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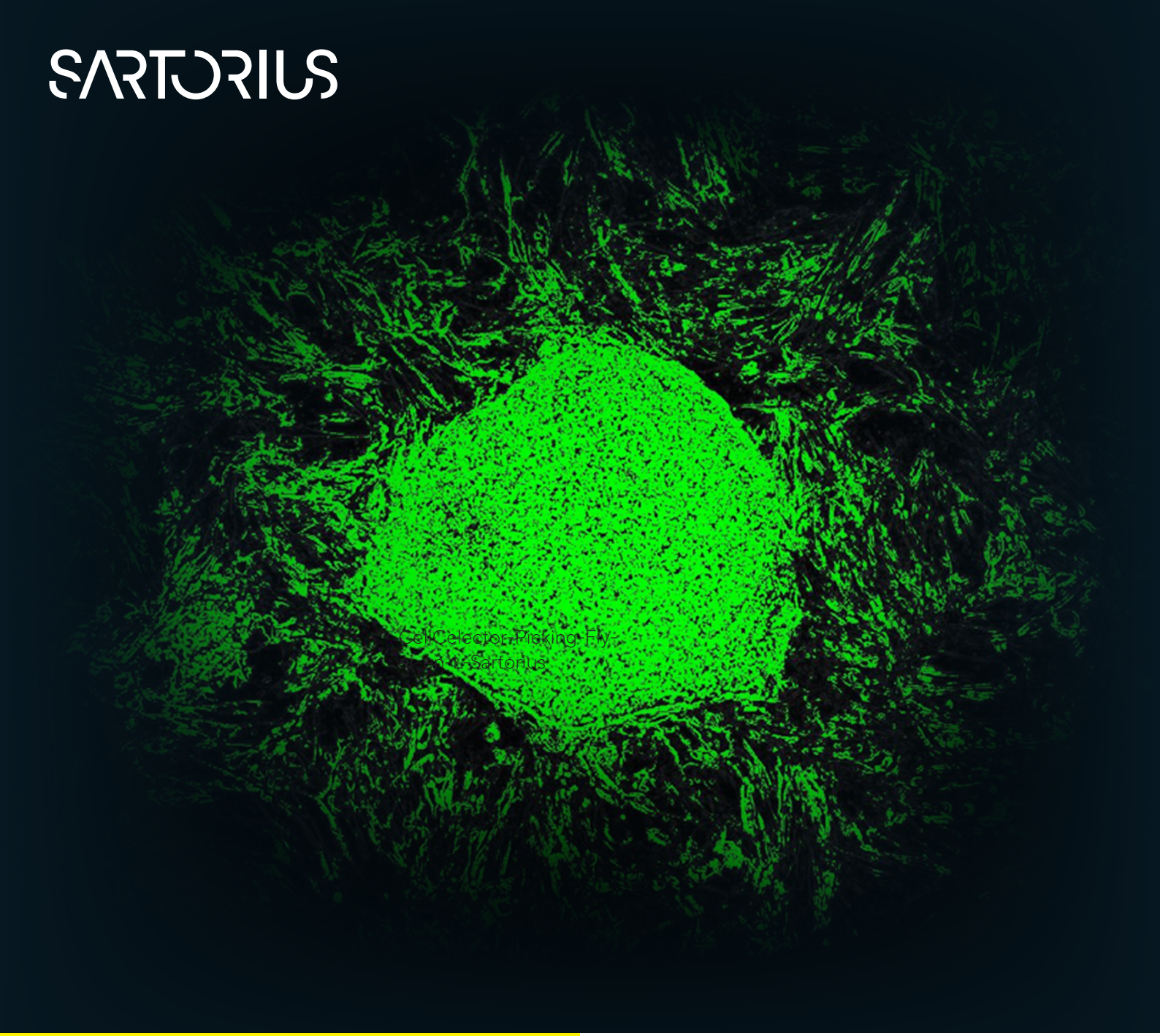


