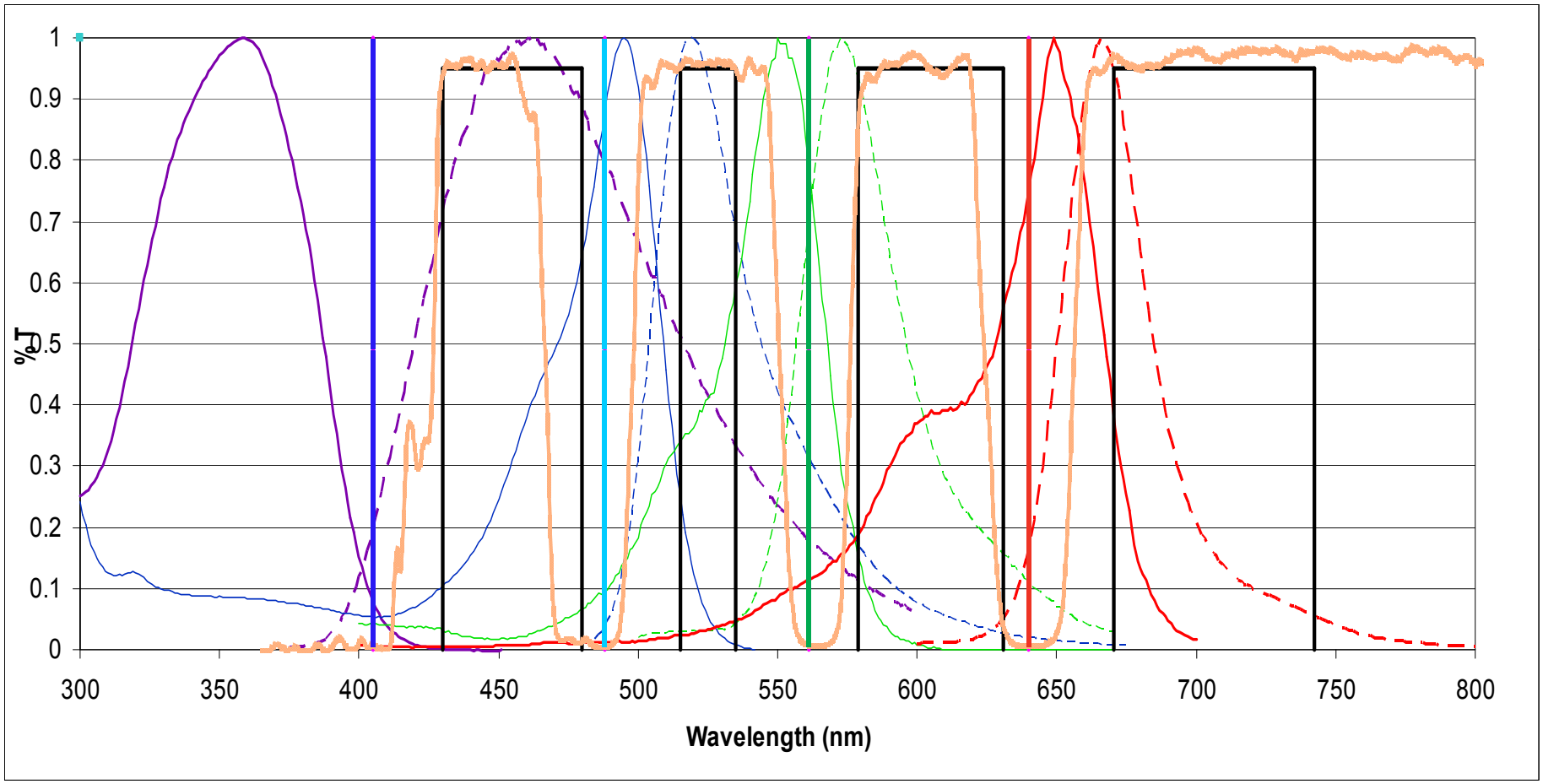
Polychroic, lasers and typical fluors data



DAPI Excitation		DAPI Emission	
FITC Excitation		FITC Emission	
TRITC Excitation		TRITC Excitation	
Cy5 Excitation		Cy5 Excitation	

Emission filters		Polychroic	
405 nm laser		561 nm laser	
488 nm laser		640 nm laser	

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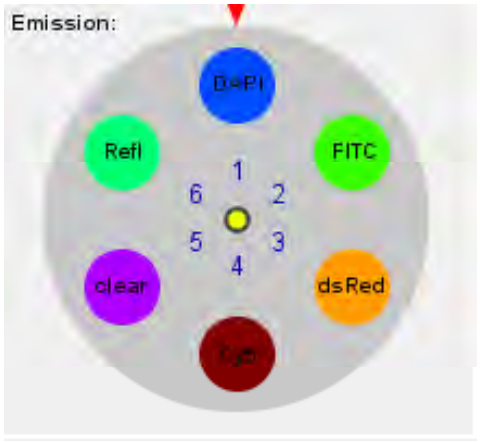
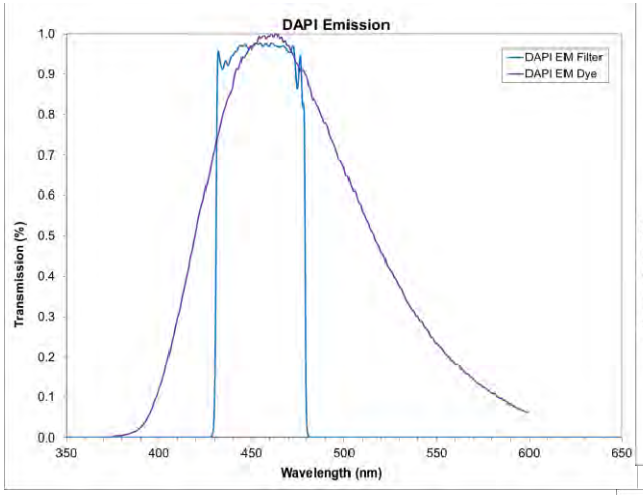
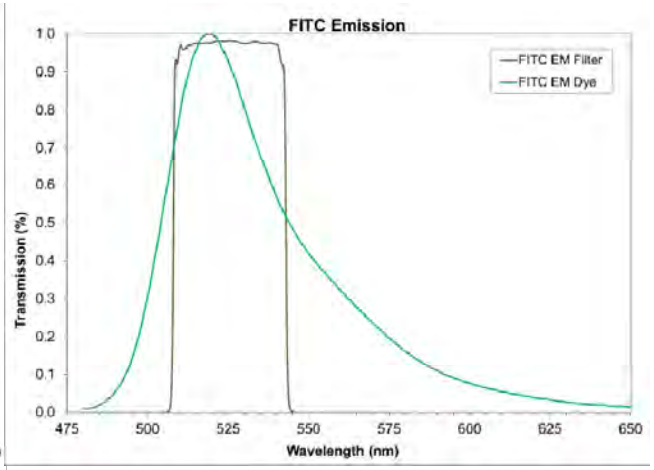
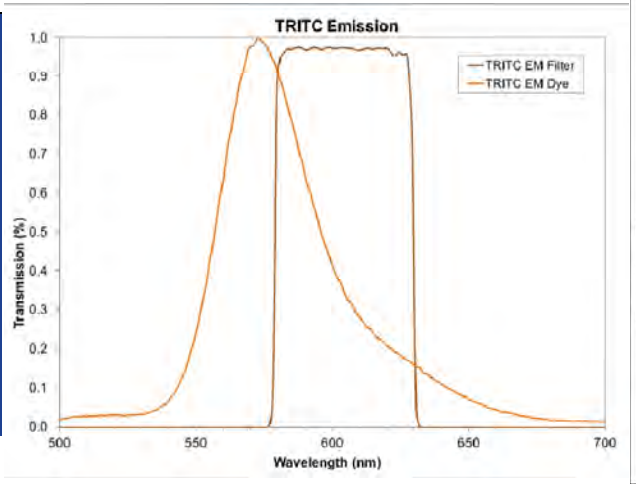
Emission Filters

