New User Training Materials

Predominantly iDev Mode

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Agenda

Day 1

- Resources review, system overview, Getting Started, & terminology
- Overview of Workflow- load YOUR training sample plate, select an assay protocol, acquire images, optimize assay protocol for 1st pass, scan plate, review results

Day 2 (ArrayScan VTI only)

- Dive deeper into the Assay Protocol: All input settings and how they're saved, more on optimizing 1st-pass settings for 2nd pass, etc.
- Interactively developing 2nd-pass input settings for a training sample. Execute 2nd-pass scan analysis.

Overall Aim: Load sample plate, develop input settings for imaging & primary analysis according to Best Practices for HCS.

– A sample plate should be analyzed, and at least a preliminary review of results completed.

Resources

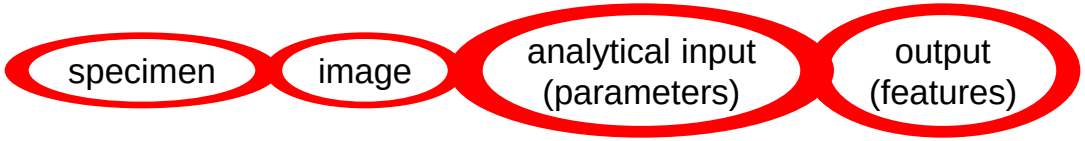
- Online Help and Tooltips
  {update to mention iDev tutorial DVD + Help integrated into iDev, plus vHCS:View & iView Help. What's the iDev equivalent of tooltips for ranges, etc?}
- Users Guides
  A disk containing PDFs for operation of the ArrayScan and BioApplications was included with your shipment. Best used as detailed reference documents.
- Cellomics Technical Support: (800) 432-4091
  or HCSTechnicalSupport@thermofisher.com
  - **Always contact Tech Support FIRST. FAS may be unavailable (e.g., training), it may be a hardware malfunction requiring an engineer, and/or it may be an IT matter in which your FAS has limited expertise. Tech Support will triage as appropriate.**
- To contact Technical Support, please take note of:
  - **Build number of your software: Build _____**
  - **Serial number of your VTi, using the last 3 digits of your instrument's serial # (see tag on instrument): _____**
- Reagent technical support : perbio.eurotech@thermofisher.com
- HCS Reagents Technical Support: 800-874-3723 **is the place to go for answers to Cellomics assay kit questions not found in ClubHCS**
- Your FAS (Field Applications Scientist): _____

  mobile _____ **email** _____
  - **Your FAS knows all Cellomics products, Wants to know your biology & applications, & can recommend certain BioApplications for certain tasks, much more expedient than your learning the details of all BioApplications.**
- Your Key Account Manager
  - **This Sales professional is dedicated to your success and meeting your needs:**

_____ _____

HCS Data “Quality”

- “GIGO”: “garbage in, garbage out”
- HCS assay data’s “legitimacy” depends on the suitability & quality of an assembly of steps.
- “A chain is as strong as its weakest link.”
  - Quality and suitability of…



∴ After good sample preparation is achieved, the 1st step in HCS is getting a suitable image. But this is circular/bidirectional between adjacent links…

specimen ↔ image ↔ image analysis input ↔ output

Typical assay development principles apply:

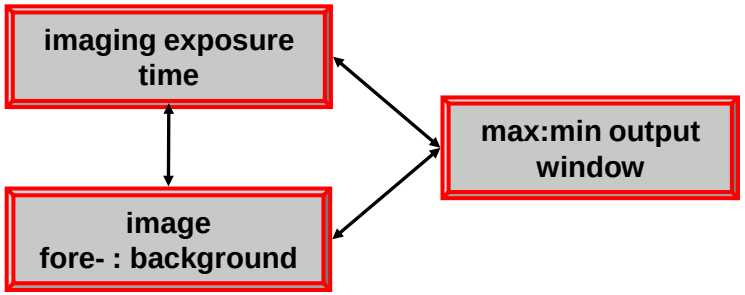
The outputs are not meaningful unless the inputs are.

Certain input/outputs can be *progressively* optimized. For example, Reference Level-based, derived, non-intrinsic features (e.g., %HIGH_, %Responders, %<2N…) can be attended to after “raw”, intrinsic features are solidly based on robust input settings.

- HCS principles don’t differ from scientific assay development generally:
  - maximize/optimize signal:noise ratios
  - maximize/optimize max:min response windows
  - maximize/optimize speed vs. resolution of S:N & max:min
- …these are determined by sample quality, imaging, and analytical input parameters
- images’ foreground:background ↔ output max:min responses
  - images’ foreground:background depends on both…
    - specimen quality, including labeling reagents and support matrix (e.g., plastic vs. glass well bottoms)

    -and-
    - imaging exposure times (eventually, background will rise with foreground, approaching point of “diminishing returns”)
  - output max/min response windows depend on both …
    - image foreground:background (sample quality, exposure times, etc.)

    -and-
    - analytical input parameters



A Brief Cellomics Glossary

- **Object-** the item(s) in an image that you wish to analyze. This can be a cell, a nucleus, a colony, or an organism
- **Object Identification**- the process of determining which pixels in an image are part of an Object or part of the Background. This is done by creating or modifying a Threshold.
- **Threshold-** the intensity value that delineates object pixels from background pixels. Maybe specified directly or indirectly.
- **Field-** from microscopy's "field-of-view'- the area of a Well visible by the camera
- **Channel-** a collection of image acquisition settings for a single image; including exposure time, Dye selection (wavelength filter configuration)
- **Image Set**- one or more images acquired from a single field, comprising one image per channel
- **Primary Object**- generally the objects in the first Channel
- **Sub-objects**- an informal term for objects that belong to a primary object- such as neurites or spots assigned to a single cell.
- **Object Segmentation**- the process of separating objects that are very close to one another, for the purposes of analyzing them individually
- **Parameter-** generally, an input that you make to the software
- **Feature-** an output data point based on the image and data analysis process
  - **Cell Features**- numeric data points describing the measured properties of a single Object
  - **Well Features**- numeric data points describing the measured properties of a Well of cells/objects- most often, statistical calculations (Mean, SD, % responders, etc.)

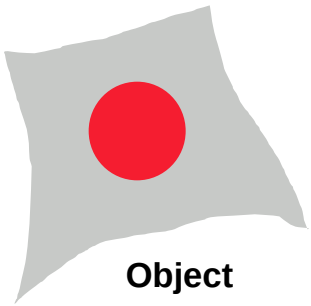
Data Hierarchy and Tiers

Many terms in HCS have historical roots that are blends of photographic imaging and cell-based screening. Often the vocabulary works well, but is not a restriction on capabilities.

- **Plate level features**- E.g., exposure times, z-offsets, Reference Levels. This "plate" structure is rooted in cell-based assays in multi-well microtitre plates. In HCS, it can mean a microscope slide, too.
- **Well level features-** E.g., Z-position, CellCount, MEAN_ObjectAvgIntenCh2, SD_ObjectTotalInten, %HIGH_RingSpotCountCh2. A well is merely a subdivision of a plate. It constitutes a container for materials delivered to it. It can also be a subdivision of a micro-slide.
- **Fields**- A term borrowed from microscopy's "field of view". When a Field size is smaller than the image-able surface of a well, multiple fields may be imaged per well. Some BioApplications report Field features. All report Cell Features linked to Wells+Fields.
- **Cell level features-** E.g., ObjectSizeCh1, RingSpotCountStatusCh2. Historically, it was cells that were plated into wells of a plate. In HCS, the fundamental unit of analysis (primary Object) is most often a Cell, and multiple features of it are measured. Some of these features may be sub-objects (e.g., spots, micronuclei, perinuclear rings, neurites), though not necessarily. So, Cell Features are a class in this hierarchy, but Cell Features may correspond to properties of colonies or whole organisms (e.g., C. elegans).
- **"Intrinsic/raw" Cell Features-** e.g., RingSpotCountCh2 is a mere count of how many spots have been ascribed to an object based on sub-objects (spots) identified by Ch2's label within a sub-compartment (ring) of an object.
- **"Simple Statistical" Well Features based on intrinsic/raw Cell Features**- e.g., MEAN_TargetAvgIntenCh2 of a well's population of objects is simply computed from the values for every selected object's corresponding cell feature TargetAvgIntenCh2. That mean has a SD_TargetAvgIntenCh2 about it, too. ObjectCount is another example; it's the sample size, n, on which the mean, stdDev, etc., are based.
- **Status Cell Features**- e.g., RingSpotCountStatusCh2. In some BioApplications, an object can be assigned to one of some Cell Feature phenotypes. For example, if an object's TargetIntenCh2 exceeds the corresponding Reference Level, it's classified by TargetIntenStatusCh2=1; else =0.
- **Population Characterization Well Features based on Status Cell Features**- Rather than a metric of a mean response, these report the fraction of a well's sampled population that fall in a phenotypic class. E.g., %HIGH_TargetAvgIntenCh2 reports what percentage of the well's sampled population exceeded a minimum Response Level. It's simply computed by tabulating the corresponding Status frequencies.
- The meaning & legitimacy of intrinsic/raw features depends on the quality of input settings that generate them. The same is true of Population Characterization features. the %HIGH value is not meaningful if relevant Reference Level inputs were not used.

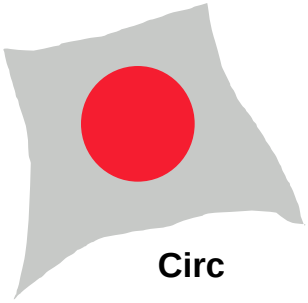
Anatomy of a Generic Cell

Ch 1



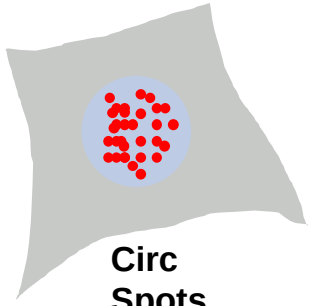
Object

Downstream Channels

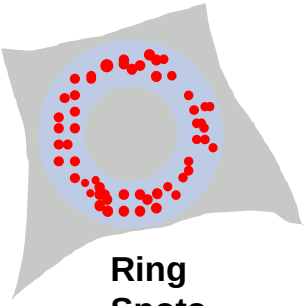


Circ

Ring



Circ Spots

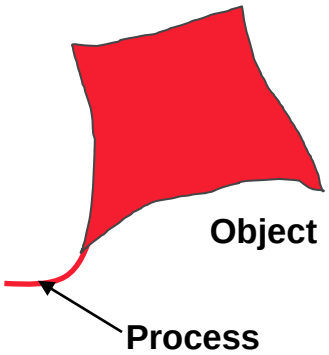


Ring Spots

Primary Object = Nucleus

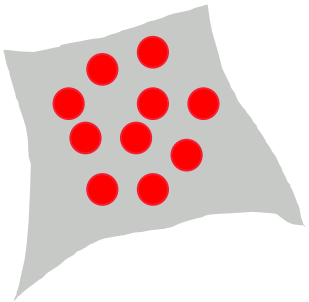
Special Design for Morphology Explorer

Ch 1



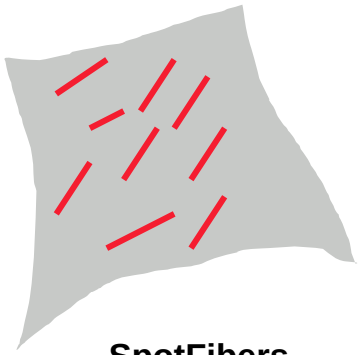
Primary Object = Whole Cell

Ch 2

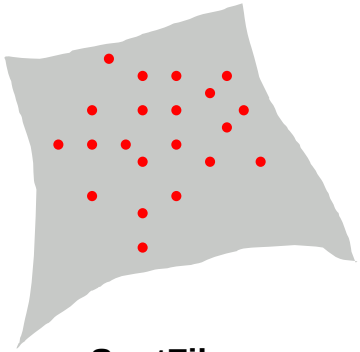


Members

Ch 3 or 4



SpotFibers (high LWR)

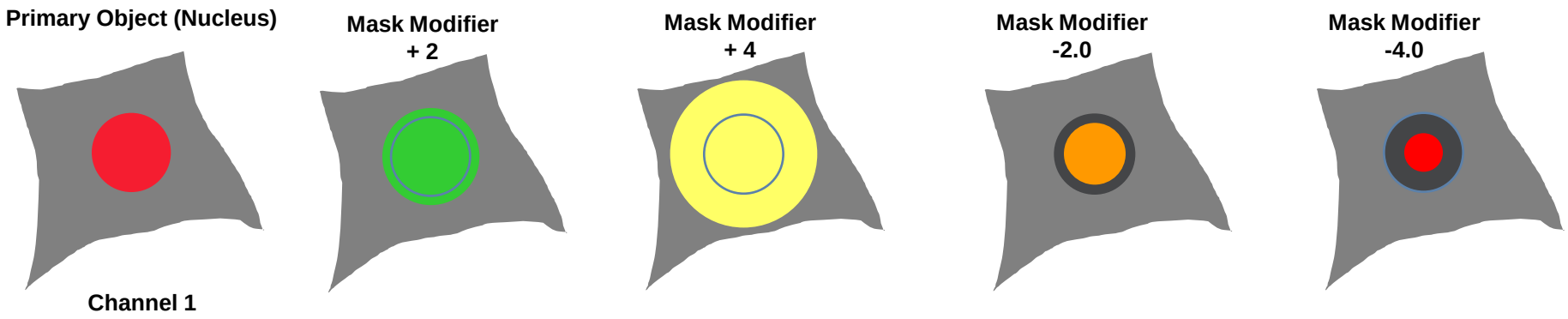


SpotFibers (low LWR)

Masks and Modifications

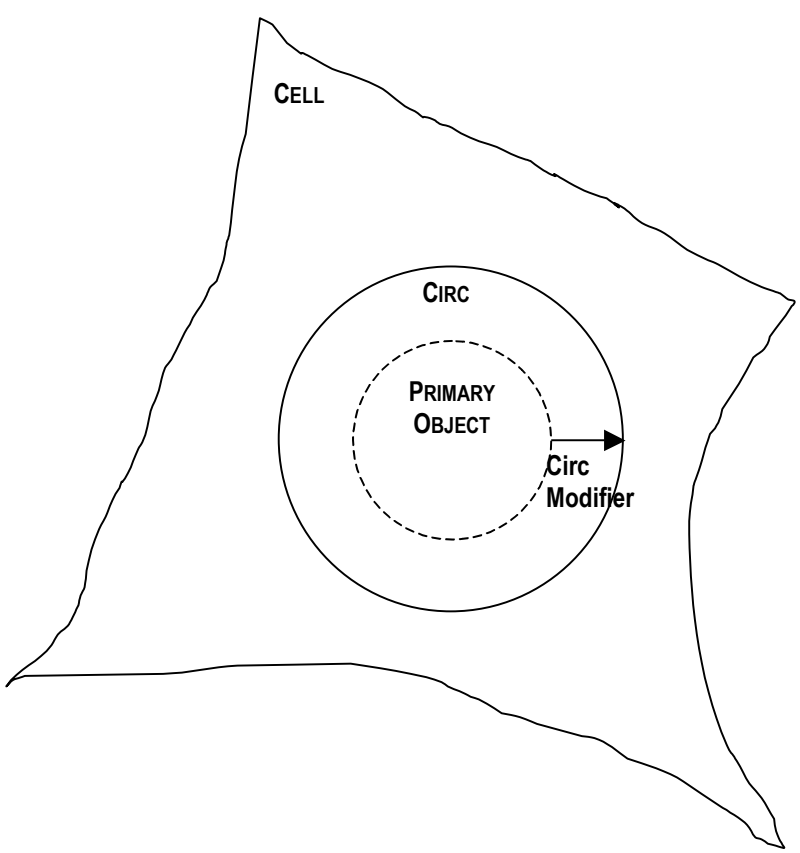
- Mask: the graphic representation of an object that is applied to separate channel in order to make a measurement in that channel
- Mask Modification: the enlargement or shrinking of the original mask
  - Positive values for the Mask Modifier parameter enlarge the mask (dilation)
  - Negative values for the Mask Modifier parameter reduce the size of the mask (erosion)
- Three major types of Mask Modifications:
  - Primary object mask (MaskModifier)
  - Circ Mask (CircModifier)
  - Ring Mask (RingDistance and RingWidth)
- The value of the Modifier is the number of pixels to dilate (positive) or erode (negative) the mask