Cell Selection and Retrieval

CellCelector Imaging and Image Analysis

Brightfield, Phase Contrast, Fluorescence Scanning and Image Processing

- Brightfield, phase contrast, and fluorescence illumination
- Well screening with autofocussing
- Thresholds, measurements and ranges, image masks and morphological filters
- Online and scan shading corrections, image overlays and measurements, particle detection options, high sensitivity modes

CellCelector Brightfield and Fluorescence

The optical unit of the CellCelector Flex consists of an inverted fluorescence microscope, a cooled charge-coupled (CCD) camera, and a motorized X/Y stage. The highly flexible nature of the platform ensures that even the most complex cellular structure can be visualized either in brightfield, phase contrast or fluorescence, with standard and custom filters available for almost any commercially available fluorochrome.

Microscope Camera
CellCelector digital microscope cameras are Peltier cooled, charge-coupled devices (CCD) which deliver excellent resolution and high sensitivity due to the following features:
- 1376 × 1032 pixel resolution
- A 6.45 µm pitch sensor
- 14-bit dynamic range
- 28 frames per second at full resolution
- Exposure times of 100 µs – 160 secs
- Three binning modes of 2 × 2, 4 × 4 and 8 × 8

Two options are available – one model for standard fluorescence imaging and another for imaging the entire range of fluorescent dyes, including those emitting in the near-infra red spectrum.

Brightfield and Phase Contrast Imaging
Brightfield imaging can be used to easily identify cellular structures which can then be superimposed on colored fluorescence images to make particle structures more visible. Phase contrast imaging is preferable to brightfield when the sample is transparent, colorless or has very fine details.

Fluorescence Imaging
Light is transferred from a fluorescent light source via a 3 mm liquid light guide cable, into the microscope, and diverted up towards the sample. Two fluorescence light engines with different configurations are available:
- 6 LEDs with an excitation filter to generate light at the desired excitation wavelength
- A single white light source which creates a consistent white light spectrum

Filter Selection
Depending on fluorescent light source, three emission filter set options are available:
- Individual emission filters for single fluorescent channels
- 1 single multi-band emission filter for faster multi-channel fluorescence image acquisition
- A filter wheel with up to 8 single band emission filter positions for the automated selection of specific emission wavelengths, minimizing any potential cross-over between fluorochromes

Fluorochrome Selection
A maximum of 5 fluorescent channels (Table 1) are therefore available, depending on instrument configuration, allowing for a total of 14 different fluorochromes to be used in tandem. Common fluorochromes can be selected from a pre-configured library within the CellCelector software, with additional fluorochromes entered manually or imported in bulk. Corresponding excitation and emission filters can be configured, and additional filters easily added for the detection of non-standard fluorophores

Illumination Channel Intensity
Fluorescent excitation light intensity can be altered depending on the fluorescent properties of the source material. This can be especially useful for dimly fluorescent objects, whilst decreased intensities are recommended for light sensitive dyes with an increased bleaching hazard. Illumination channel intensity is indicated as a percentage of the maximum intensity value.

Objectives
Different fluorescent and non-fluorescent objectives are available, ranging from 1.25 × to 40 × (Table 2). There are two main considerations to follow when choosing the correct objective:
- **Working distance:** distance between the objective and the cells. Objectives with large working distances allow cells to be visualized whilst being placed on membranes or increased distances above the base of the plate
- **Focal depth**: the higher the objective magnification, the lower the focal depth of the images (i.e. the smaller the area the objective can provide sharp, focused images)