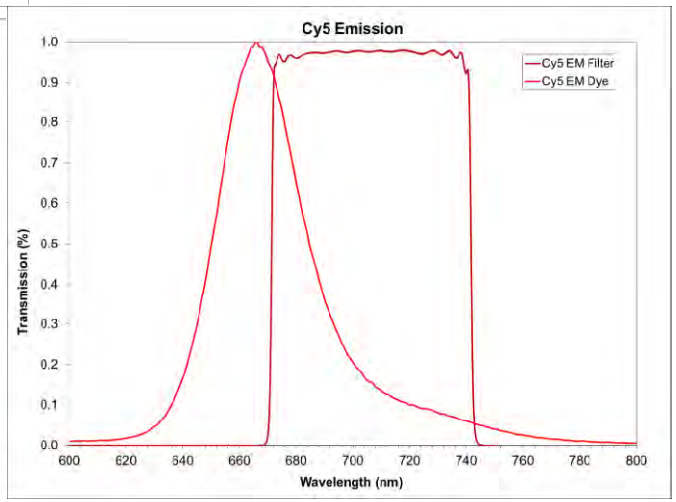






Laser Line	Channel	CW (nm)	BW (nm)	Blocking (OD6 avg, OD5 abs)
405nm	DAPI	455	50	300-420nm; 490-900nm
488nm	FITC	525	20	300-505nm; 545-900nm
561nm	TRITC	605	52	300-569nm; 641-900nm
640nm	Cy5	706.5	72	300-660nm; 753-900nm





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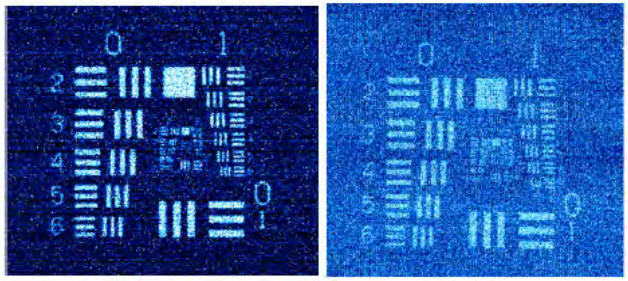
sCMOS Camera benefits

sCMOS camera essentials:
- Scientific grade CMOS chip
- 2560 x 2160 pixels (5.5 Megapixel)
- <2 electrons read-noise
- 33K electron well-depth
- 50-55% max QE

sCMOC vs. standard CCD

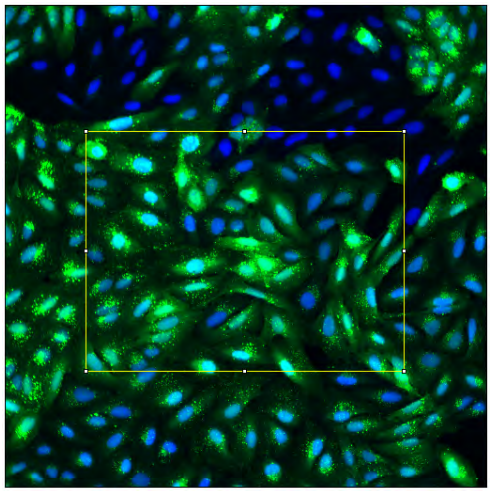
Lower image noise



sCMOS (*1.5 e⁻ noise*) Interline CCD (*5 e⁻ noise*)

sCMOC vs. standard CCD

Large Field of View



IN Cell Analyzer 6000 image of FYVE assay (merged DAPI & FITC)
Area in the middle is standard 1.3 megapixel CCD FOV

New generation of CMOS cameras offers:

- Large field-of-view (whole well imaging for 96 well plate at 2X)
- Sensitivity comparable with scientific grade CCDs
- 5 x less noise in comparison with industry standard cooled CCDs
- **Rolling shutter feature enables new line confocal technology**

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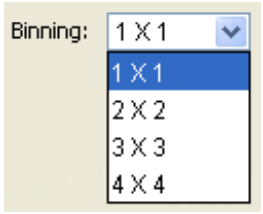
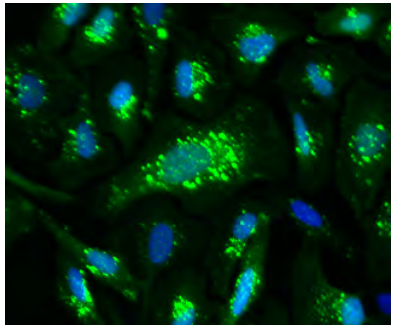
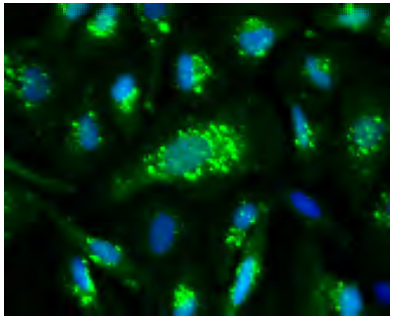
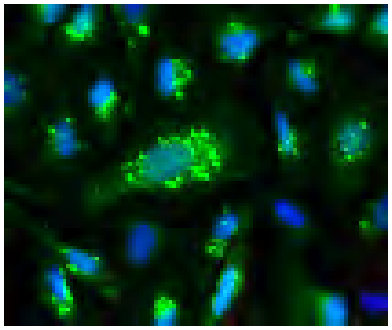
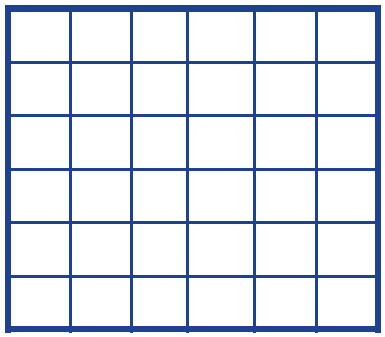
IN Cell Analyzer 6000 Binning