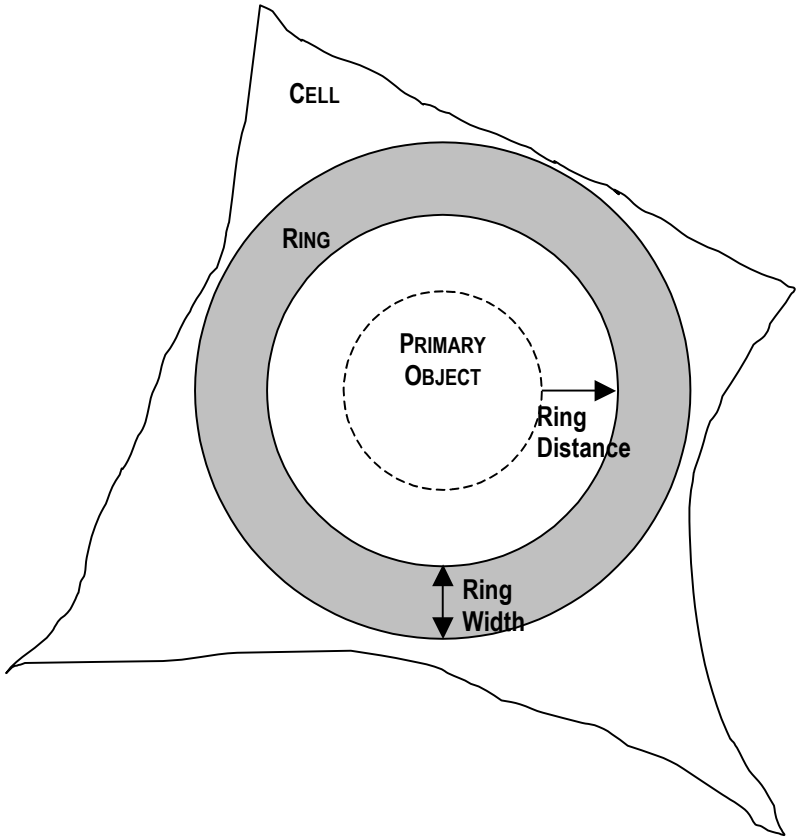
- Adjustment of Mask and Circ Mask looks like this:



Adjustment of Circ & Ring Regions

- Ring Mask is described by 2 parameters: Ring Distance and Ring Width
- Adjustment is always relative to the Primary Object Mask





Getting Started

1. Power on instrument.
   - ArrayScan VTI: Single green switch on right side or front of instrument. (For VTI Live cell chamber module, see Appendix.)
   - Cell Insight:
2. Power on computer.
   - Order of 1 & 2 does not matter. But…
   - Do NOT power off instrument (or components) while Scan software is open.
   - If do, close software & re-boot computer.
3. Log on to Windows on the computer:
   - May not be able to use same login as at your desk PC.
   - May need to be part of a Cellomics domain group.
   - Only YOUR network administrator/IT support staff can help you with this. That person is:

     _____

     THIS LOGIN IS VERY IMPORTANT for full functionality.
   - ***Never let IT staff change name of computer without consulting Cellomics***. Some functions (e.g., licenses) could be lost & require full re-installation.

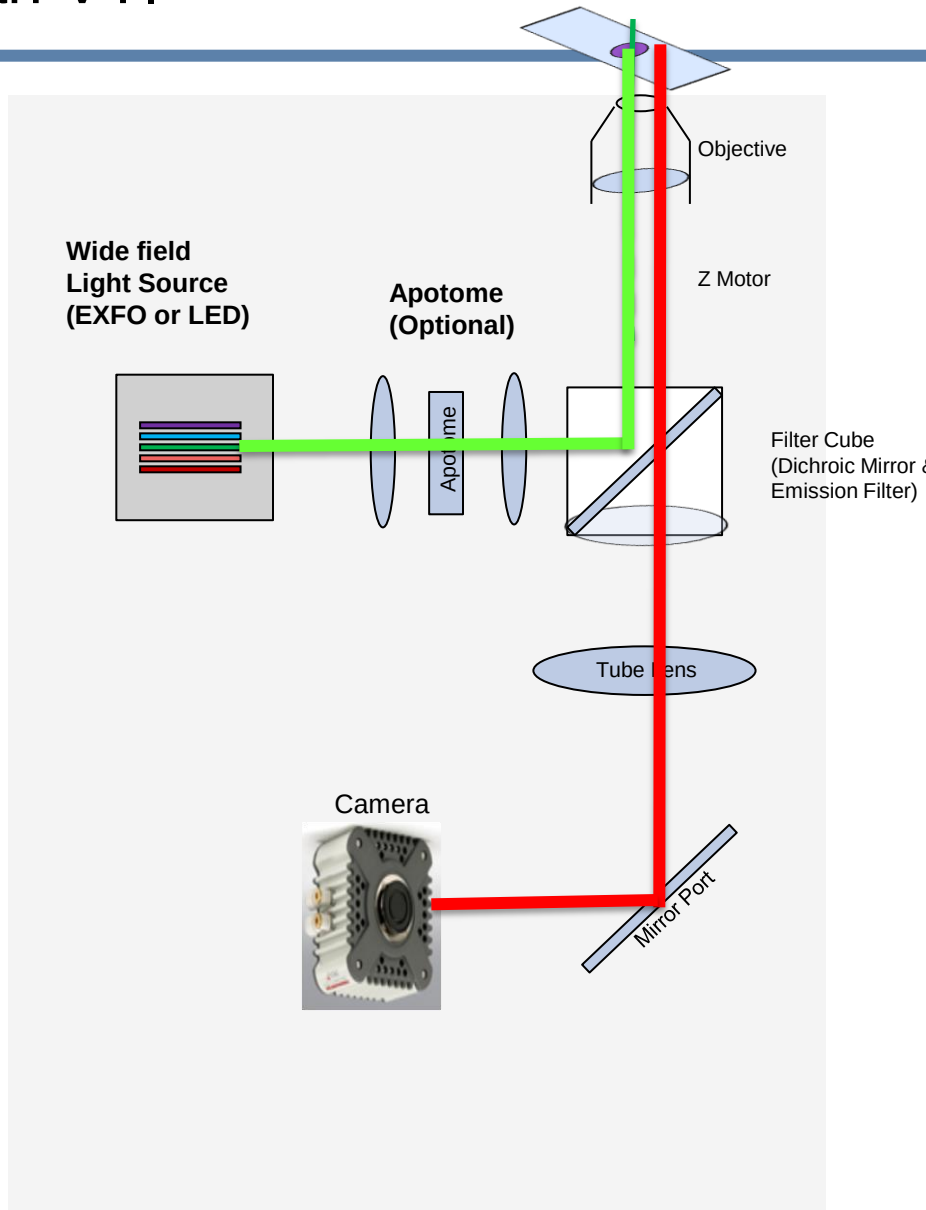
4. Launch Cellomics software.
   a) **Instrument**: Shortcut like "Cellomics ArrayScan Instrument" or "Scan"
      To start software in **Disk Mode**, have instrument powered off. (or add –canned to shortcut Target)
   b) **vHCS:View**

- Either (a) or (b) will prompt you for a **Cellomics login**.
  - **NOT same as Windows login**.
  - Default is "cell/cell". Yours?: _____
  - Your system admin may have/should set up each user with their own Cellomics login (see ArrayScan/Scan Administrator tab in the ArrayScan/Scan Operator's Guide) to personalize your files, results, & activities on the Cellomics system.
  - NOT a secure (as Windows or your network's) login, so keep simple, and know that others can see it.

5. **Suspect problem?** Use **HCS Administrator** software.
   - If some Cellomics software behavior is suspect, launch your HCS Administrator> Diagnostics> Run Diagnostics.
   - All Results☑? You're OK. Otherwise, may need your IT staff's support. Email Report, or drag down row buttons to select all, then Ctrl+C to copy (then paste and email, etc.). Or PrntScrn keyboard button (then paste, etc.)
   - Diagnostic results also useful for learning AppSvr name, acct name, network bandwidth, server path & free space, database name & location…

How it works on the ArrayScan VTI

Prior to the Confocal Module, normal widefield mode uses an epifluorescence light path



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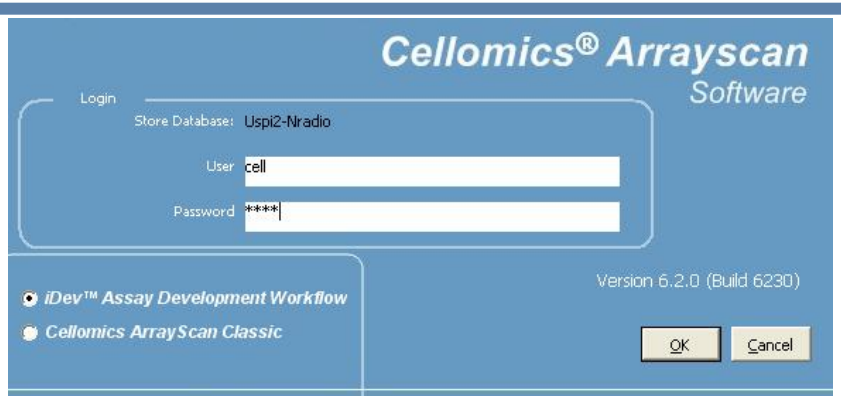
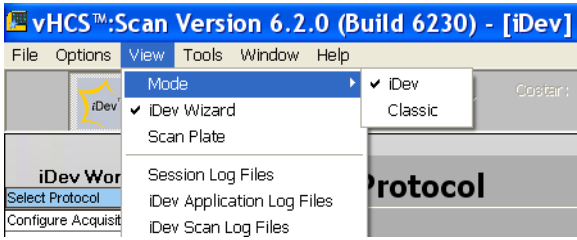
Scan Login Screen – iDev vs Classic

Choose iDev or Classic during login.

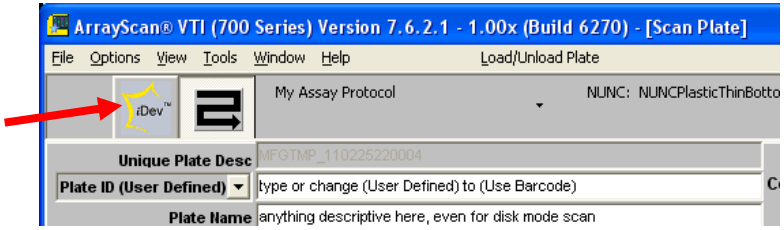
This booklet is iDev-focused, so choose iDev.



After login, to change modes, use **View> Mode>**



After login, you start in the Scan Plate window, which presumes you are an experienced user – an inappropriate place to begin training. Click the iDev button shown at right to go to the Select Protocol window of iDev Workflow.



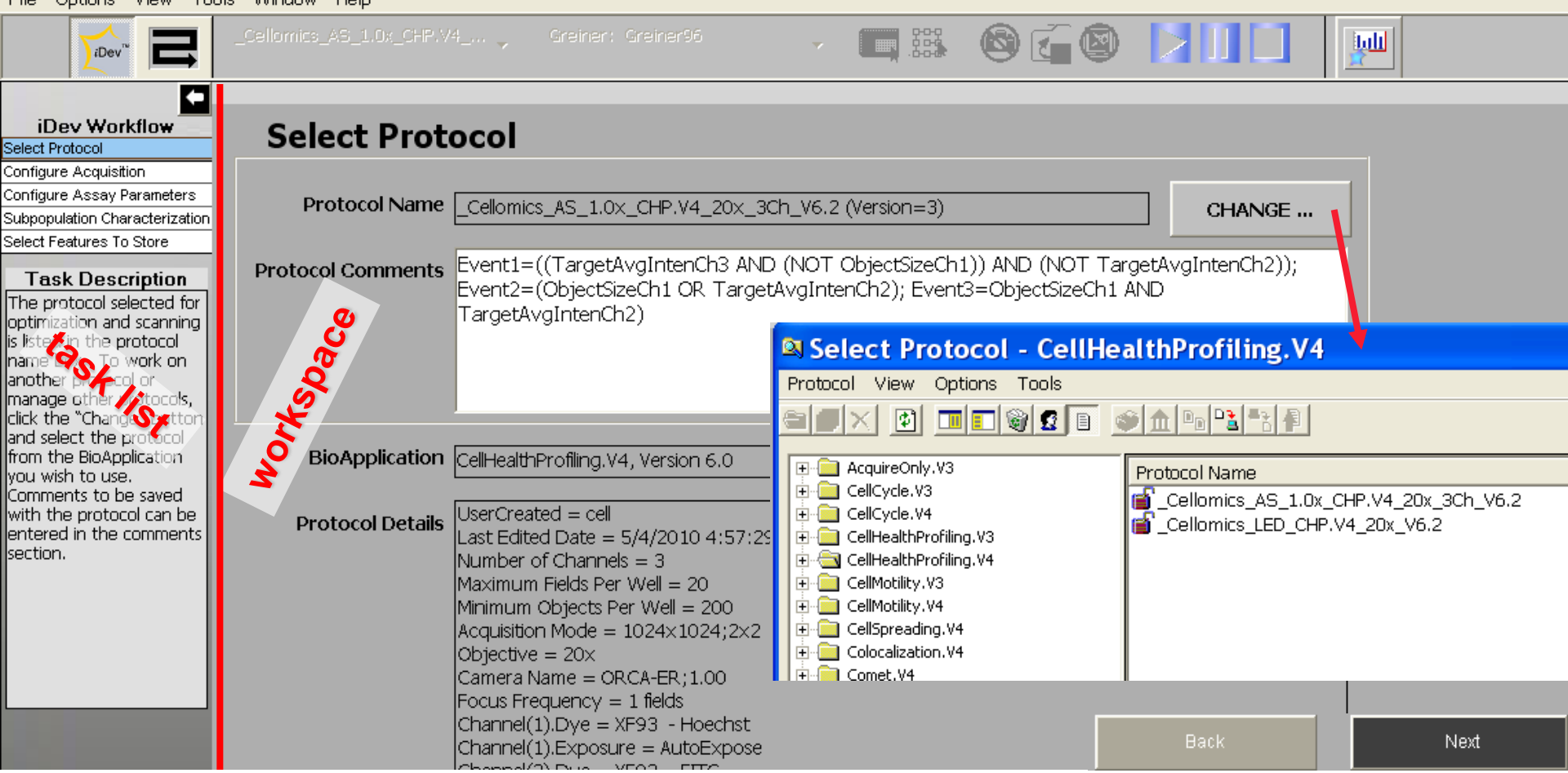
"Assay Protocol"

What's saved as an Assay Protocol?

- Objective (magnification)
- Imaging resolution
- What color fluorophores to image
- Exposure (CCD integration) times
- Autofocusing settings
- How many images taken per well
- BioApplication image processing algorithm settings

What's NOT saved in an Assay Protocol?