Illumination Channels	Excitation (nm)	Emission (nm)	Example Fluorochromes
Brightfield			
Phase Contrast			
Blue	395 | 25	434 | 32	DAPI, Hoechst, Alexa Fluor 350, Alexa Fluor 405
Green	475 | 28	519 | 26	GFP, Alexa Fluor 488, FITC, Cy2
Red	555 | 28	595 | 33	Alexa Fluor 546, Alexa Fluor 555, TRITC, Cy3, PE, mKO, mOrange, TR, Alexa Fluor 568, Alexa Fluor 594, mCherry, Cy3.5
Far Red	635 | 22	680 | 40	Alexa Fluor 647, APC, Cy5
Near-infra red (NIR)	730 | 40	810 | 90	Alexa Fluor 750, Cy7

Table 1: Overview of the different fluorescent and non-fluorescent channels with corresponding excitation and emission wavelengths for the 5 fluorescence channel option, and a non-exhaustive list of available fluorochromes.

	Objective	Illumination	Numeric Aperture	Working Distance(mm)	Resolution per pixel	Application Examples
Fluorescence	1.25×	BF and FL	0.04	5.0	8 µm	Low magnification cellular overviews
	2×	BF and FL	0.08	6.2	5 µm	Large organoids or colonies
	4×	BF, PhC and FL	0.13	17	2.5 µm	Stem cells, clone picking, spheroids, organoids
	10×	BF, PhC and FL	0.30	10	1 µm	Cell line development or single cell cloning
	20×	BF, PhC and FL	0.45	6.6 – 7.8	0.5 µm	Intracellular applications
	40×	BF and FL	0.60	2.7 – 4.0	0.25 µm	Intracellular applications
Non-fluorescent	1.25×	BF and FL	0.04	5.0	8 µm	Low magnification cellular overviews
	2×	BF	0.06	5.8	5 µm	Large organoids or colonies
	4×	BF and PhC	0.13	16.4	2.5 µm	Stem cells, clone picking, spheroids and organoids
	10×	BF and PhC	0.25	8.8	1 µm	Cell line development or single cell cloning
	20×	BF and PhC	0.40	3.2	0.5 µm	Intracellular applications
	40×	BF and PhC	0.55	2.2	0.25 µm	Intracellular applications

Table 2: Overview of the different fluorescence and non-fluorescence objectives, with corresponding numeric aperture and working distance specifications. BF: Brightfield, FL: Fluorescence, PhC: Phase Contrast.

Microscope Stage

The microscope stage contains two motors for movement along the X and Y axis and can be moved automatically within the software. In addition, the stage can also be manually positioned using the joystick to rapidly identify individual cells or colonies of interest.

Stage Delay

During the scanning process, liquid within the source plate can move as the position of the microscope stage is altered, the extent of which is also dependent on other variables, such as well size, depth and medium volume. In order to obtain homogenous overview scans with phase contrast, it is therefore necessary that the medium layer remains of a uniform thickness within the whole well. By introducing a manually adjusted delay between the stage movement and subsequent image recording, a consistent liquid level can be maintained in the well to produce optimal images irrespective of well size or medium volume.

Microscope Stage Adapters

A key feature of the CellCelector is that all common forms of labware can be used to culture cells or colonies for subsequent picking. No unique or expensive consumables are required. Consequently, a wide range of microscope stage adapters have been developed to ensure maximum flexibility (Table 3).

At least one adapter is required to facilitate scanning. Adapters include:

- Petri Dish Adapters
- Standard Microplate Adapters
- Nanowell Plate Adapters
- Glass Slide Adapters

All adapters are precisely inserted into the stage using grub screws with a spring stop, and are specifically constructed to securely hold all sizes of microtiter plates and petri dishes. These features are necessary to prevent any unintended movement of the source vessel on the stage and facilitate successful imaging and object transfer.