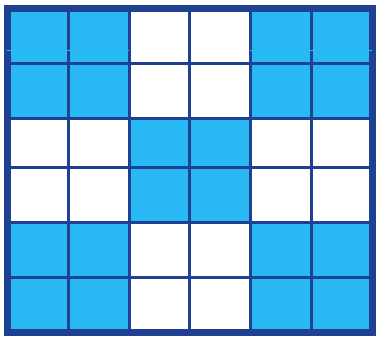


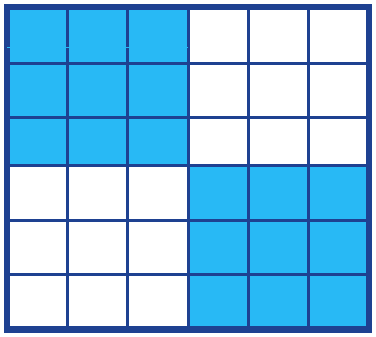












1x1 bin readout
Image file size = 8Mb

2x2 bin readout
Image file size = 2Mb

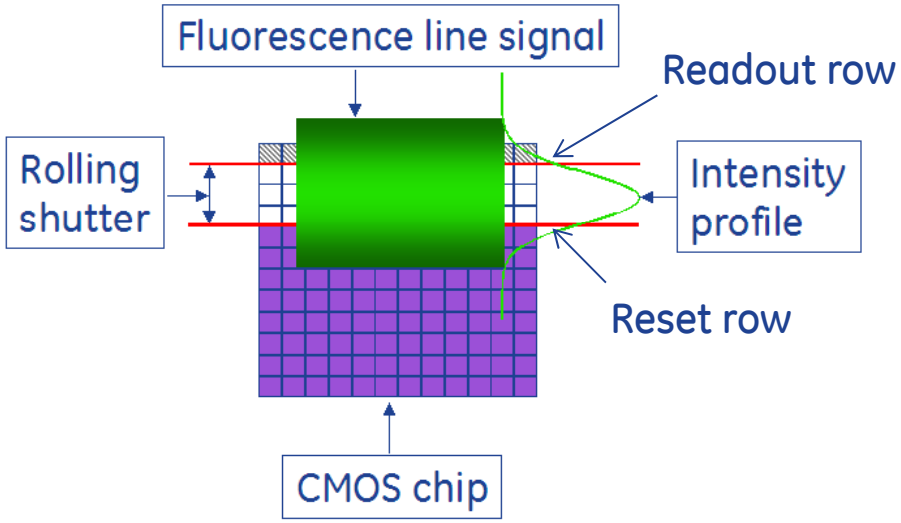
3x3 bin readout
Image file size = 0.9Mb

Increase Sensitivity, Decrease Storage size, Increase Speed, Decrease Resolution

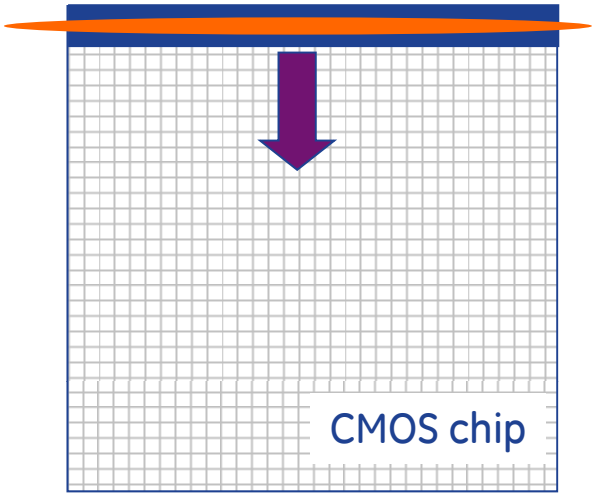
IN Cell Analyzer 6000 calculates the *mean* value for the binned pixels to avoid image saturation while at the same time reducing noise (vs. CCDs, which sum the values)

imagination at work

Rolling shutter – what is it?



Laser line and rolling shutter arrangement



Typical CMOS cameras have two modes of operation: global shutter and rolling shutter.

Global shutter is similar to conventional CCD operation: all pixels of the chip are collecting light during whole exposure time, i.e. the entire chip is exposed at the same time.

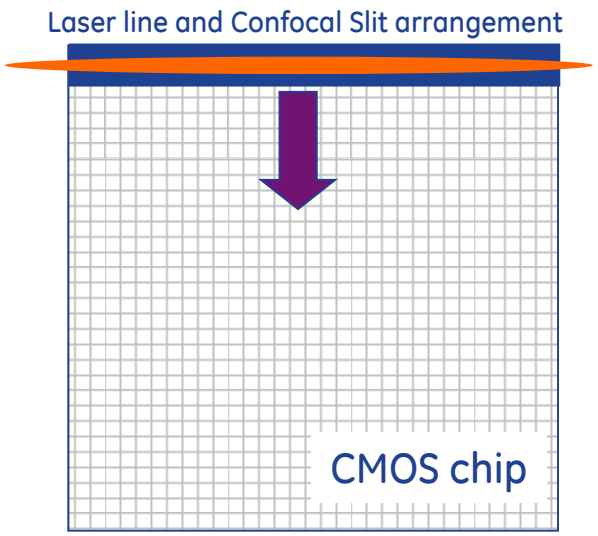
Rolling shutter is what is implemented in the IN Cell Analyzer 6000. It works differently from a global shutter in that the pixels do not collect light at the same time. All pixels in one row of the imager collect light during exactly the same period of time, but the time light collection starts and ends is different for each row. The top row of the imager is the first one to start collecting the light and is the first one to finish collecting.

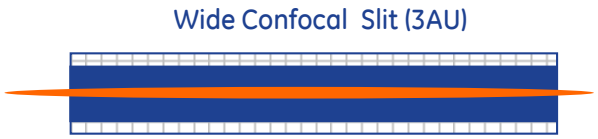
The start and end of the light collection for each following row is slightly delayed. The total light collection time for each row is exactly the same, and the delay between rows is constant. The time delay between a row being reset and a row being read is the integration time.

Summary: The rolling shutter exposes different portions of the CMOS chip at different points in time, "rolling" through the chip from the top to bottom. It's not an actual physical moving shutter. Instead, the sensor is telling different pixel rows to become light-sensitive at different moments in time, and as this process proceeds until the entire chip is exposed.

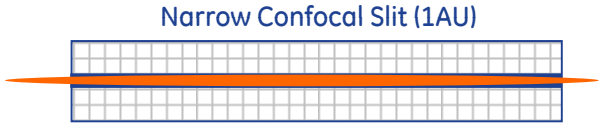
imagination at work

Camera





- Thicker optical slice
- Lower z-resolution
- Brighter images



- Thinner optical slice
- Higher z-resolution
- Dimmer images

Excitation	Emission	Image	Open Aperture	Exposure	Laser Power	Aperture (AU)	Z Slices	Z Step	Z Section	
UV	DAPI	2-D	☑	0.100	100%	3.0	1	0.00	0.00	

Excitation	Emission	Image	Open Aperture	Exposure	Laser Power	Aperture (AU)	Z Slices	Z Step	Z Section	
UV	DAPI	2-D	☐	0.100	100%	1.2	1	0.00	0.00	

imagination at work

IN Cell Analyzer 6000 confocal modes

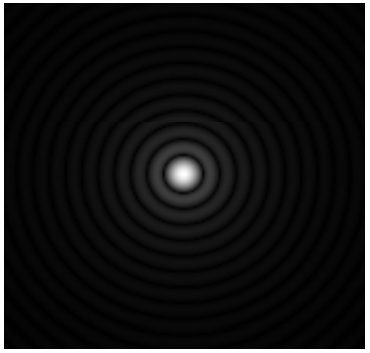
- IN Cell Analyzer 6000 operates in two confocal modes: **Open Aperture** and **Adjustable Confocal**