- Plate Type Form Factor
- Which wells of a plate to analyze
- Which field of a well to start in
- Whether to save images
- Maximal image displays during scan
- Reference Well selections

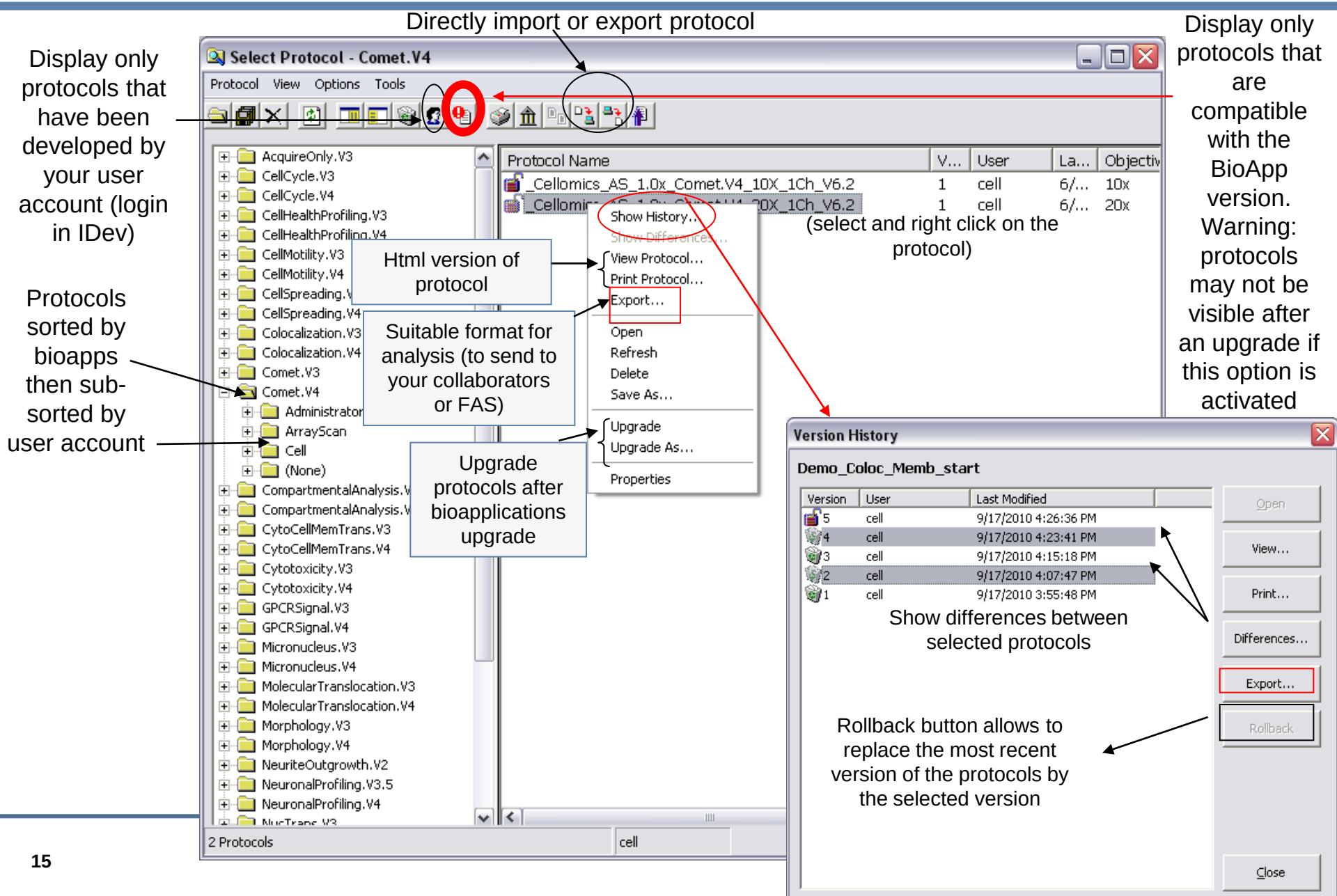


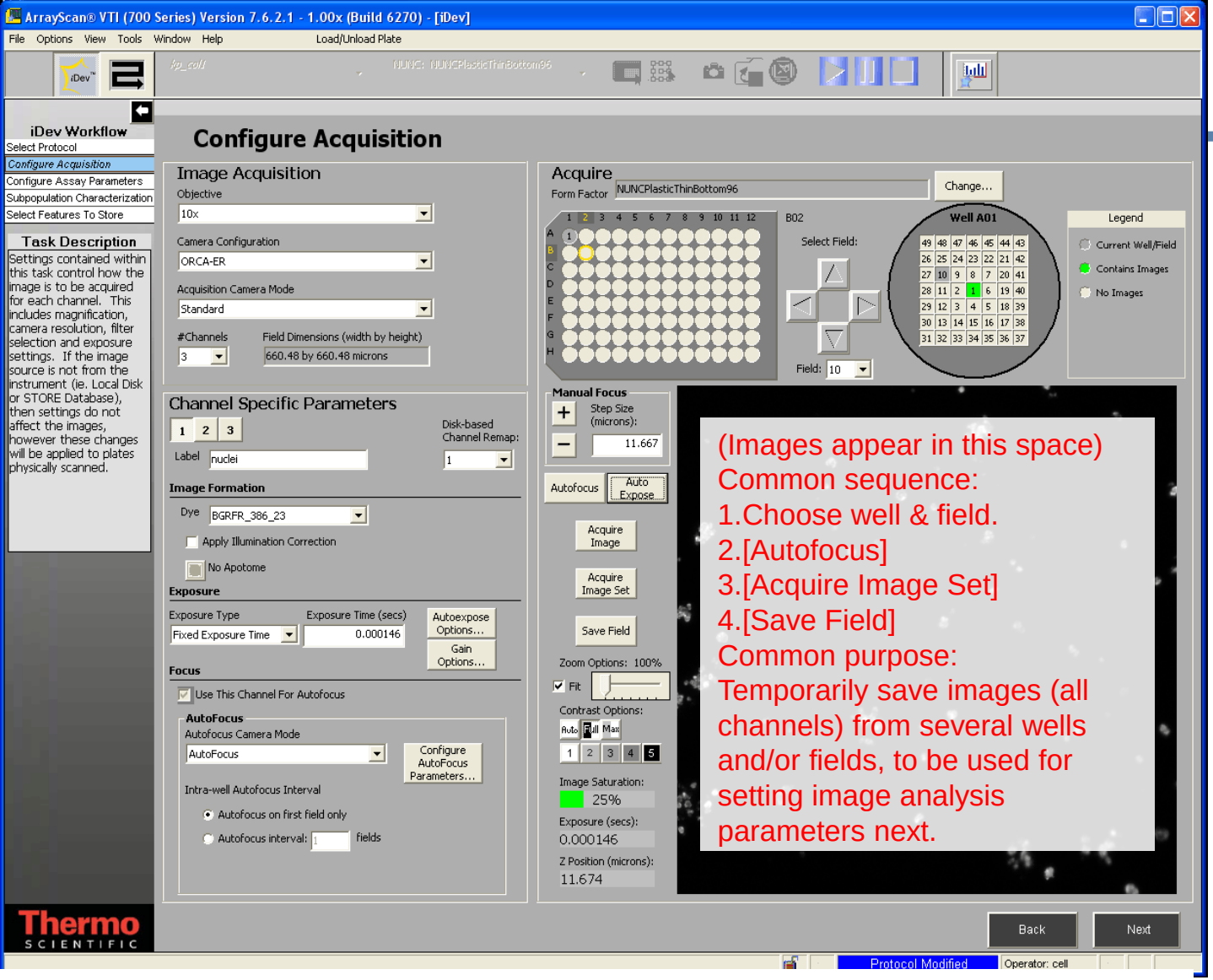
Software divided into two areas: **iDev Workflow** **task list** (& Task Description), and the selected task's **workspace** (specific to each task).

Primary tasks in the iDev Workflow list can be selected either by clicking a line in the list, or the [**Next**] button at the lower right.

Only ≥V4 protocols are compatible with iDev. The Select Protocol dialog (a.k.a., Protocol Manager; shown above, after clicking [**Change…**]) can be used to upgrade from prior versions (select a protocol, right-click, Upgrade…).

Protocol Manager





Details about settings and controls in this window are presented in subsequent sections of this guide. Although the images saved by **[Save Field]** are only stored temporarily, all settings in **Image Acquisition** and **Channel Specific Parameters** panes are saved in the Assay Protocol. (Nothing in the Acquire pane is saved 'permanently'.)

Note titles of 3 top-level divisions in this window. Details about settings in the **Image Acquisition** and **Channel Specific Parameters** panes are presented later.

(A) In the **Acquire** pane, verify or [**Change…**] the **Form Factor**.

(B) To load sample, click **[Load Plate]** on highest menu bar. **[Retract Plate]** after placed.

(C) Set/verify exposure parameters (detailed later).

(D) Acquire Image Sets & Save Fields as described at left.

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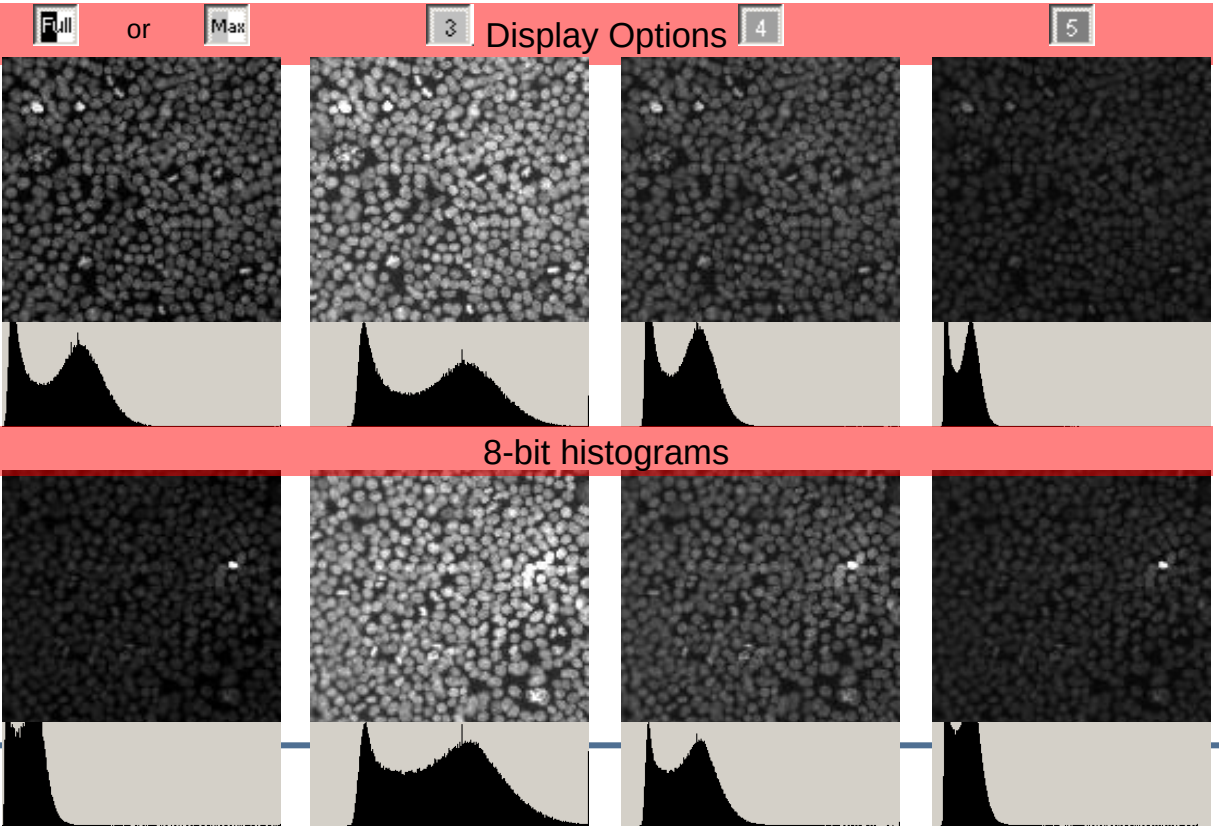
Contrast Options – common illusory image comparisons!

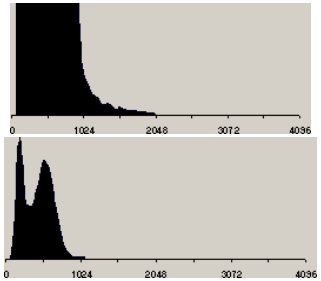
- There are only 2 images on this page – top & bottom row. Across each row are different display adjustments of the respective image. Top row's field was directly adjacent to the field in the bottom row!
- In 1st column – Full or Max – do you think the bottom field is dimmer? Do the 12-bit histograms suggest that?
- The 1st column illustrates the common illusion of Contrast-Stretching—an auto-adjustment of brightness/contrast done on an image-by-image basis. Contrast-stretched images canNOT be legitimately compared for visual intensity differences!

(In Brightness Clip controls elsewhere, Full (the default) = "Automatic High & Low Input Clip", Max = "Automatic High Input Clip".)

- **Beware: Full Contrast Stretch is the default display adjustment!**
- **For image-to-image intensity comparisons, Contrast-Stretching should be deactivated, and Bits-To-Display selected.**
- **Histograms under images are 8 bits—256 gray levels—the maximum # of grays display-able on a monitor. Bitshift 3 (i.e., bits 3-10, or grays 8-1024 from the 12-bit source image) illustrates the best spread of the gray levels across the 8 display bits. For the top field, Bitshift 4 resembles the Contrast-Stretched image, whereas for the bottom field, it's Bitshift 5.**

(In Brightness Clip controls elsewhere, Input Clip Mode = Manual is used instead of Bits-To-Display bitshifts.)

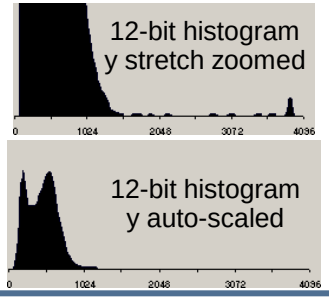




12-bit histogram y stretch zoomed to see low frequency pixels

12-bit histogram y auto-scaled

- In the bottom field, the bright spot shows up on the 12-bit y-stretch histogram. When auto-re-mapped to 8 bits, those high values prevent expansion of the display histogram.



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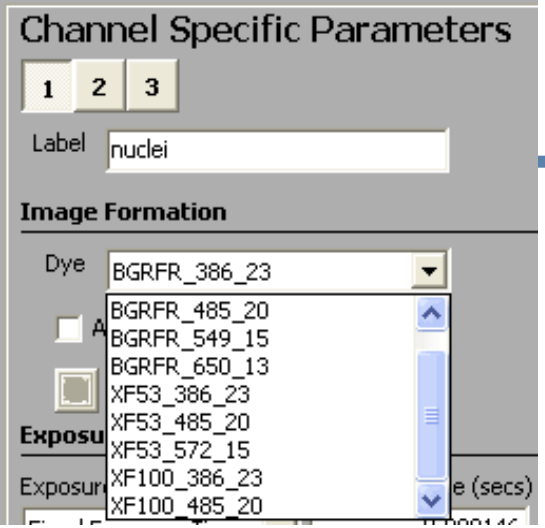


Channel Specific Parameters- “Dye” (filter)

Label: Merely ‘free-form’ text to serve as image captions in later windows.

Illumination Correction: Advanced topic. Regular use unnecessary.

- Consult Appendix B of the *User’s Guide* tab within your *Operator’s Guide* binder, which explains why **scans are faster if all fluors in a sample can be imaged by the same XF or BGRFR set.**
- All else being equal, **Cy5** channels are usually the weakest (because of both excitation and detection optics). Therefore, **use a Cy5-like dye only if a 4th color is needed**, and use to label/ report the most abundant target, etc.
- Other dyes/fluorophores are certainly possible; consult Cellomics Support or online filter sites.