Adapter Type	Size Specifications	Adapters can Hold
Petri Dish	35 mm	Two Petri Dishes with diameters of between 32 – 40 mm
	50 mm	One Petri Dish with a diameter of between 48 – 50 mm
	60 mm	One Petri Dish with a diameter of between 52 – 55 mm
	89 mm	One Petri Dish with a diameter of between 82 – 88.5 mm
	90 mm	One Petri Dish with a diameter of between 87 – 88.5 mm
	93 mm	One Petri Dish with a diameter of between 86 – 92.5 mm
	100 mm	One Petri Dish with a diameter of between 97 – 100.5 mm
Microplates	1, 6, 12, 24, 48, 96 or 384 well plates	One standard microplate
Nanowell Microplates	6, 12 and 24 well plates	One Nanowell plate
Slides	Standard glass slides	Up to 4 standard glass slides on the standard microplate stage adapter
MagnetPick Slides	Magnetic Slides	Adapter to place one MagnetPick Glass slide into the standard microplate stage adapter
Custom	Custom adapters can be built based on user specifications and requirements	

Table 3: Overview of the different stage adapters available to facilitate use of all common plastic labware.

CellCelector Imaging and Image Analysis

Selecting and Defining New Plates

A wide variety of commercially available or custom designed source plates can be used on the CellCelector, negating the need for purchasing expensive instrument-related consumables. Pre-defined plates can be chosen in the software from a plate library, or new plates (symmetrical or non-symmetrical) created by the user. Plate calibration is easily performed in the software to ensure maximum scanning accuracy.

Well Scanning

In many cases it may not be possible to capture whole wells with a single image, with mutiple images therefore required to create a full overview image. Wells are scanned in either a horizontal or vertical comb or meander sequence, with a 20 µm default overlap between sequential images to ensure the absence of potential blind spots within a well. This overlap can, however, be decreased to 0 µm depending on user specifications. Images are then sequentially processed in the order they were taken to create whole well images, ensuring the highest possible well-to-well resolution and allowing for a more accurate cell identification and transfer. Different masks, thresholds or filters can then be applied to individual or whole well images.

Autofocus

Automatic focusing is an exceptionally powerful tool and is achieved by analyzing a range of different focal planes surrounding the sample to determine the optimal focus for each image (Fig. 1).

Use of the autofocus is always recommended for the scanning of specimens with higher magnifications, and for plates or wells with uneven bases. Different autofocus options can be selected within the software, including:

- **3-point-focus:** where the best focus is determined at 3 positions within the well
- **Focus maps:** where the optimal focus is determined within a well based on a pre-set number of positions
- **Center of well autofocusing:** where only one focus point is determined in the center of the well, and is useful for small wells with homogenous bases

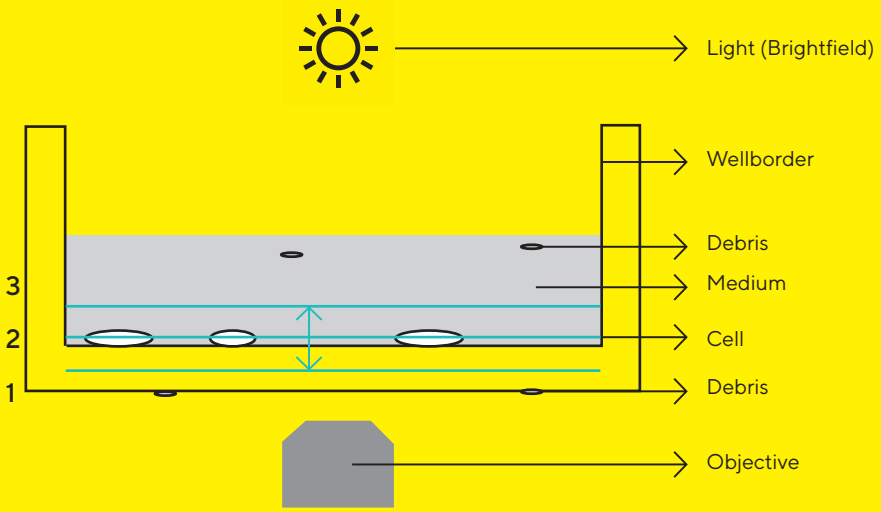


Figure 1: A graphical view of the autofocus function. The following focal planes are depicted: (1) the underside of the well bottom, (2) the inner well bottom containing cells, and (3) the surface of the medium. The system automatically searches for an optimal focus between the blue lines, which represent the focus range.