Confocal Mode	Open Aperture	Adjustable Confocal
Objectives	available for all objectives (2x–60x)	available for higher NA objectives (20x, 40x and 60x)
Adjustment	Confocal aperture is open. No user adjustable parameters.	Confocal aperture can be adjusted in a range from <1AU (most confocal) to 3 AU (least confocal)
Application	**Default mode that works best for most common applications.** **Ease to use:** no user-adjustable parameters **Brightest images:** since confocal aperture is wide open, all the light from the sample is detected by the camera	**Background rejection**: vary confocal setting to find optimum between background rejection and image brightness **3D imaging:** use most confocal setting for better rejection out-of-focus light **Note:** confocal imaging always results in light losses, thus, it should be used only if a strong fluorescent background is present or for 3D imaging.

imagination at work

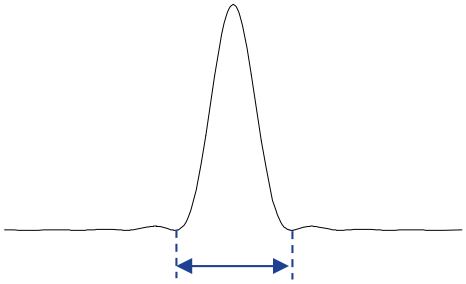
Airy Units: meaning and calculation

Airy disk



Y

X



Airy disk diameter
$\approx 1.22\ \lambda/NA$

- Airy unit is a measurement of confocal aperture width
- Confocal aperture of 1 AU has a width equal to Airy disc diameter or laser line width
- For optimal confocal image we want our aperture to pass entire Airy disk

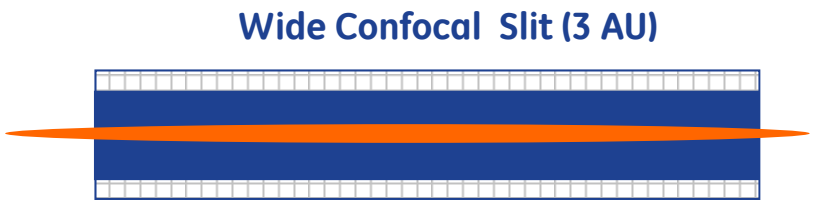
Airy Unit calculation

Example for 20x, NA=0.45 objective and 488 nm laser (note: 488 nm ~0.5 um)

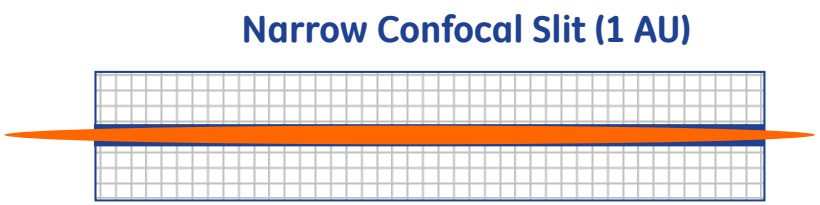
1. **Calculate laser line width using Airy disk diameter equation**
   *Laser line width on sample = 1.22*0.5 / 0.45 = 1.4 um*
2. **Calculate width of laser line on a CMOS detector = laser line width on sample X objective magnification:**
   Laser line width on CMOS = 1.4 20 = 27.1 um*
3. **Calculate 1 AU aperture width = Laser line width on CMOS / pixel width. sCMOS camera has 6.5 um pixel size**
   1 AU aperture width in pixels = 27.1 / 6.5 = 4 pixels

imagination at work

Aperture size effect



- Thicker optical slice
- Lower z-resolution
- Brighter images



- Thinner optical slice
- Higher z-resolution
- Dimmer images

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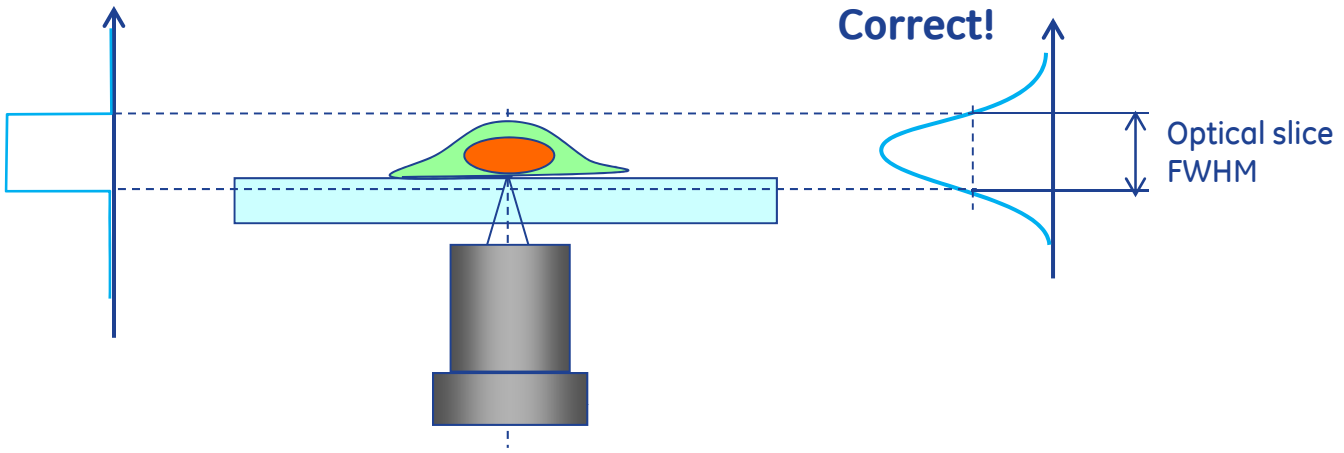
What is “optical slice” thickness?

Wrong!



The optical slice or section does not have sharp edges as in a slice of bread. Z-intensity profile has a Gaussian profile, and is shaped like a bell.

The exact Z-intensity profile depends from many factors like objective NA, optical alignment, confocal aperture size, refractive index of media and sample. So, practical thickness may deviate form theoretical estimation by 30% or more. A good first order approximation is:

$$\text{“Optical slice” thickness (FWHM)} = \sqrt{\left(\frac{0.88\cdot\lambda_{em}}{\left(n-\sqrt{n^2-NA^2}\right)}\right)^2+\left(\frac{\sqrt{2}\cdot n\cdot PH}{NA}\right)^2}$$

The thickness of the confocal sections varies with:
(1) objective (numerical aperture)
(2) size of the confocal aperture
(3) wavelength of the light

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IN Cell Analyzer 6000 confocal resolution

- **IN Cell Analyzer 6000 confocal performance:**
  - Confocal resolution using standard objectives is close to cell thickness (~ 10 um)
  - IN Cell Analyzer 6000 is a High Content Imager optimized for imaging of cell populations.

Practical estimation of IN Cell Analyzer 6000 confocal slice thickness

FWHM = theoretical value + 50%

#	Objective	1 AU	3 AU
1	4x, NA=0.2	Wide field	
2	20x, NA=0.45	9 µm	20 µm
3	40x, NA=0.6	5 µm	11 µm
4	20x, NA=0.75	3 µm	7 µm

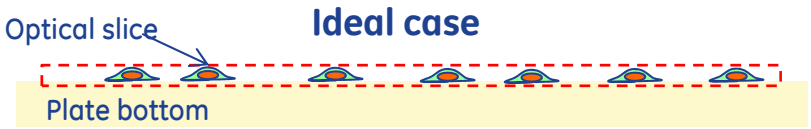
imagination at work

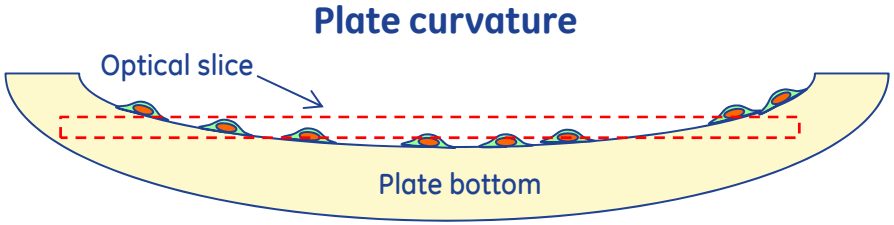
How to optimize confocal setting?