Dye/ Fluorophore Color Classes

Hoechst (_386_23)	FITC (_485_20)	TRITC (_549_15)	Texas Red (_572_15)	Cy5 (_650_13)
DAPI	fluorescein	rhodamine		DRAQ5
Whole Cell Stain Blue	GFP, EGFP Alexa Fluor® 488 Whole Cell Stain Green	Whole Cell Stain Orange Alexa Fluor 546 Alexa Fluor 555	Alexa Fluor 594 ~Alexa Fluor 568	Whole Cell Stain Red
	YOYO®-1 YO-PRO®-1 SYTOX® Green	DyLight™ 549 ~BOBO™-3 Cy3™	~YO-PRO™-3 ~SYTO®17	Alexa Fluor 647 ~TOTO®-3
Coumarins	Fluo-4 Calcein BODIPY®-FL	DiI (DiIC18(3)) BODIPY-TMR		DiD (DiIC18(5))

	XF100	BGRFR XF93	XF53	XF32	XF110
Hoechst (_386_23)	good	good	good		
FITC (_485_20)	good	good	good		
TRITC (_549_15)		good		sensitive	
Texas Red (_572_15)			good		
Cy5 (_650_13)		good			sensitive

Exposure Type: Fixed Exposure Time vs AutoExpose

- **Fixed Exposure Time**: Specified time for a channel is used throughout imaging and scans. This is the recommended method.
- **AutoExpose**: still must specify an Initial Exposure Time, which will be used as a starting point to automatically determine a time that meets your AutoExpose Options. This method is not recommended until you completely understand this process (see below).

- Key image analysis steps are tightly dependent on image characteristics that are, in turn, largely determined by exposure times.
  - Matching exposure times to existing input analysis parameters is often more productive than adjusting the more numerous analysis parameters.
  - Instrument components change over time, so AutoExpose Options that can compensate will prove useful, even when the preferred Fixed method is selected and used during plate scanning.
  - ArrayScan/vHCS:Scan *Classic* only (not iDev): Even when stored images are re-loaded, their % of dynamic range will be reported by the Autoexpose routine.

"Target" guidelines for what % of dynamic range ("Peak Target"; see next page) may be suitable for types of fluorescence labels.

25%: Good for mainly **"structural"** labels (e.g., counter-stained **nuclei**). Still affords some range for differentiation between, e.g., interphase, mitotic/apototic nuclei. ~20% can be tried for minimizing autofocus time & maximizing speed. Typically also sufficient for "binary" assays where the interest is whether the label is there or not, yes/no—e.g., mitotic index, negative viability ("dead dyes"), micronuclei, and others.

35-40%: For "**intensity**" quantifications. Also reasonable starting range for "structural+intensity" labeling—e.g., nuclei in assays needing more discrimination (e.g., DNA content/cell cycle).

55-65%: Good for multi-modal structural labels where accurate identification of more than one intensity level is of interest—e.g., some neuronal cell bodies are considerably brighter than neurites that also must be accurately identified. High dynamic range may also be required for some indicators of cellular metabolism, physiology, health, etc.

Best Practice: When trying higher %, be on guard for saturation. Optimal exposure ranges are worth careful investigation. Large improvements often emerge particularly in "intensity" assays. If your BioApplication has multiple channels measuring the same thing, fill them all with variable exposure times during assay development pilots.

Recommended default practice for setting exposure times

1. In your Autofocus channel, set…
2. Exposure Type = Fixed Exposure Time
3. Exposure Time = 0.1 sec (in absence of better knowledge)
4. [AutoExpose Options…]
   a. List of wells to Autoexpose = a1
   b. List of fields to Autoexpose = 1

   (The above 2 settings are irrelevant for Fixed Exposure Type, but the software requires an entry to access the remaining settings.)

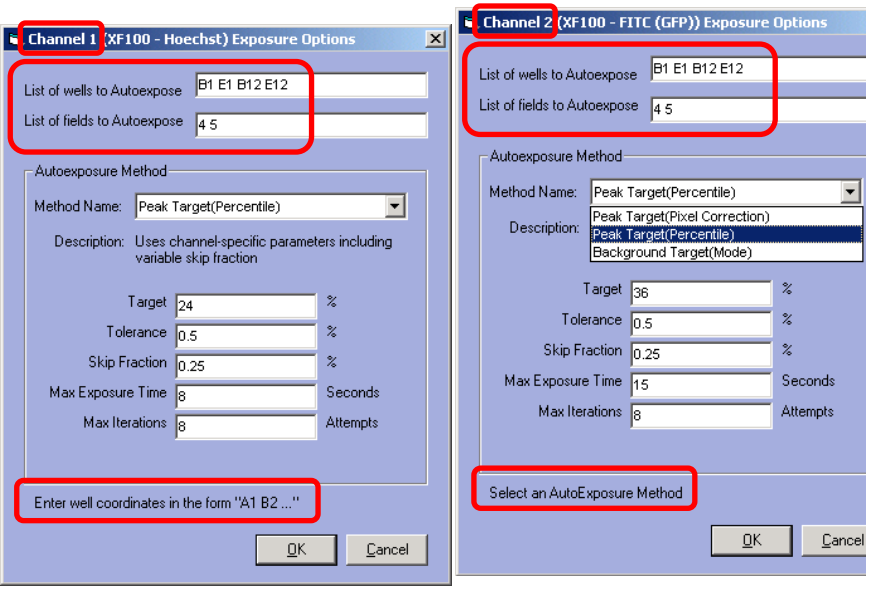
   a. Method Name = Peak Target(Percentile)
   (the different methods are summarized on next page)
   b. Target = {per Target guidelines on prior page}
   c. Tolerance = 2 %
   d. Skip Percent 0.5 %
   e. Max Exposure Time = 2 Seconds
   f. Max Iterations = 8 Attempts
5. [OK]
6. Get an in-focus image (you may need to adjust Exposure Time if AutoFocus fails) from a well appropriate to Target guidelines (e.g., if this is an “intensity assay” type of channel, choose wells where the brightest signal is expected).
6. Is this a “representative” image, in your experience?
   If current field is unusual (many outlier bright regions, sparse, overgrown, dark…), find another more representative field.
7. Image Saturation [bar]: Is it green (i.e., within Tolerance of Target)?
   a. If no, [Auto Expose] (one click at a time until bar is green).
   b. If yes, test that a few other representative fields are close to the Target (±5-10% is normal variation).
   c. Each time you click [Auto Expose], the Fixed time changes, so be reasonable with expected variation, note the time differences each time you click [Auto Expose], and consider a happy medium.
8. Test your accepted exposure time in multiple fields of multiple wells, especially spanning experimental conditions where brightness differences might be expected.
9. Repeat for all other channels, changing the Target according to Target guidelines if necessary.

Save all these exposure settings with your Assay Protocol. If the quality of your samples changes at later dates, you should merely repeat this process of clicking [Auto Expose], so that your images span a similar dynamic range as your original sample(s).

AutoExpose Options: caveats & best practices

AutoExpose Options

1. Whereas Background Target(Mode) Method refers to image histograms, **"Peak" does *not* refer to a *histogram* peak**; after Skipping specified brightest pixels, Peak is histogram's ***X***-axis intensity of remaining brightest pixel.
2. Peak methods good for targeting brightest signals as % of camera's 4095 grey-level dynamic range.
3. Background… method: When signal is expected to be absent, still can get useful exposure time (typically low % so when fore-ground appears in unknowns, it extends into useful range).



If a scan is executed with an Assay Protocol specifying AutoExpose as Exposure Type, before the whole scan begins, the system…

1. Goes to 1st well in List of wells to Autoexpose.
2. Goes to 1st field in List of fields to Autoexpose.
3. Autofocuses (using Configure Focus…> Autofocus Options> Camera Exposure Time> Focus Exposure Time For Autoexpose—note, AutoFOCUS setting!).
   - ***If Autofocus fails, so does that field!*** Proceeds to next.
4. Execute Autoexposure Method.
   a) Failed any channel? Try in next specified field (i.e., in example, field 5 is used only if field 4 fails).
   b) Succeeded for all channels? Temporarily hold determined exposure time(s) in memory.
5. Go to next well, as applicable, repeat, hold time.
6. Compute mean of all "held" times (i.e., where succeeded) & use it for respective channel(s) for whole plate scan.

Benefits:

- If reagents, optics, etc. vary over time or from plate to plate, this Exposure Type may be compensatory.

Caveats:

- No automatic way to know if specified wells/fields are in good condition in every plate. And final exposure time(s) is only from successful wells. Images from AutoExpose are not saved.
- Screeners may *want* **not** to have assay variables masked by system compensations.
- Users need to remember what Assay Protocols have set as wells/fields to autoexpose and whether the same for every plate used with that Protocol.

An Intermediate – Best Practice: Use [Autoexpose] button in Configure Acquisition on fields deemed representative by you, but specify Fixed ultimately in the saved Assay Protocol.

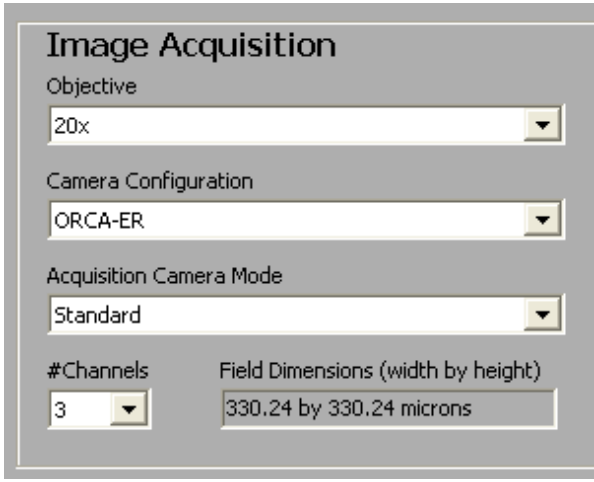
Image Acquisition pane

1. **Objective**: Lenses installed on system appear in drop-down list.

   To install different objective: Handle objectives with great care (expensive to replace), and *avoid touching glass lenses with fingers* or otherwise scratching.

   - ArrayScan VTI: Close ArrayScan software if open. Then, Windows Start button> All Programs> Cellomics> Objective Change Wizard. Follow instructions.
   - Cell Insight: Tools> Maintenance> Move To Objective Change Position.

   Later Configure Assay Parameters task, understand benefit of Use Micrometers Option menu command and impact of Objective setting on area- & length-based features and Selection Parameters. See BioApplication Guides.



2. **Camera Configuration-** on an Instrument you will have only one choice; on Toolbox clients choose the camera that you have or plan to acquire images with.

3. **Acquisition Camera Mode**: Generally, "**Standard**".
(Specifies image resolution by on-camera pixel binning during image acquisition. Under guidance of Cellomics support, HiRes may be suggested for greater pixel resolution, but longer exposure times [e.g., 4-fold vs Standard] are needed [potential bleaching], image processing may be up to 4-fold slower, so overall scan times will be slower.)

4. **# Channels**: BioApplication-dependent: For some, the standard # of channels can be scaled back, and/or additional "gating" channels can be added. See BioApplication Guides.

AutoFocus settings

a) **Use This Channel For Autofocus**: Generally, choose a channel imaging a dye whose quantitative fluorescence is not critical and/or is relatively photostable (e.g., a nuclear Hoechst stain for demarcating the nucleus).

b) **Autofocus Camera Mode**: "AutoFocus" often specified in default Assay Protocols. "Standard" may yield faster scan times if Acquisition Camera Mode also Standard, but depends on samples imaged in Focus Channel; determine empirically or consult Cellomics Support.

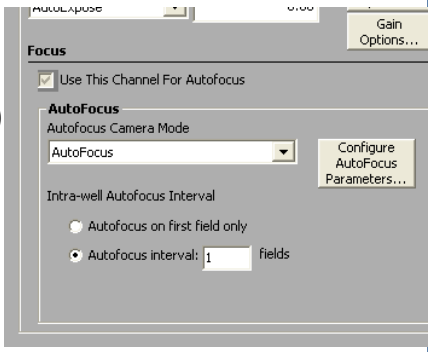
c) **Intra-well Autofocus Interval**

- "on first field only" rarely recommended. Autofocus Interval is illustrated in your *User's Guide* (within *Operator's Guide*).
- Determines the points within a well where a focus adjustment is performed.Conservative **generalizations** to start:
  4 fields@5x 3 fields@10x 2 fields@20x 1field@40x.
- ***Affects scan times & quality***, depending on #fields scanned per well (Scan Limits).

a. Therefore, best optimized during development of an assay by careful review of all images acquired during a scan (at least review images from wells with highest # of fields acquired).

b. Variables to consider include

1. sample's cell density (too sparse? "empty" fields take extended time)
2. sample's quality (occasional debris or stacked cells? maybe more frequent Interval)
3. objective magnification and NA (inversely proportional to Interval)
4. standard- or thin-bottomed plates (thin wavier? maybe more frequent Interval)
5. plate's well density (384, 96, etc.; esp. if thin-bottomed, lower density wavier)
6. users' and algorithms' analytical tolerances…



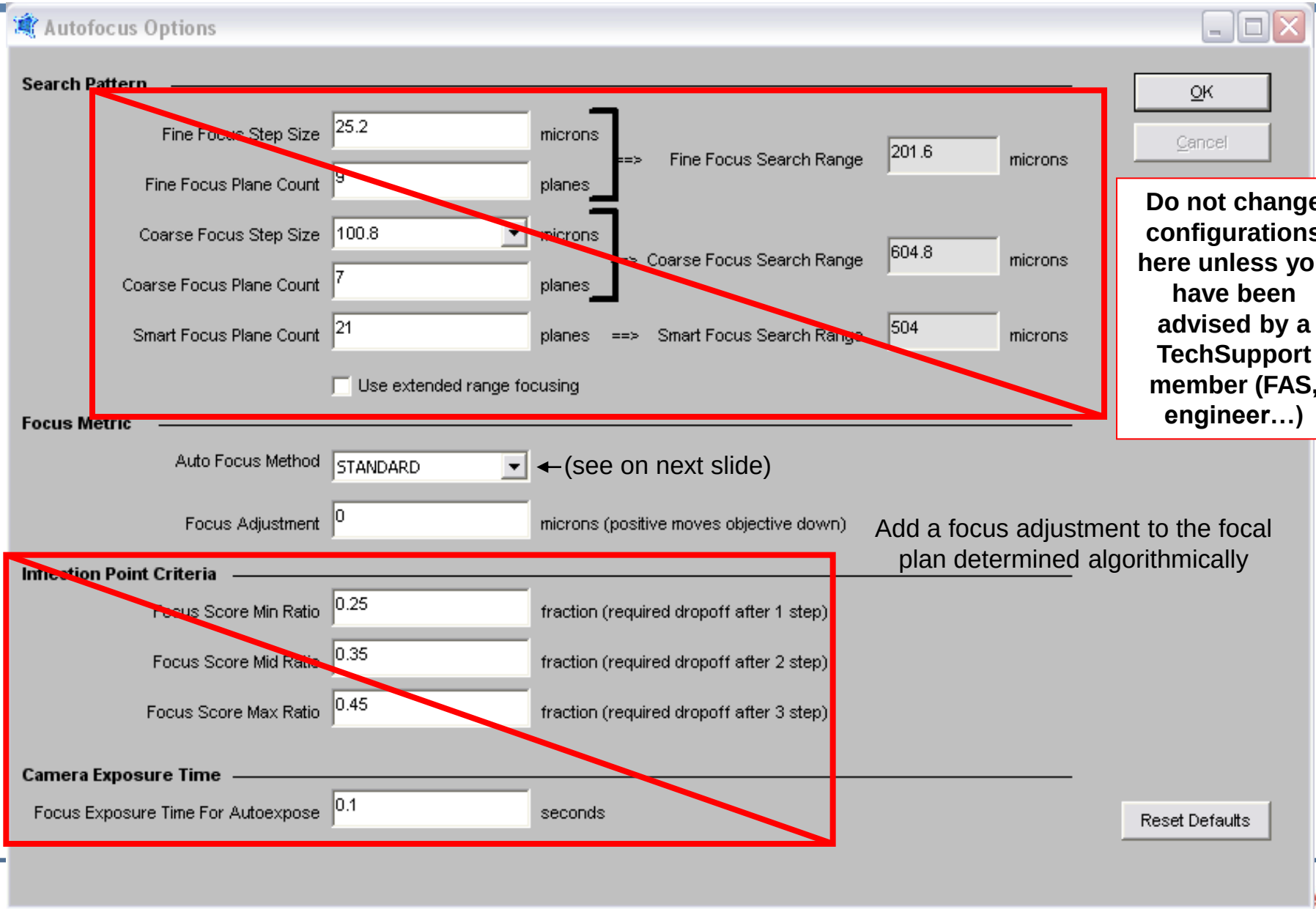
d) **Configure AutoFocus Parameters… Generally, use defaults.**