Image Generation – Live Images and Snapshots

Live images can be observed when the camera is activated, allowing snapshots of the current image to be taken. Snapshots can then be taken with all specified imaging configurations, such as fluorescence and phase contrast, and overlayed to create a final overview. The snapshot manager lists all available snapshots from each assay, allowing for a rapid overview of multiple plates, wells, or specific areas of interest.

Based on the live image, the exposure time can be automatically set so that optimal results are achieved at the current stage position.

Exposure Time

The exposure time refers to the length of time when a camera's shutter is open for image recording. With longer exposure times, more light will be collected by the CCD photo sensor, in making the image brighter, intensifying both the signal and background. However, for weak signals, increasing the exposure time can increase signal brightness, as real signals are captured and poor signals are not intensified, resulting in no additional increase in image noise.

Automatic Gain Display

Automatically optimizes the contrast of the image and enhances the visualization of poor signals in the live image.

Image Sharpness

Image sharpness for specific regions of interest or areas with poor contrast can be controlled and optimized during live image recording and is easily controlled using a slider bar, thus ensuring a more accurate and defined object picking.

Thresholds

Thresholds can be set for the automated detection of objects which meet defined intensity threshold values (Fig. 2). Reference images containing target objects are used to highlight all objects that meet threshold values, and thresholds can be adjusted at any time if too many or too few objects are detected. Lower and upper limit thresholds can easily be set for each channel based on specific assay requirements. Images are then reprocessed to highlight only those cells or objects which satisfy all defined criteria.

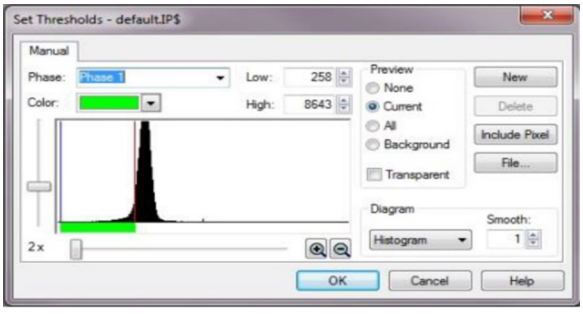
Measurements and Ranges

Further gates can be set based on particle information during the analysis stage. Common parameters include area, diameter, elongation, shape factor, sphericity, border distance, grey value mean and nearest neighbor distance variables. Over 140 different parameters are available with short descriptions provided for each parameter within the software.

Image Masks and Morphological Filters

Image masks can be created and optimized to exclude any interference with the detection of target objects. In addition, over 30 different morphological filters or processing algorithms can be implemented for enhanced image processing. Each filter examines the vicinity of a single pixel and analyses the shape of the object, before modifying, completing or removing image points, smoothing out irregularities, or removing single noise signals. Images can therefore be processed to provide as much – or as little – detail as desired (Fig. 3 and 4).

A



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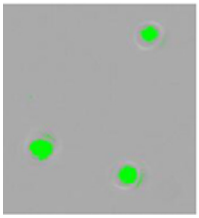

C

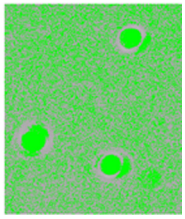

Figure 2: (A) Intuitive setting of image thresholds within the CellCelector software, (B) Thresholds set correctly with only cells being detected, and (C) Threshold set too high with background also detected.

Online Shading Correction

Shading Correction allows the correction of inhomogeneities or differences in the image illumination. The use of a shading correction is always recommended if a uniform image illumination cannot be achieved due to the oblique incident of ambient light or impurities in the system. Whilst implementation is very helpful for brightfield and phase contrast images, this function is normally deactivated in the case of fluorescence images. When the shading correction is activated, correction images are offset against the current image so that a homogenous image illumination is obtained.