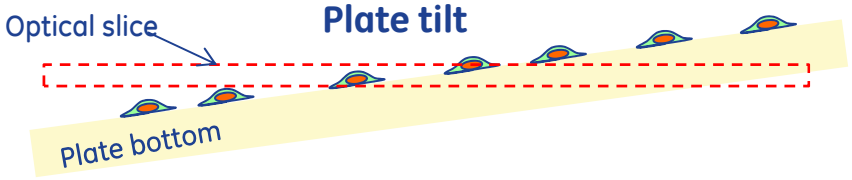
- **Sample preparation is KEY for confocal imaging!**
- **Confocality does not fix light scattering by sample, plate tilt, plate bottom curvature– it only removes out of focus light**
- **Confocal optimization tips:**
  - Always start with wide aperture of 3AU and reduce if needed
  - If aperture is too small, image may be too dim or part of the image may not be seen correctly

Example: For 20x/NA=0.45 objective optical slice thickness for 1 AU is ~ 9um, and field of view size = 650 um

- Sample tilt more than 0.8 degree will bring part of sample out of confocal slice
- Plate curvature more than 9 um over 650 um area will create problems with imaging







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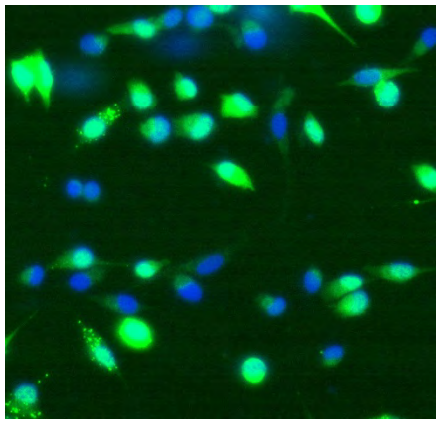
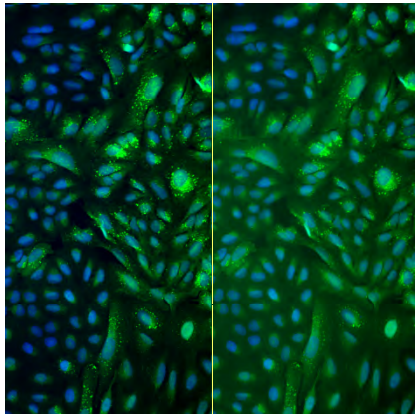
Flexibility to Meet a Range of Imaging Conditions

Dim monolayer cells



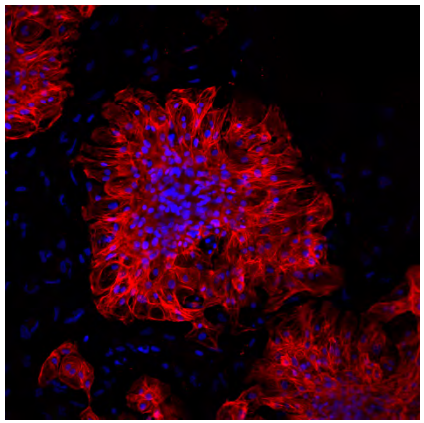
Solution: Non-confocal imaging allows detection of more light

Cells in media with strong background



Solution: Moderate confocality to remove background

Thick 3D samples



Solution: High confocality for precise optical sectioning

Aperture width

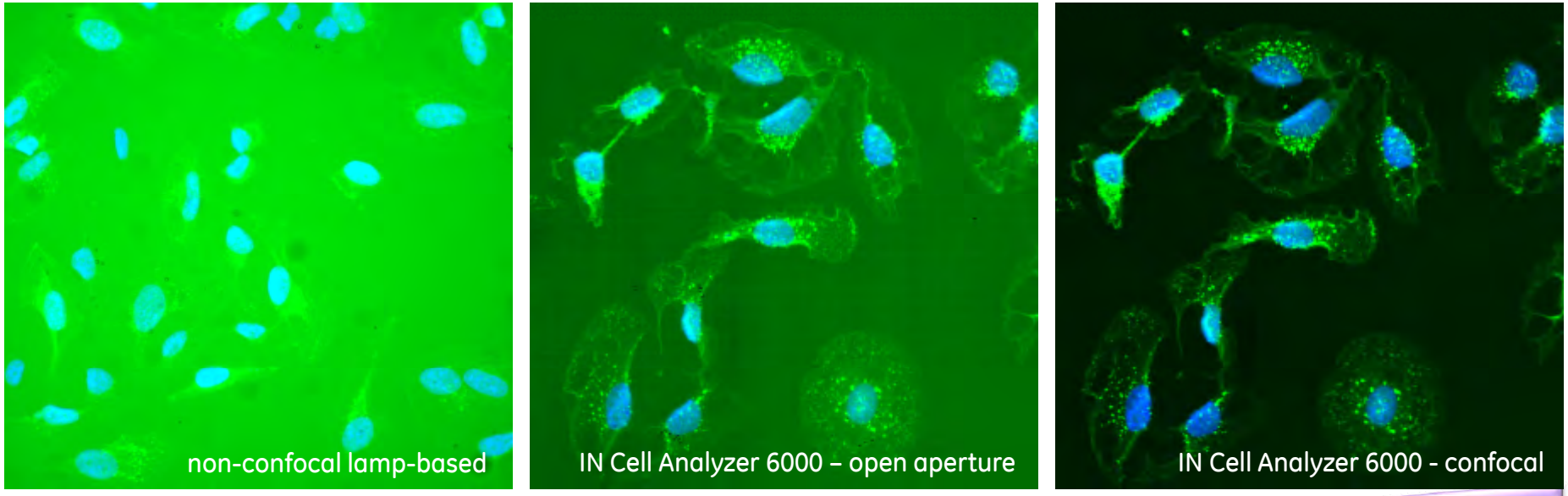
Open Aperture | **Aperture: 3 to 1 AU** | **Aperture: 1.5 to 0.5 AU**

Different applications demand different aperture settings to maximize image quality. IN Cell Analyzer 6000 adjusts in order to maximize signal detection and optimize output.