- Autofocus may consider a Focus Channel image out-of-focus (/sparse) & prevent BioApplication analysis (though save the field's images). AutoFocus Method = RELAXED or ASV40SF avoids this interception (the case for *all* Disk Scans, since Autofocus isn't used).
- AutoFocus Method = RELAXED or ASV40SF may be useful when:

1. High-NA objective on standard thick plates (see User Guide Appendix "Plate Types & Objective Selection");
2. Object intensities in Focus Channel vary widely;
3. Sparse fields are potentially common;
4. Multi-layer cells are potentially common;

- AutoFocus Method = RELAXED or ASV40SF *also* ***have additional effects (e.g., focus accuracy may be less), so it's <u>not recommended</u> as default.***

Configure Focus



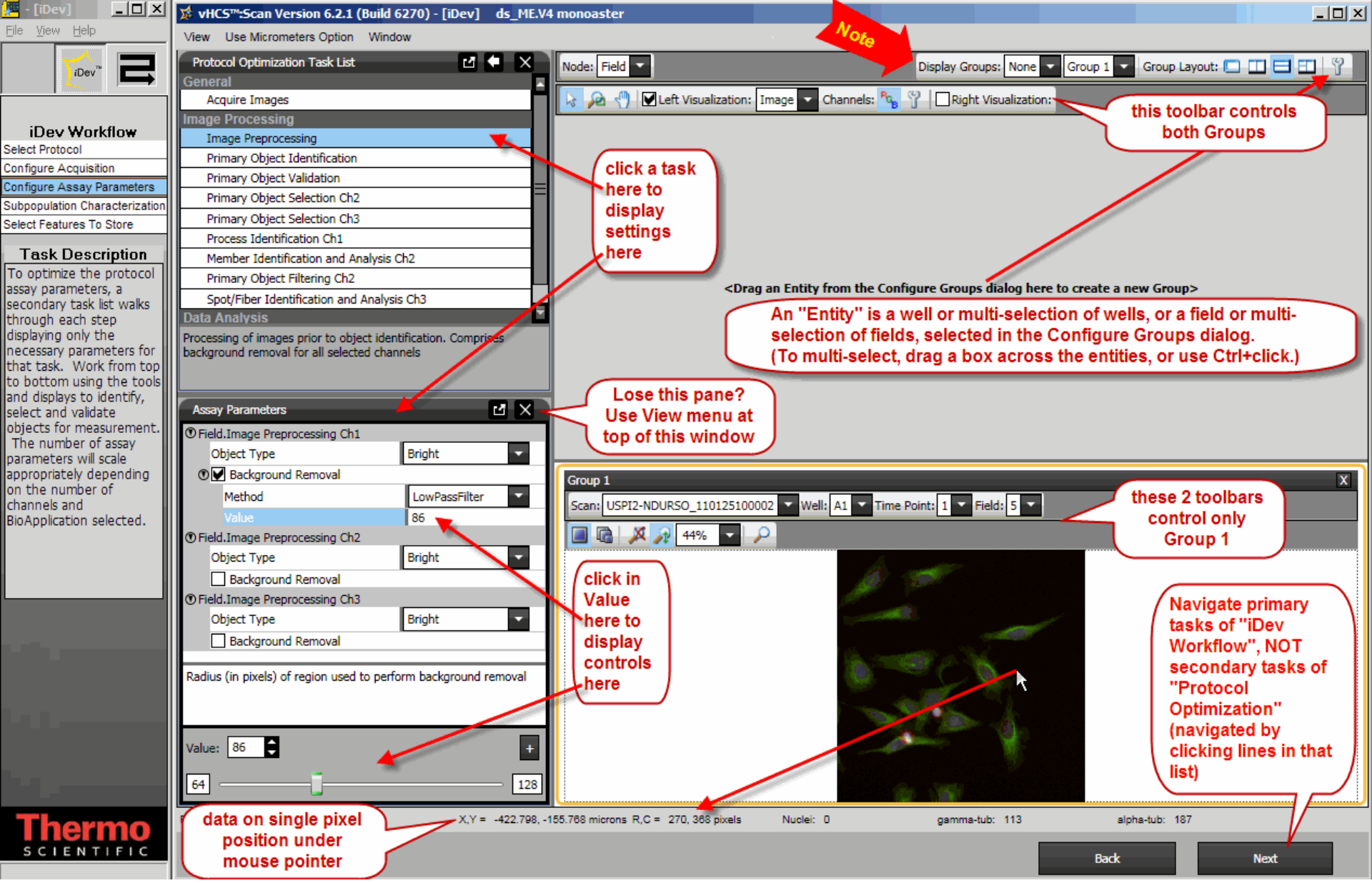
[Next] to Configure Assay Parameters | Live- vs Disk-mode

When have I used **[Save Field]** enough in the **Configure Acquisition** task to go [Next] to the **Configure Assay Parameters** task?

Answer: When you have representative sampling of your assay development sample. To answer this question reasonably…

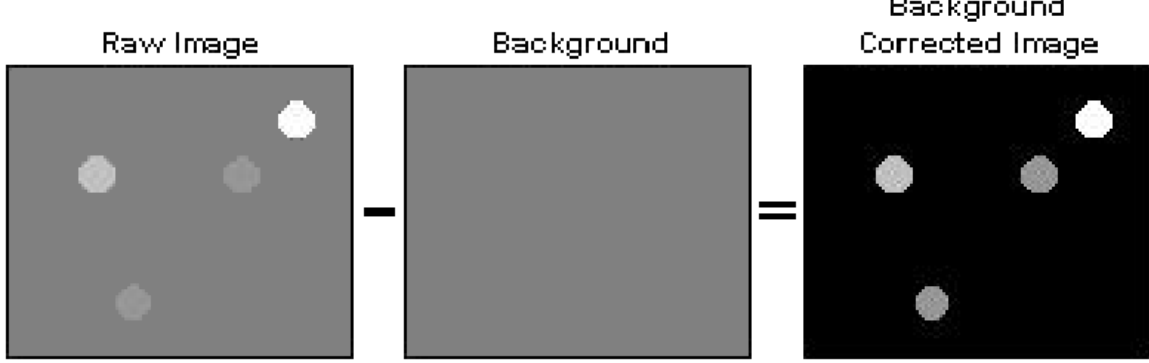
1. You have to be familiar with your assay development sample, such as the variation from field-to-field (if any) and well-to-well, in both positive and negative controls, etc.
2. You have to appreciate the subsequent tasks in the workflow, especially…
   You have to understand **Groups** in the **Configure Assay Parameters** task.
   1. A **Group can contain no more than 10 fields** ("entities" are fields), whether from different wells (or the same), and/or different plates (see Disk mode at right).
   2. Fields from the same well cannot be split into different Groups.
   3. You can create >2 groups, but only 2 groups can be active for the Workspace at the same time.
   4. You do not *have* to configure >1 group to use fields images from different wells or experimental conditions/controls. You can put fields from very different wells into one group for simplicity, if ≤10 field will suffice for your purposes. But only 1 field can be displayed at a time (rather than the max of 2). Also, if you wish to use data visualizations (graphs or tables), you cannot see separate data from the pooled conditions.

1. Scan software's 2 modes for getting images:
   a. **Live-mode** Scan Setting: This is possible only when the software is running an instrument (i.e., NOT on a Toolbox). It's not called "Live" because the samples are live cells, but because images are acquired from a live camera.
   b. **Disk-mode** (a.k.a., "disk scan" mode): Images from previous scans are loaded from a disk location, rather than the camera. This is the only possible mode on a **Toolbox** (i.e., *not* on instrument's PC). However, the software on ***instrument's PC can also be used in Disk scan mode***, either by (a) launching the Scan software when the instrument is powered off, (b) using the camera button on the toolbar in Scan Plate view. (A 3rd is via a Shortcut on the Desktop made by copying the Scan shortcut and editing its Properties to add –canned to the end of its Shortcut Target string; rename this copy to Disk Mode).
2. **Configure Assay Parameters**: 1st step depends on mode:
   1. **Live mode**: Configure Assay Parameters task begins in Configure Groups dialog, from which you can choose the image sets of wells and fields you saved temporarily by the [Save Field] button in the Configure Acquisition task.
   2. **Disk mode**: Configure Assay Parameters task begins in **Acquire Images**, where the **Source** can be plates already in your **Store** database (expand **Search Criteria** if required/desired), or stored separately outside your database somewhere on **Disk** (change **Source** to **Disk**, and **Change Directory…** as needed). After select and Add Plate, you advance to the Configure Groups dialog, as for Live-mode.
   3. Whether in Live or Disk mode, you can add additional plates to the Configure Groups dialog using [Add] in the Plate pane of that dialog, if you want to configure groups using images from different plates/sources.



In the example above, note the Group Layout is 2 Groups. Only the bottom Group (Group 1) has been configured (Configure Groups dialog not shown). The “Workspace” is everything to the right of the Protocol Optimization Task List.

Image Preprocessing: Background Removal

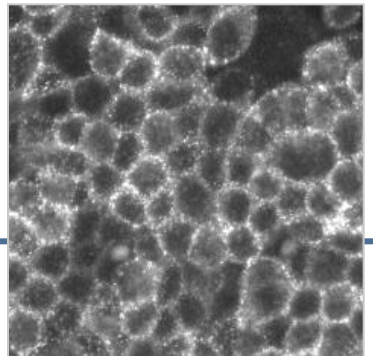


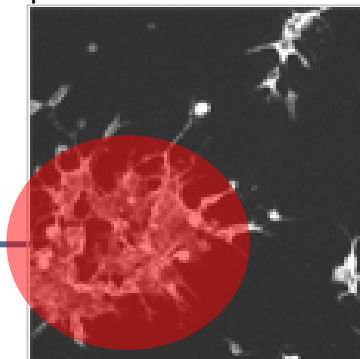
Best Practice: Use whenever possible!

Background image constructed essentially by blurring **Raw Image**. Value inversely proportional to how much blurring. NOT an intensity value itself—it's spatial

1. Always use it, whenever you can! And, when you can,…
   Best Practice:
   Use Fixed Threshold for Object Identification Method (if you can).
2. When can I *not* use it?
   When any image may have near-confluent fluorescence (maybe):
   a. Imagine the Raw Image is full of objects; what would the blurred Background image look like?
   b. …And the Background Corrected Image?
   c. In sum, has to be some *background* in every image. Why?...
3. Background Removal is a "global" Assay Parameter—i.e., when activated, it is performed on every image in that channel
4. High impact on all aspects of processing & analyses
   a. Performed BEFORE Object Identification (except spots/neurites)
   b. All output feature values vary with Value (incl. spots/neurites)
5. Can I see the Background-corrected images? Yes, use Configure Channels dialog. However, only the raw image is ever saved. (Background image can never be viewed.)
6. Choosing a Value: Display the BGRemoved image at low Gamma
   a. "Aggressive" Values (low) ~ diameter of largest typical objects. (unit is pixels, so "bridge" across all fluorescence patches)
      a. for reliably monodisperse objects/signal.
      b. Aggressive low values afford "localized" correction
   b. "Conservative" (large Values) for heterogeneous objects/signals.
      a. Greater than 2x largest diameter?
      b. Heterogeneity too unpredictable? Consider Max Value (255)

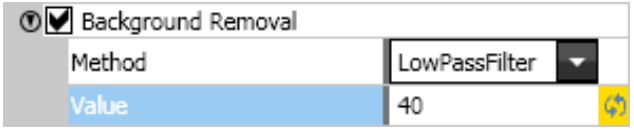
7. Choosing a Method: (see BioApplication Guides for illustrations)
   a. **LowPassFilter**
      i. Best for object identification channels.
      ii. Caution for intensity-critical channels because over-corrects (often ~20%), so some true foreground signal can be lost.
      iii. Caution for unpredictable object sizes (like some colony samples).
      iv. Poor for dime edges of "whole cell" graded fluorescence.
   b. **3D_SurfaceFitting** (do NOT use Values ≤ 32; ≥ 64 best)
      a. Best for intensity-critical channels (preserves foreground better than LowPassFilter, often under-correcting ~10%).
      b. Best for preserving dim edges of "whole cell" graded fluorescence.
      c. Poor for "dirty" samples (because so gentle, ascribes haze as BG).
      d. Caution: Values too low can cause unpredictable artifacts.





How does the Background correction low-pass filter method work?

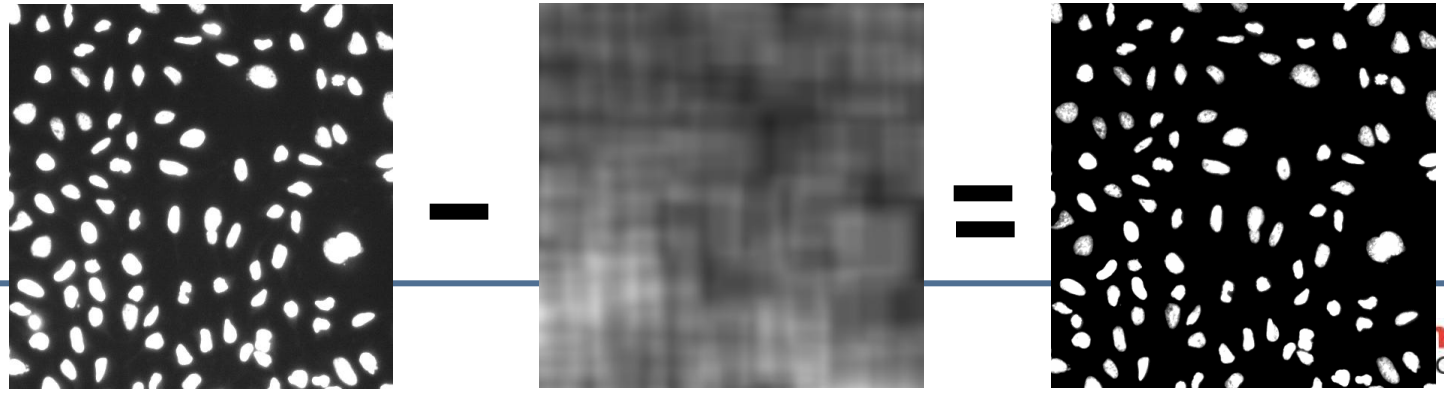
- Activate Low Pass Filter method for Background correction and select a value:



- In this example, for each pixel in the image an square area of 2x40 pixels large centered around this pixel will be defined.

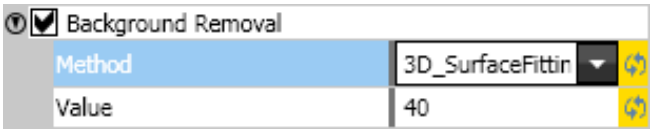
- The average intensity of this area is an estimation of the Bgnd level at this pixel and will be subtracted to the pixel intensity value

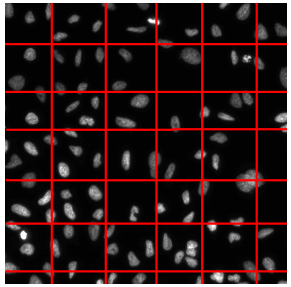
- Eventually, it equivalent to subtract to the original image an image “smoothed” with a filter of a size of 81 pixels. Ex:



How does the surface-fitting method work?