Scan Shading Correction

In contrast to online shading correction, the scan shading correction is an image processing step that is applied during the scan - between image acquistion and storage. While online shading correction is normally deactivated in fluorescence applications, scan shading correction is recommended especially for weak fluorescence images with a high background. The use of scan shading correction can help to improve analysis and detection.

Particle Detection Options

Precise object detection is critical in order to achieve optimal picking results. Consequently, numerous particle detection parameters are available. In areas of increased cellular density, objects can be analyzed on a pixel-by-pixel basis to identify single cells. The outermost pixels are automatically analyzed to determine the sharing of adjacent borders or diagonal positioning (Fig. 5), whilst particles at the edge of scans can either be included, excluded or truncated, and objects with non-homogenous intensities can be filled to ensure the most accurate detection possible.

High Sensitivity Mode

The High Sensitivity Mode is often used for the gain of weak fluorescence signals or for specimens displaying a minor bleach, requiring only low intensity illumination. The activation of this function produces a digital gain of all signals that are received, and can be increased twofold, fourfold, or eightfold.

Image Overlay and Measurements

Additional information can be added to an image, such as annotations, measurements or shapes (Fig. 6). The addition of an overlay does not change the image and can be deleted or hidden at any time.

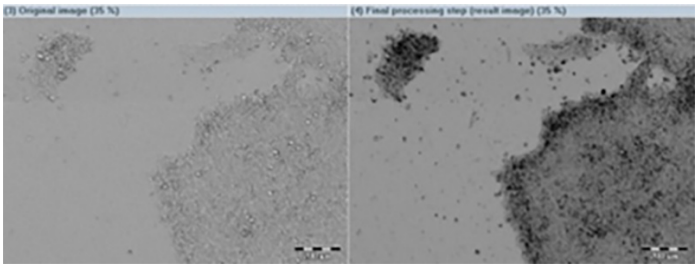

Figure 3: Use of an erosion filter on a stem cell colony.

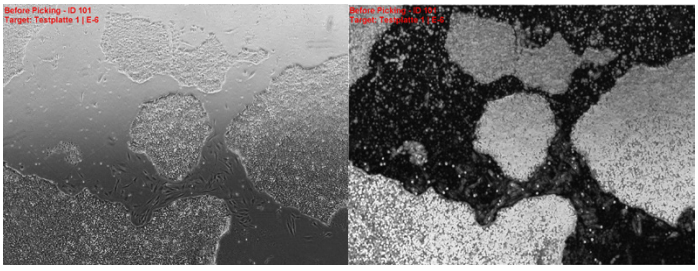

Figure 4: Use of a gradient filter on a human stem cell colonies cultured without feeder cells.

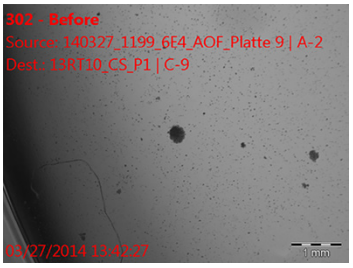

Figure 5: Image overlay with annotations describing source vessel, plate and well number, cell type, destination plate, date and time.

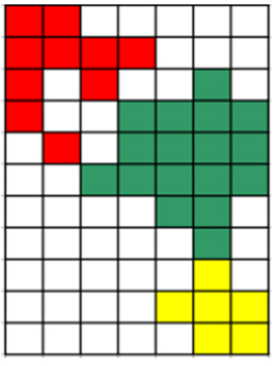

Figure 6: The Adjacent Borders standard setting means that only the particles with common borders will be considered as one particle. The image on the right shows the green and yellow areas which would be considered as a single object due to the common border, whilst the red particles would be considered as a different particle. If the "Include Diagonals" option is selected, a combination of the red, green and yellow areas would be considered as a single object.

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