Fluorescent Background rejection

Confocality removes fluorescence from unbound probe

EGFP-FYVE cells in medium containing 0.6ng fluorescein per well



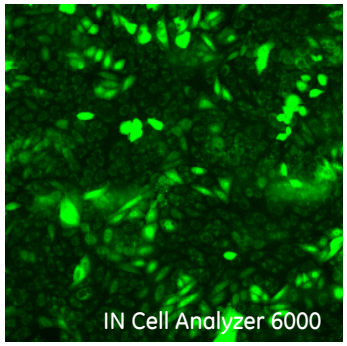
SSMD (assay quality): **2.05** **2.92** **5.41**

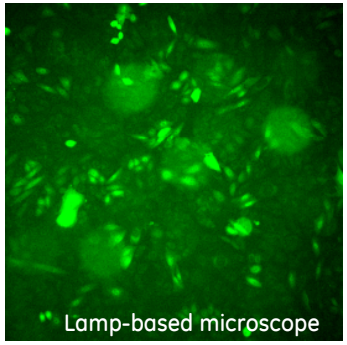
Eliminates wash steps
Improves assay quality
Ideal for high throughput screens

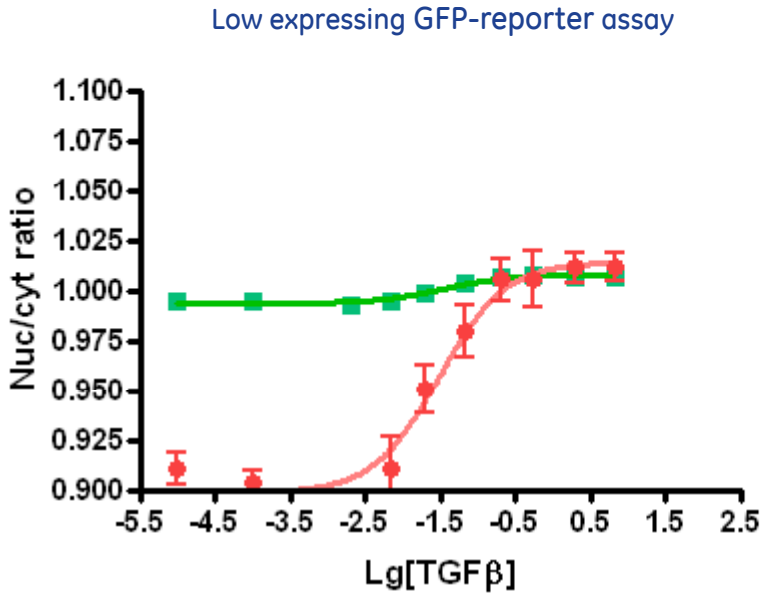
imagination at work

Sensitivity –
imaging a low intensity signal assay

- high power lasers
- high sensitivity low noise camera







lamp-based microscope, 1x1 binning

6000, open aperture, 1x1 binning

1.5-fold increase in SSMD (assay quality) when imaged on IN Cell Analyzer 6000

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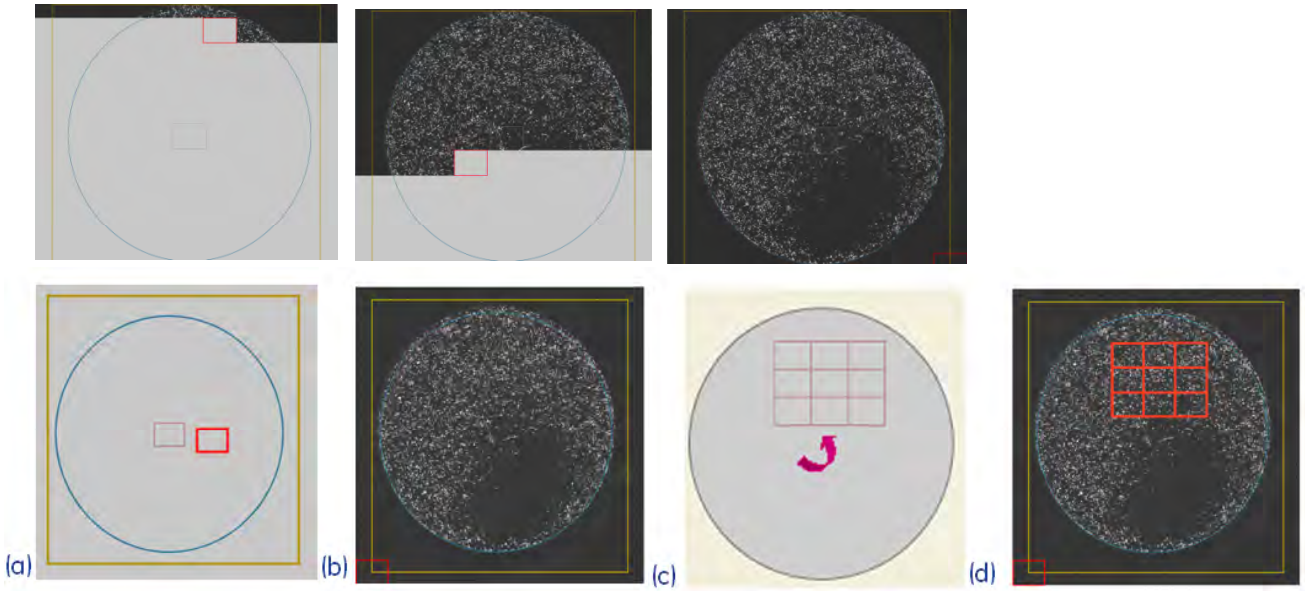
Preview Scanning Feature



Use Preview Scan to decide where to place the FOV

Preview ROI can be as large as an entire plate!

Interactively move the fields to select imaging region

Home-in on desired features – “rare events”

Avoid artefacts – e.g. patches devoid of cells

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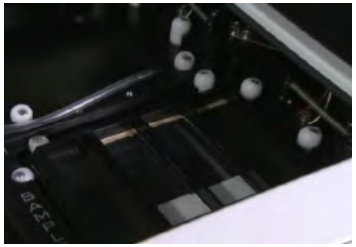
Flexibility - Slide Imaging

Faster and simpler acquisition from slides



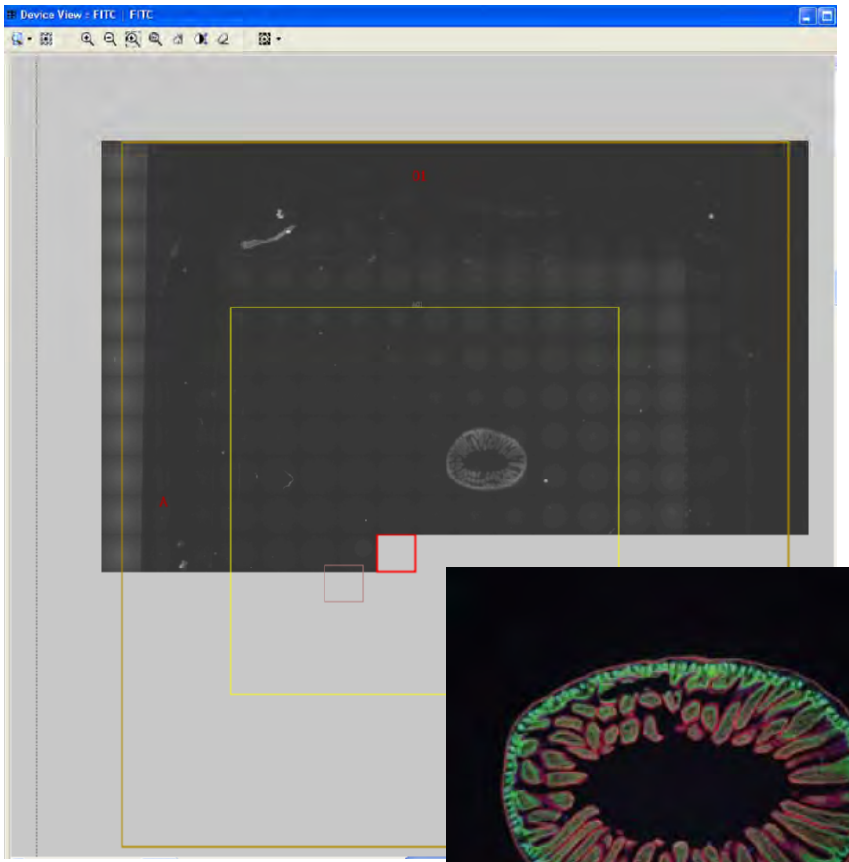
Capacity - 4 slides on the stage

Slide set-up - simple to understand & makes focus strategy easy

Preview Scan - makes finding the ROI easier

Overlapping Images - specify overlap; import into IN Cell Investigator for stitching