- Activate 3D-Surface Fitting method for Background correction and select a value:
- In this example, the image will be virtually cut into squares of 2x80 pixels large
- In each square, the background estimation will consist in picking the minimum intensity value



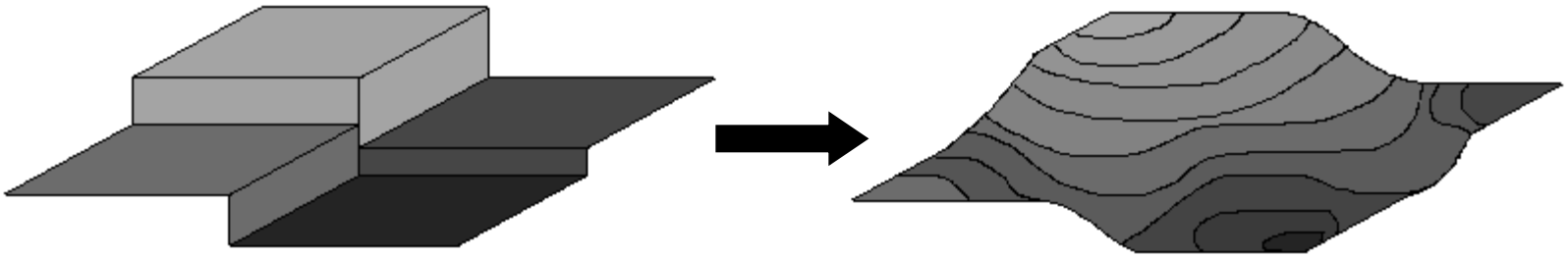


Prefer values that divide 512 (size of pictures in standard configuration) to get a strictly square cut-out

- Think to a 3D plot where intensity at each point of coordinates X,Y could be represented as an altitude
- The obtained surface will be smoothed (surface-fitting) and then subtracted to the original image to get the corrected image

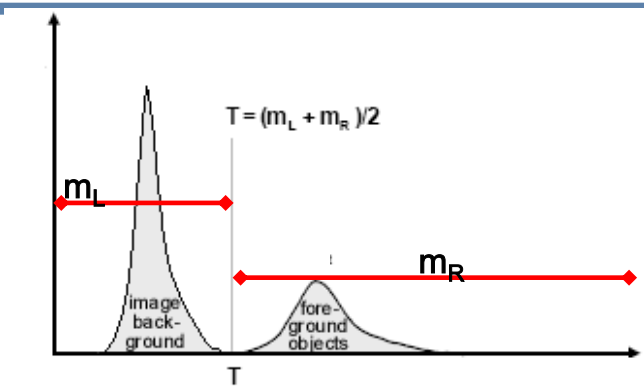
First background estimation

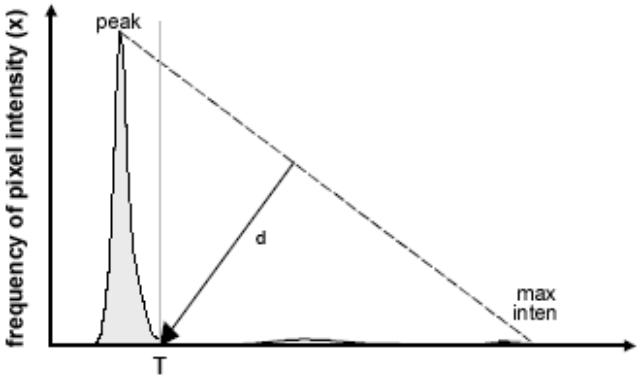
Surface-fitted background image

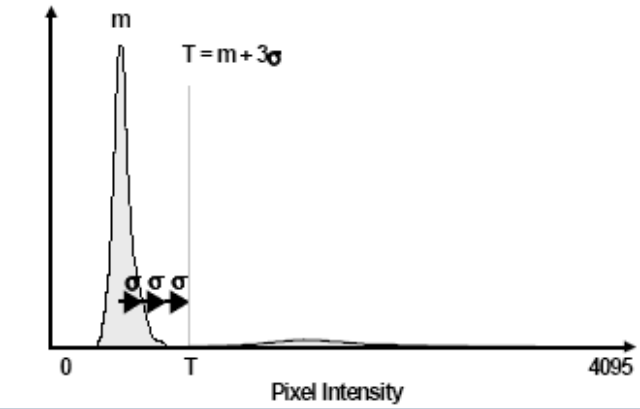


Object Identification Methods & Values

- **Object Identification** is, fundamentally, identifying foreground objects as patches of pixels that are brighter than background pixels.
  - An image comprises pixels; each pixel has an intensity value.
    - A frequency distribution histogram of these pixel intensity values is called the "**image histogram**" and is one form of information in an image.
    - Does an image histogram represent spatial information from the image?
  - Analyze image histogram info → intensity threshold → identify objects as foreground vs. background. Advantage:
    - 100's or 1000's of images from one assay have variations in foregrounds and/or backgrounds, so histograms vary from image to image.
    - Flexible, systematic method of analyzing each can still → comparable object identification results amidst the variations.
- Method: **IsodataThreshold**
  - Assumes bimodal histogram, determines T by iteratively computing threshold between means on left (m_L) and right (m_R) sides of histogram until T does not change any more.
  - Good for bimodal histograms (expect every image to have similar densities of foreground objects?)
    - What happens in fields that are sparse, or fluorescence very low?
    - What happens in fields with bright fluorescent outliers, debris, or artifacts?
- Method: **TriangulationThreshold**
  - On image histogram, hypotenuse "drawn" from mode (tallest peak) to max intensity.
    - T is value on x-axis where longest normal (perpendicular line) between hypotenuse & histogram's curve occurs.
  - Good for potentially sparse fields.
    - What are possible outcomes when fields are potentially dense?
    - What are possible outcomes when fields have bright fluorescent outliers (consider size of such objects, and "tightness" [kurtosis] of their peaks)?
- Method: **3sigmaThreshold**- calculated threshold is 3 standard deviations above the calculated Mean of the distribution.
- **Value**: Shifts auto-computed threshold (T_{calc}) according to equation below.







$$T_{final} = T_{calc} \times (1+Value)$$

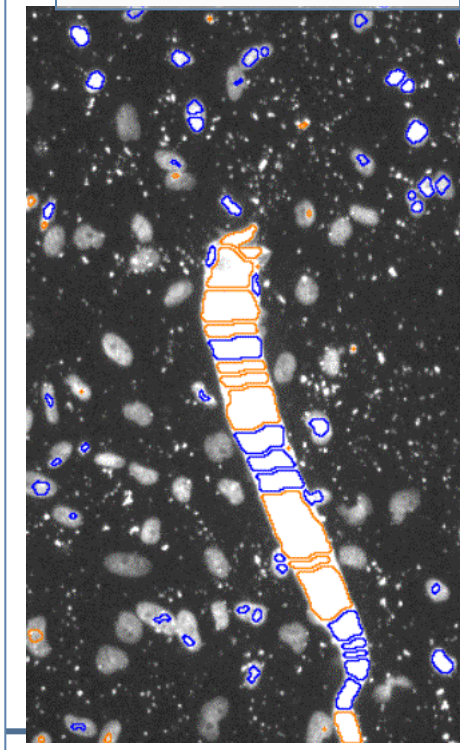
Object Segmentation in Object Identification

ObjectSegmentation : Just when you must!

Why only when you must? It's an approximation, so subject to imperfections.

1. When *must* I use it?
   a. When a prohibitive majority of cells are simply too crowded to identify individually, *and* individual identification is required.
   b. e.g. let your biological assay needs guide you.
2. How else can I deal with clustered cells?
   a. Seed wells to achieve lower final densities, and/or more appropriately dispersed cells (some tips are provided in Cellomics' FAQs).
   b. Use a different cell type, if possible (a handful of cell types are known for their tendencies to cluster; e.g., HEK293, HepG2, MCF7).
   c. Reject them using Object Selection Parameters. In such a case, weigh…
      i. [time to acquire more images] versus [risk of inaccurate Object Segmentation];
      ii. whether Rejection of clusters introduces bias (e.g., target phenotype expressed *only* in clusters?). Compare results using [Object Selection Parameters that Reject all but single cells] versus [Rejection of only/mostly single cells].
3. What Method & Value should I use? Determine empirically. Some guides…
   a. Shape: Best for clusters of 2 or 3 cells, where geometry of borders are visual cues.
      Start Value ~radius (1/2 the diameter) of a typical Object.
   b. Intensity: Best for "whole cell" fluorescence, & maybe nuclear clusters of >2 or 3 cells.
      i. Use of Smoothing critical for avoiding over-segmentation.
      ii. Value easiest to determine empirically, but see illustrations in BioApplication Guide if interested.

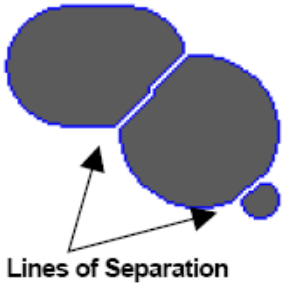
Image illustrates some caveats of ObjectSegmentation. Some of such debris segments can be Rejected if Object Selection Parameters are optimized, but not always. Also, other caveats less obvious include over-segmentation of a large or elongated single object.



ThermoFisher SCIENTIFIC

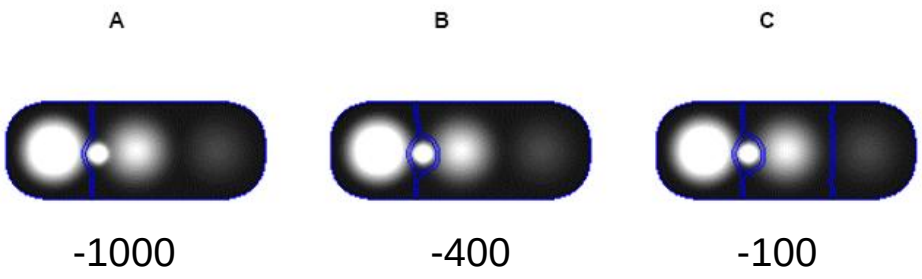
Object Segmentation

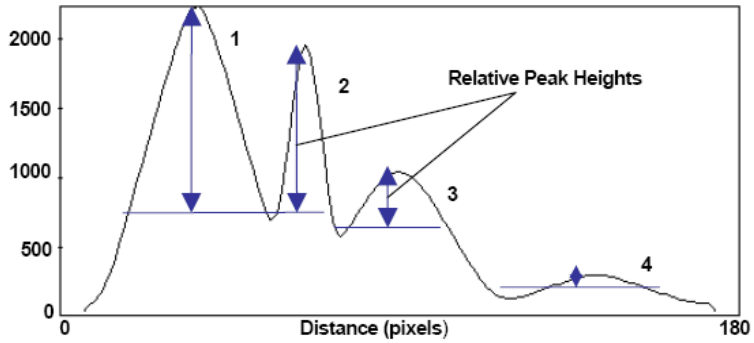
- Shape (Classic = positive) – Value = radius (in pixels) of smaller object to segment



Segmentation = 7
(radius of R3)

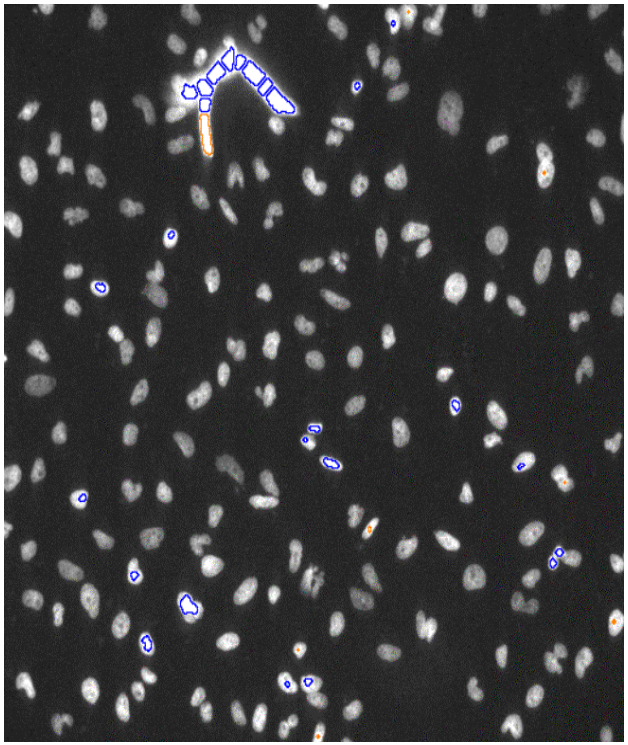
- Intensity (Classic = negative) – Value = minimum relative height of intensity peak

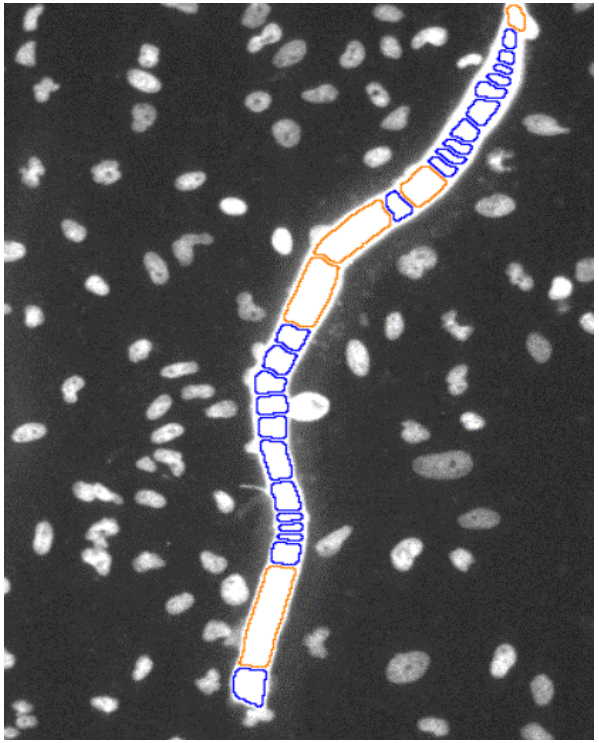


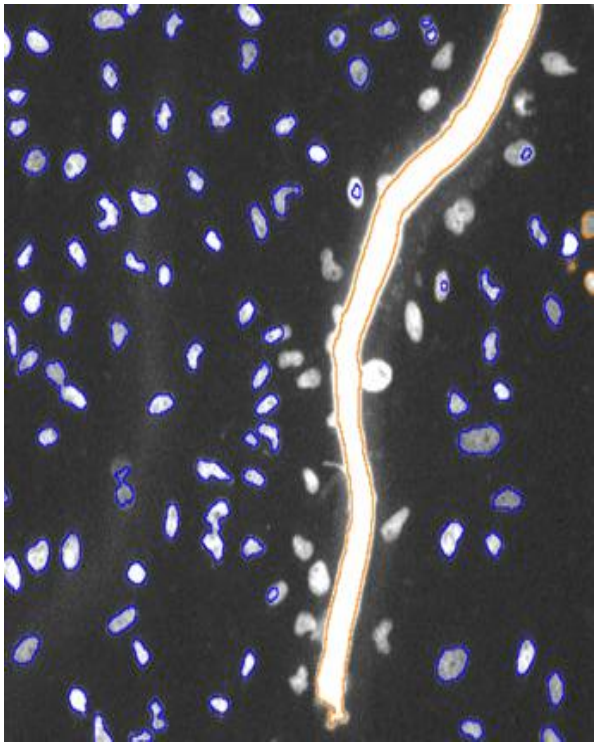


Caveats & benefits of different Object Identification Methods and Assay Parameters

Images show caveats of Isodata (or PeakTriang or other histogram-based, dynamic) Object Identification Methods, as well as a caveat of the ObjectSegmentation Assay Parameter, and a benefit of the Background Removal Assay Parameter.