Rapid SWAF – fast algorithm significantly shortens run times



Preview Scan a large area of the cover slip

Acquire ROI of interest

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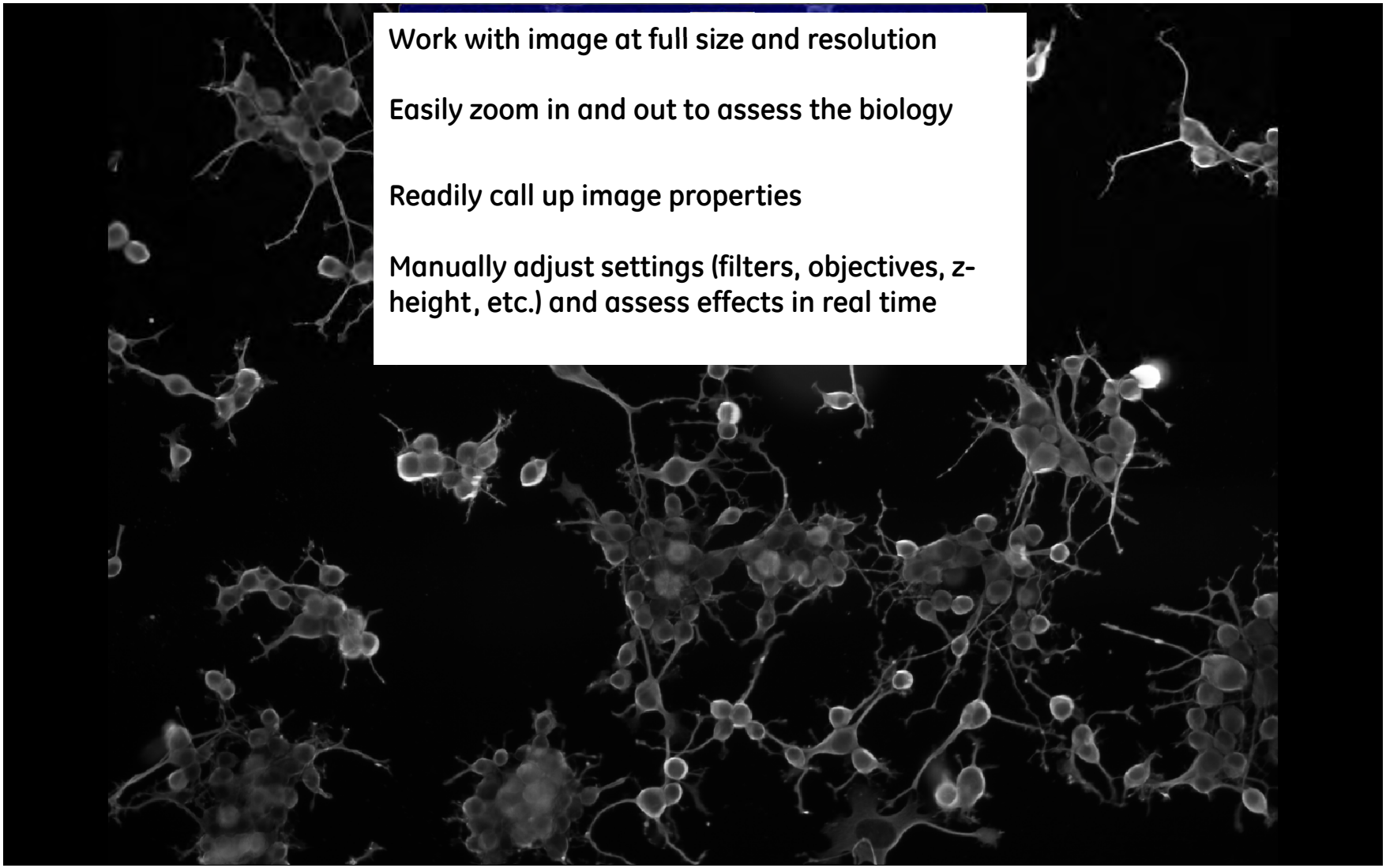
Manual microscopy mode



Work with image at full size and resolution

Easily zoom in and out to assess the biology

Readily call up image properties

Manually adjust settings (filters, objectives, z-height, etc.) and assess effects in real time

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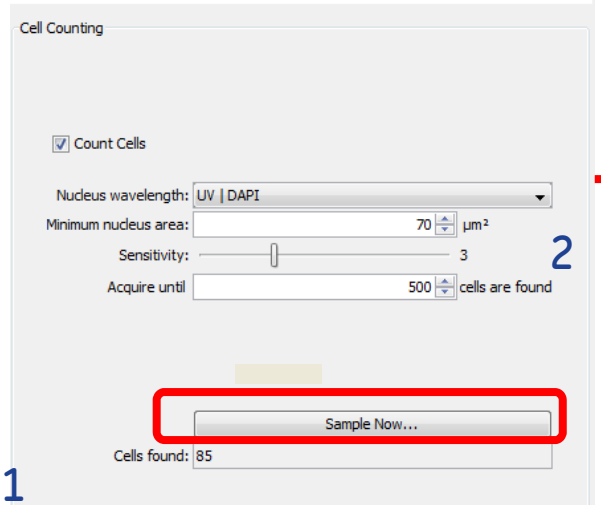
On-line Cell Counting - GUI



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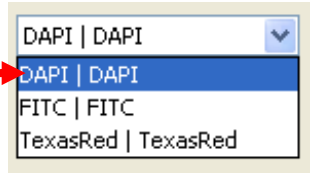

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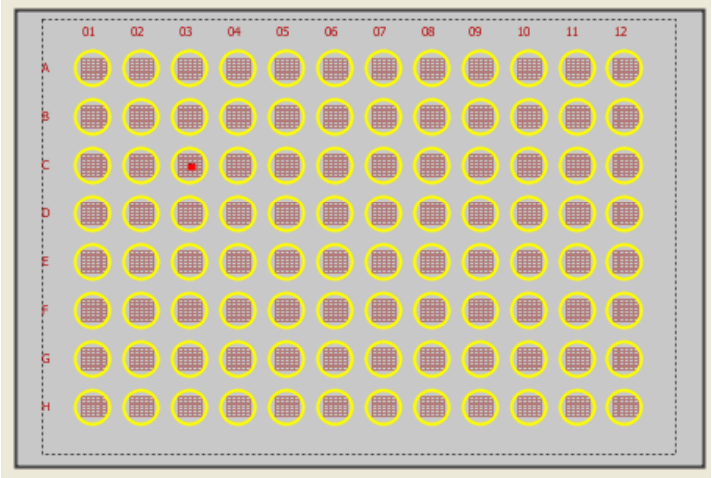