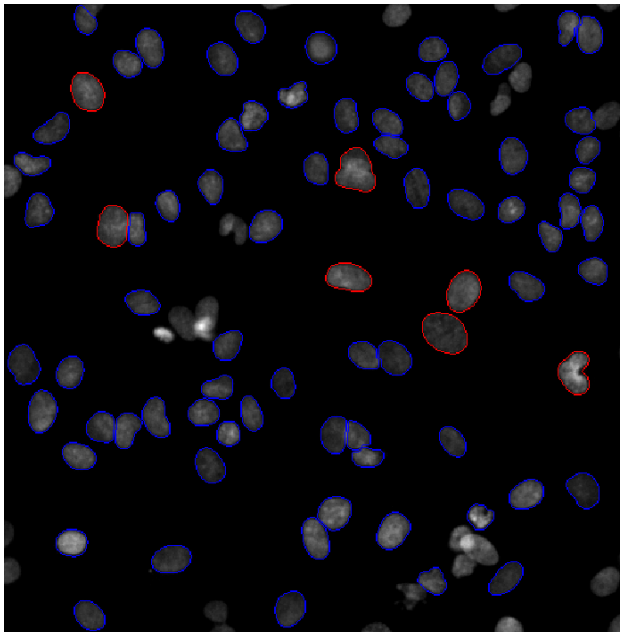


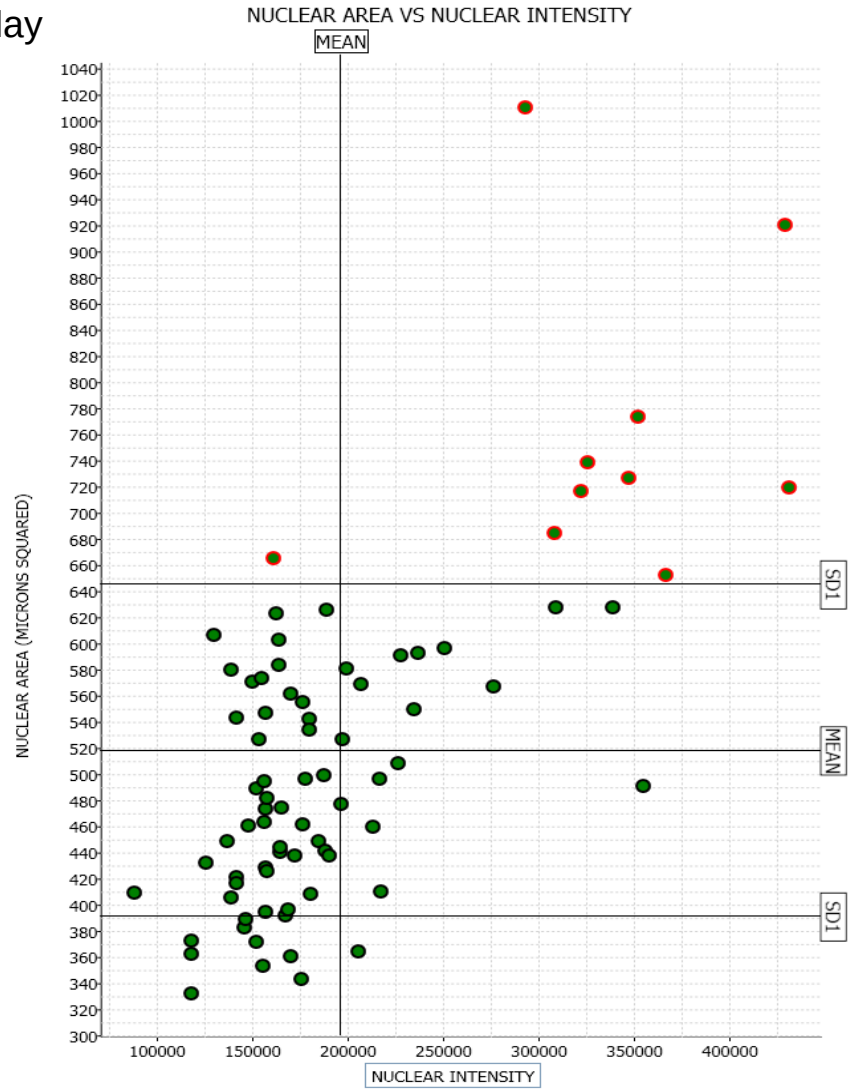
Interactive graphical displays

- Secondary visualizations are possible, that enable the display of scatter plots, histograms and data tables.

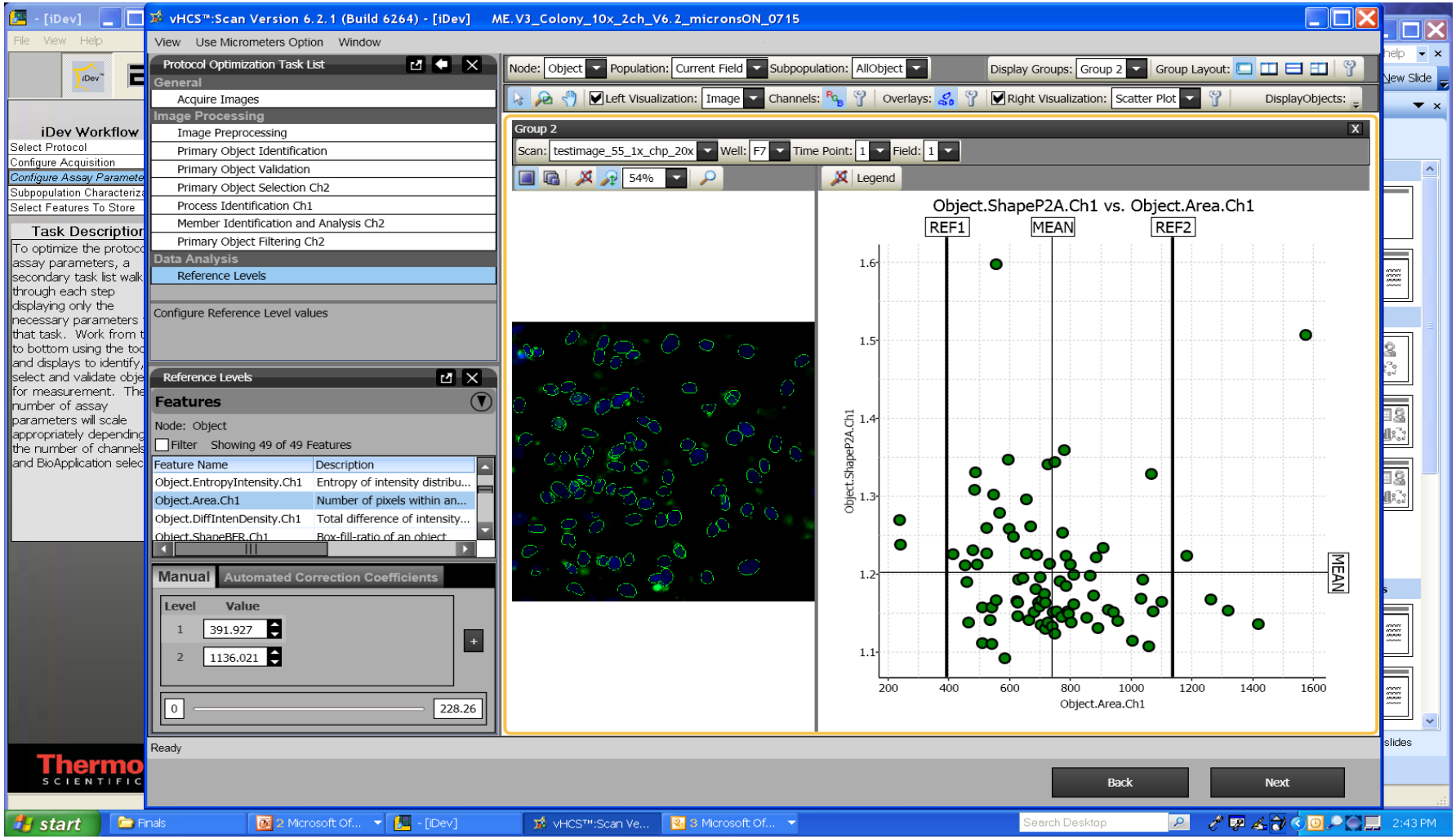
- Tables and graphs directly interact with the image. In this example, large nuclei (> 1 SD of the mean) were selected on the graph and identified by a red overlay on the graph.

- Graphical displays can be modified to show additional information (range, SD, etc.) using the configure icon.





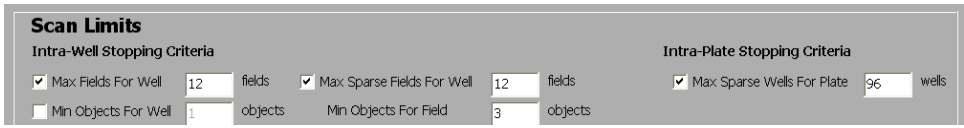
Setting Reference Levels Manually



Subpopulation Characterization - Scan Limits

1. Subpopulation Events and Reference Wells: Advanced topics; see BioApplication Guides.

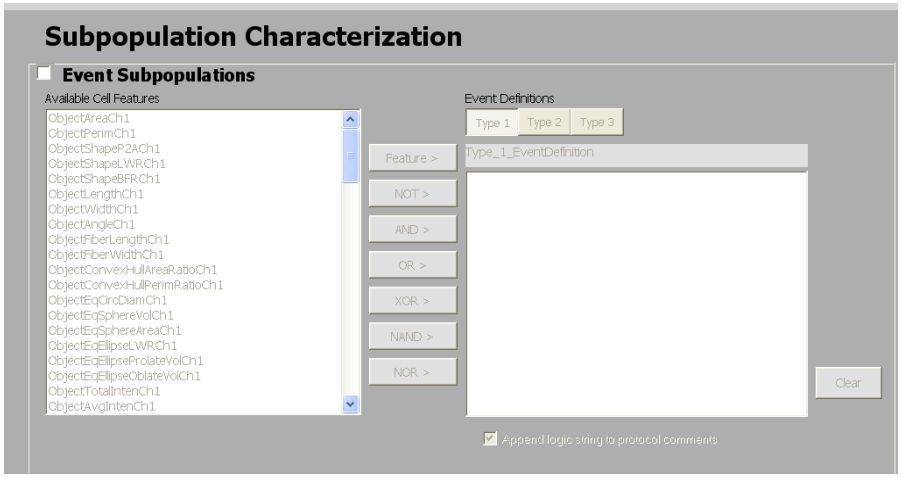
2. Scan Limits pane, Intra-Well Stopping Criteria section:
   a. When will the ArrayScan stop scanning a well, and proceed to the next well?
      i. When at least one check-able Intra-Well Stopping Criteria is met or exceeded.
   b. A “Sparse Field” has fewer objects than the Min Objects For Field (always keep >0).
   c. Max Sparse Fields For Well: Sparse Fields must be contiguous to hit this limit.
   d. All Scan Limits can have ***dramatic*** impacts on scan time. Think through logic very carefully.
   e. How many objects is enough?
      i. Must be answered by assay developer’s statistical requirements, which can be arbitrary and vary between organizations..
      ii. Cellomics has some recommendations, but these are in the context of specific assay statistical requirements, arbitrarily selected, though common.
      iii. Here’s a **Good Practice**:
      Early in assay development, scan all possible fields in model wells to create a comprehensive, stored image set. Create a series of batch rescans using the Disk Scan Wizard (or ArrayScan software in Disk Mode) to rescan the images, where the only variable between scans is decrementing # of fields per well. Such a batch is programmed using Plate Protocols, where, in this case, the Plate Protocols differ only in one regard—their Assay Protocols differ only in Max Fields For Well, with all other Scan Limits boxes unchecked. Examine the results of such batches to determine a minimum sample size at which StdDev is stable for principal output well features.
      iv. NOTE: An “object” in the context of Scan Limits is BioApplication-dependent.

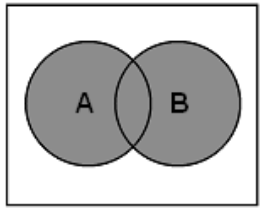


3. Scan Limits pane, Intra-Plate Stopping Criteria section:
   What use is Max Sparse Wells For Plate?
   a. In mass sample prep operations (e.g., automation), if most cells are lost by unsuitable liquid handling or a treatment undergone by all wells, or a necessary labeling regent was not suitably applied…
   b. When **scanning a stack of plates**, more efficient not to scan “bad” plates.

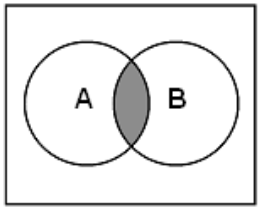
Setting Events

- Boolean operation
- Defining a subpopulation by using any combination of cell features
- Reporting characteristics of your subpopulations
- Event definition is achieved through the construction of logic statements employing upper levels for specific Cell Features and a set of defined logical operators.
  - ANDNOT and ORNOT are obtained by combining AND + NOT and OR + NOT respectively.