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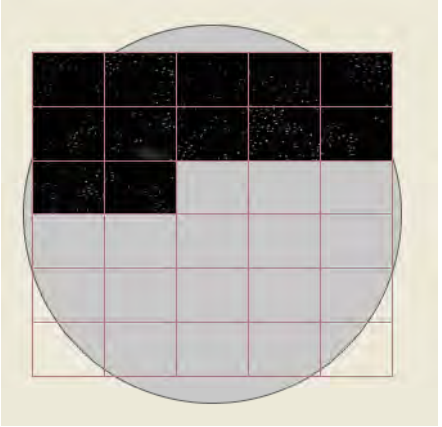

5

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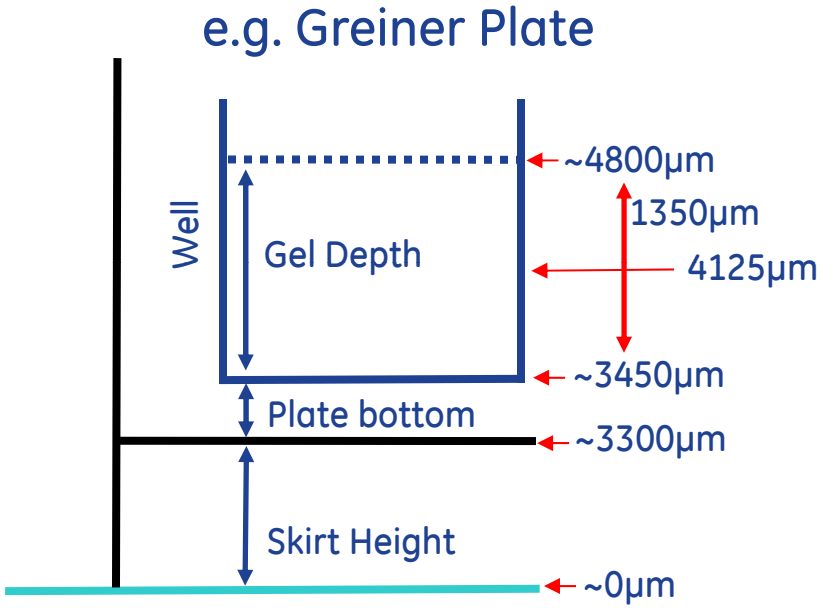
3D Imaging example



3D focus position = central image of stack (focus position can be HWAF + any offset or SWAF initial focus position (+ any offset))

For example we want to image the whole of the gel; bottom = 3450µm (found using HWAF); top = 4800µm (found manually by moving up in Z).

The centre of this stack will be 4125µm and the depth (section) of the gel is 1350µm. We would like step sizes of 50µm, this equates to 27 slices to cover the section (odd number of slices recommended).

To centre the stack around 4125µm you can use SWAF or HWAF;

Using SWAF - set initial focus = 4125µm, static mode = 0 and 27 slices of 50µm

Or

Using HWAF - the laser will automatically take you to the bottom of the gel = 3450µm, then use offset value of half the section thickness= 675µm and 27 slices of 50µm

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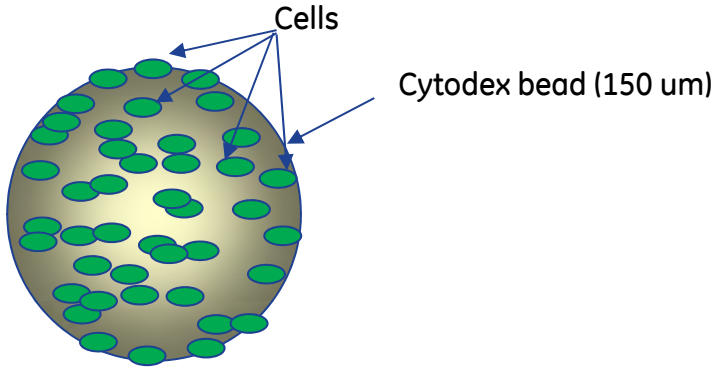
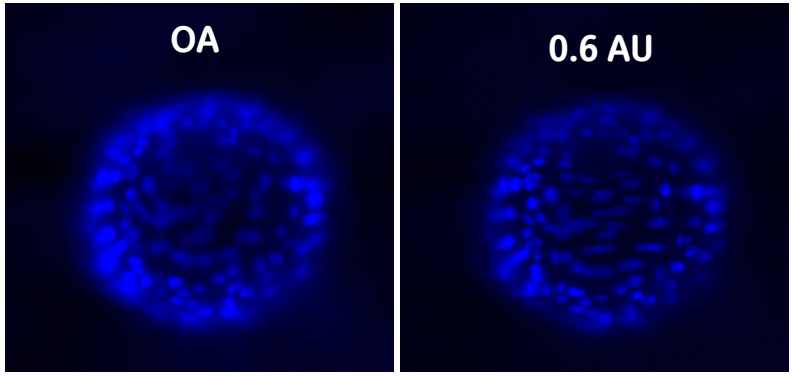
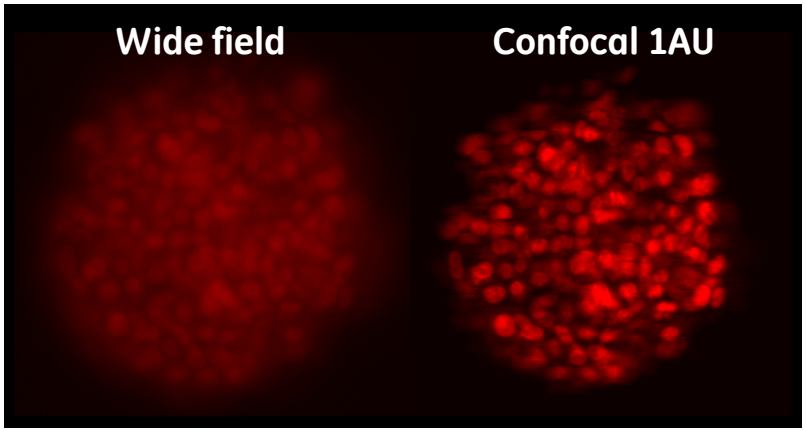
IN Cell Analyzer 6000 – 3D biology

Example 1: Cells on Cytodex beads





Example 2: Cell clump imaging on wide field instrument and IN Cell Analyzer 6000



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The IN Cell Analyzer system and the In Cell Investigator software are sold under use license from Cellomics Inc. under US patent numbers US 5989835, 6365367, 6416959, 6573039, 6620591, 6671624, 6716588, 6727071, 6759206, 6875578, 6902883, 6917884, 6970789, 6986993, 7060445, 7085765, 7117098, 7160687, 706591, 7235373, 7476510, 7853409, 7853411, 8050868, 8062856, 8103457 ; Canadian patent numbers CA 2282658, 2328194, 2362117, 2381344; Australian patent number AU 730100; European patent numbers EP 0983498, 1095277, 1155304, 1203214, 1348124, 1368689; 2044555 Japanese patent numbers JP 3466568, 3576491, 3683591, 4011936, 4693382 and equivalent patents and patent applications in other countries.

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