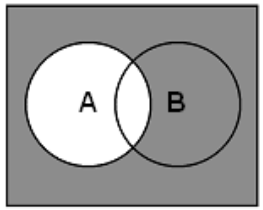




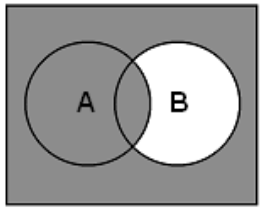
A OR B



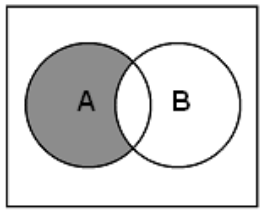
A AND B



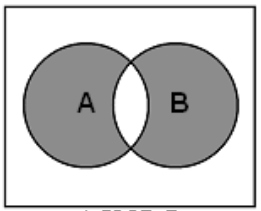
NOT A



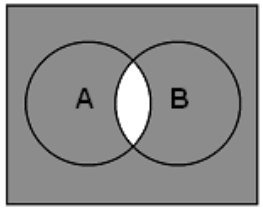
A ORNOT B



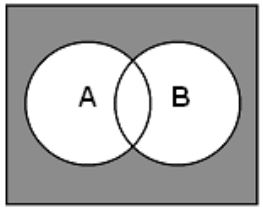
A ANDNOT B



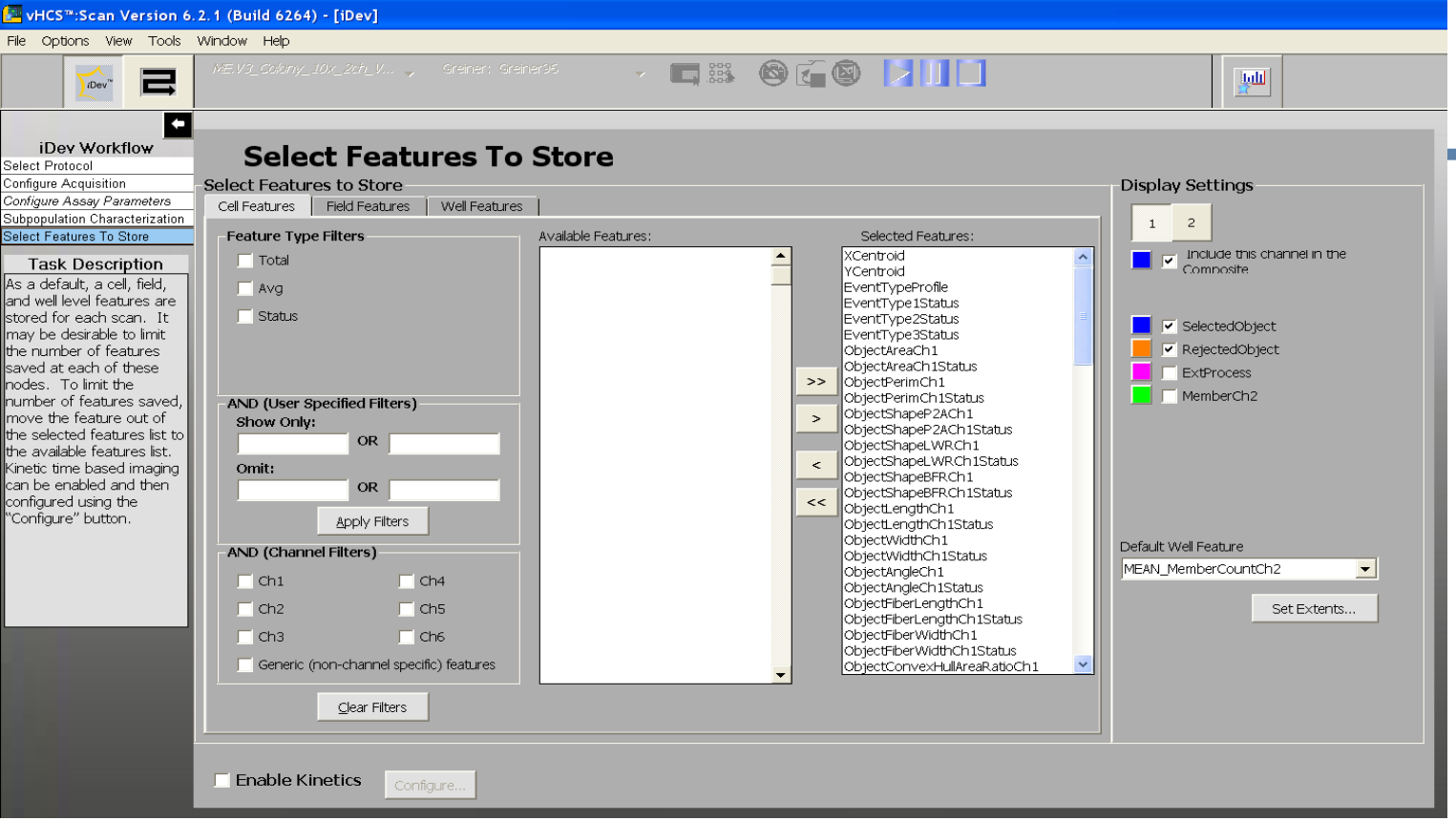
A XOR B



(NOT A) OR (NOT B)
Same as A NAND B



(NOT A) AND (NOT B)
Same as A NOR B



Features To Store: If they are not selected for your protocol, they will not be stored as data. (You can subsequently disk scan images after saving a protocol version in which more features are selected to store.) Use the filters for efficiency. For example, if you have not set Reference Levels meaningfully, Omit "status" and "event" in Cell Features, and %HIGH, etc. in Well Features.

Over extended use of your system, your database can grow large quickly if you save many Cell Features for screens. You may wish to save few or no Cell Features in large screens? (Well Features do not consume substantial database space.)

Display Settings: When you review data and images, the overlays and composite colors selected here will be stored – NOT the overlays and colors used in prior tasks of iDev Protocol Optimization.

Reviewing results in vHCS:View

Launch via Start> Program Files> Cellomics> vHCS:View

A. Highly developed software with user-friendly GUI (graphical user interface):

1. Almost complete redundancy between menus, toolbar icons, and right-click context-sensitive pop-up menus.
2. Uses Windows conventional Ctrl+click & Shift+click functions for selections on text lists, spreadsheets, etc.
3. Uses Windows conventional Ctrl+C (copy) & Ctrl+V (paste) from spreadsheets.
4. Full on-line Help menu.

B. Best Practices – Tips for fast, easy use & learning

1. Frequently right-click on GUI elements (graphs, images, diagrams, spreadsheets) for command options on single GUI elements.
2. Use the Windows menu on the menu bar for managing arrangements of multiple windows/views and navigating between them.
3. Check menu bar menus for additional command options, and when multiple GUI element types are displayed (e.g., a view with multiple graphs).
4. Notice the interconnections of interactive GUI elements (e.g., click a point on a graph, and note spreadsheet highlight changes with it).
5. Be fearlessly exploratory—except for Delete Plate(s). Aside from Delete, you cannot change any data using vHCS:View: It's only a tool for viewing data in different ways.

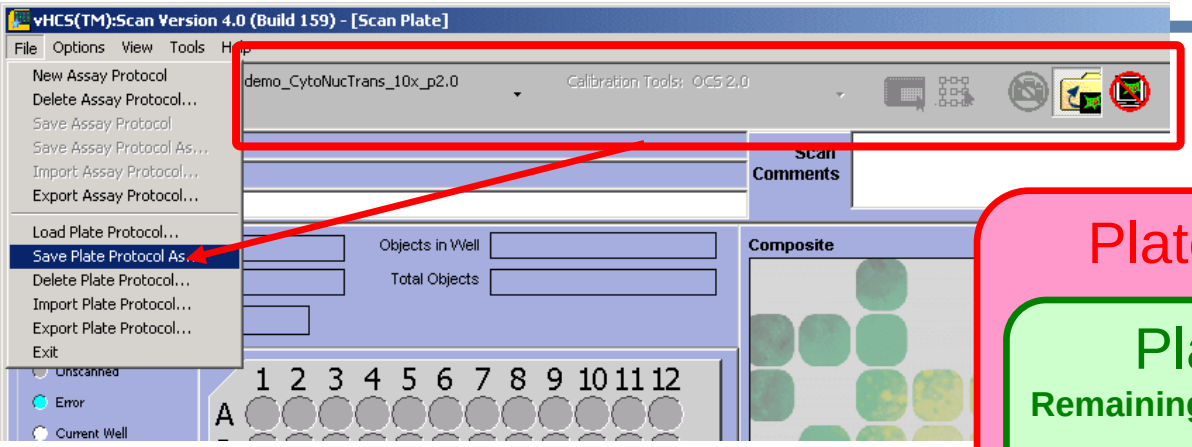
Find a Plate result of interest:

- **Well Details**
  - Row/Col mode (so lay out plates accordingly)
  - Use down arrow key to "flip" through Feature list
  - double-click anywhere on graph (except points) to Customize
  - Copy/export spreadsheets or graphs
  - Methods (bar, points, points+lines)
  - File> Report
- **Cell Details**
  - toggling overlays
  - Maximize images (and de-max to restore; don't click ☒ or Cell Details closes)
  - making New plots
  - hidden graphs B & C
  - scatter vs histograms
  - maximize, copy, export spreadsheets/graphs
  - select spreadsheet column heading, right-click, Sort…, useful to find where to use subpop cutoffs or other assay params, as are histograms/scatter plots.
  - show can call Cell Details from multiple highlighted Well Details wells (contiguous)

Other common uses besides Well & Cell Details:

- **Multi-Plate View**
  - Graph menu> Plot on Single Graph
  - Spreadsheet menu> Export to…
- **Multi-Feature View**
  - Graph menu> Plot on Single Graph (only good for similar scales, and rough with multiple rows)
  - Spreadsheet menu> Export to…

Plate Protocols



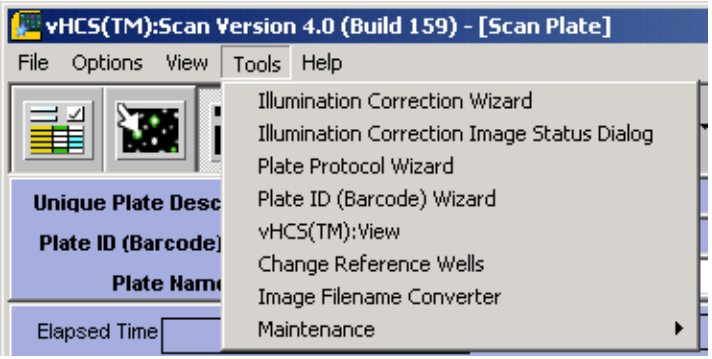


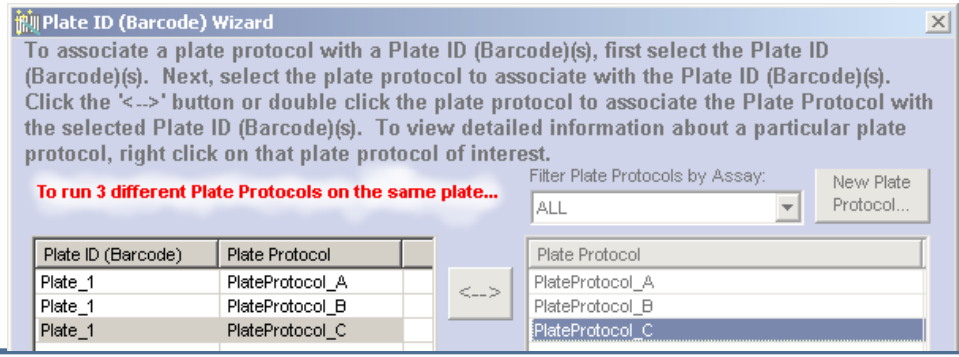
Plate ID / Barcode (when)

Plate Protocol (where)

Remaining scan settings *not* in Assay Protocol:

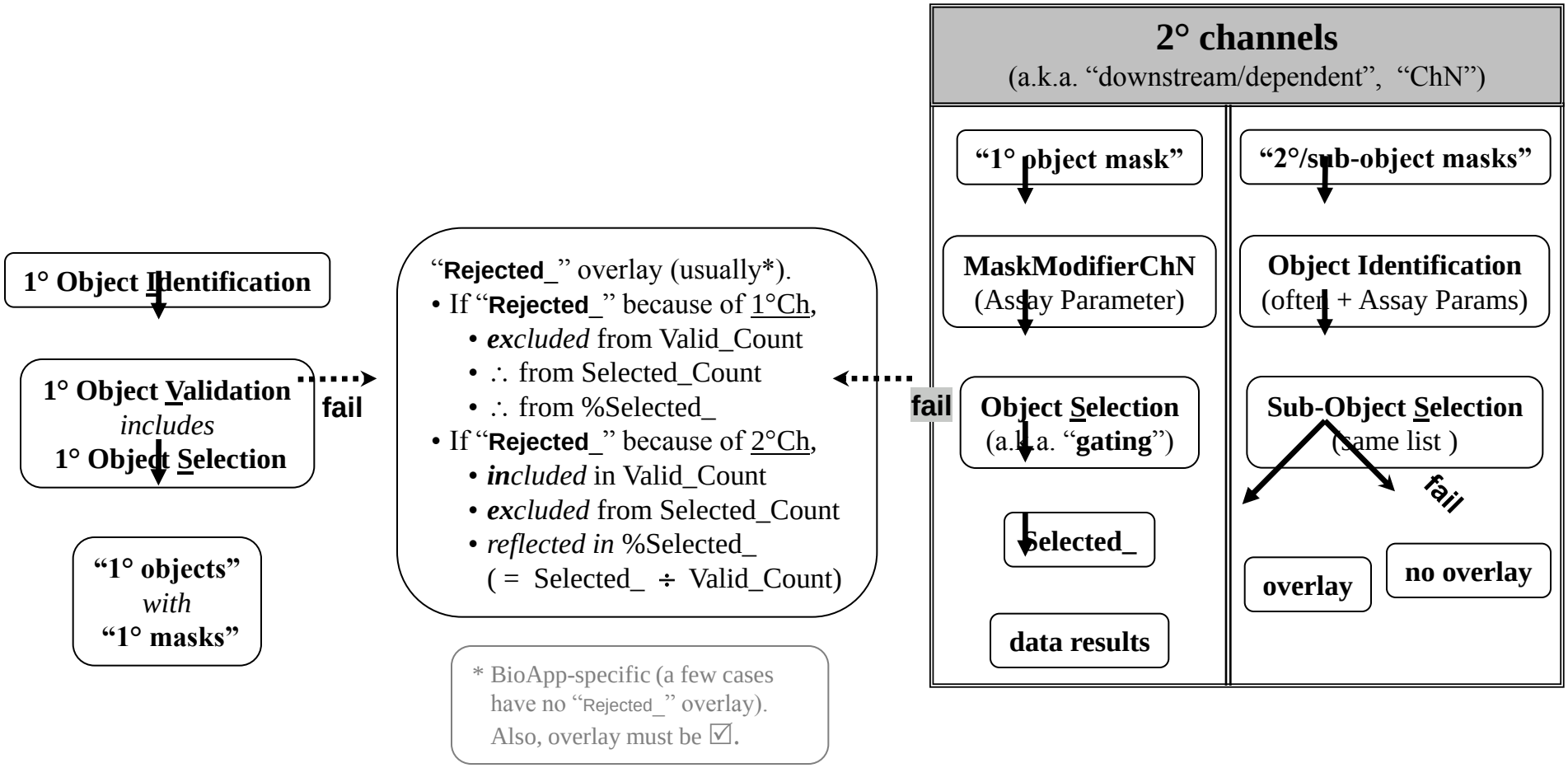
- Plate Type (Form Factor)
- Scan Area (which wells on plate)
- Starting Field Offset (within each well)
- Store Images (saves space for quick tests)
- Display More (reduced GUI graphics; reduce time)

Assay Protocol (what/how)



ThermoFisher SCIENTIFIC

"Rejected" Overlays – multiple reasons

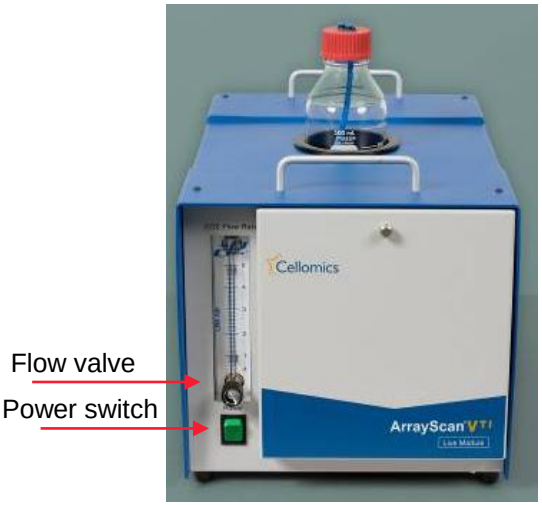


Appendix III: Starting up the VTI Live Cell Chamber (LCC) hardware

Your live cell chamber has been calibrated to your laboratory conditions. Physical adjustments to the settings on the CT controller are therefore not required, nor recommended.

- DAILY SETUP: start at least 45 minutes before you plan to put cells into the chamber
- t = -45'
  - Switch on CT unit: Green switch on front of CT unit.
  - Distilled H_2O level in humidifier (500mL bottle) 200-400 mL (>450mL may bubble into gas inlet and contaminate chamber.)
  - Make sure flow valve in lower left of CT unit is OPEN.
  - Start CO_2 flow into CT mixer from 100% source supply.
  - Open regulator at source gradually, because H_2O begins bubbling.
  - Check CO_2 settings:
    - From regulator: Max 15 psi, or 1 bar.
    - On CT controller Unit: CO_2 flow should be ~1.8 LPM; check silver bubble within clear acrylic gauge embedded in CT unit.
    - Use knob at bottom of gauge to adjust if necessary. NEVER shut down this valve completely.
  - Open ArrayScan software: LCC status is shown at bottom right of window. If you do not see status boxes, follow steps in blue text at right.
- t = 0
  - Use the ArrayScan software (Tools> Configure Live Cell Chamber…) to adjust temperature and CO_2, if necessary.
- LCC shut-down
  - Close the ArrayScan Software by using File…Exit.
  - LCC Control Unit power can be switched off while ArrayScan remains on. Leave the flow valve as is.
  - Stop the CO_2 supply at its source.

CT controller



1. **Go to Tools…Configure Live Cell Chamber and click Enable. If grayed out,**
2. **Close AS software, then go to C:/program files/Cellomics/Tools/ customer tools/ArrayScanConfig.exe to make sure the LCC hardware is enabled. Check the boxes for both temp and CO_2, Save configuration.**
3. **Once this is done, you will get a message if you start AS software with the LCC Controller powered off. Just hit OK if you don't want to use the LCC. To avoid the message you have to go to this configuration tool again to disable it, then en able it again to use.**