

INSTRUCTIONS FOR COURSE WORK 4 (HIGH-CONTENT SCREENING)

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Purpose of the work

Become familiar with a high-content screening instrument and its software. Learn how to perform image analysis in HCS platform. Gain an understanding about the possibilities and limitations of the system.

Background

What is HCS: High-content screening (HCS) means cell-based high-throughput screening. In this technique, chemical compound or RNAi libraries are typically used for finding molecules/genes with desired cellular effects (cell division, expression of certain genes, morphology etc.). An automated fluorescence microscope and sophisticated image analysis software is needed for HCS. Automated sample preparation, image acquisition, analysis, and storage enable handling of very large numbers of samples and images much faster and with less errors than would be possible manually. The response of each individual cell is recorded, which means that the amount of data generated is very large. Different kinds of software tools are used for data analysis (e.g. statistics and visualization software).

How to perform HCS: Cultured cells are plated on 96- or 384-well plates, and each well is treated with a different chemical compound, RNAi, or some other treatment. After an incubation period, the cells are fixed and stained. A robotic arm will move the plates, one by one, onto the stage of an automated, inverted fluorescence microscope. The microscope will take several images of each well and the software will immediately analyze the images. Based on intensity thresholds, image pixels on each fluorescent channel will be segmented into being part/not being part of an object. Additional gating will separate the objects into positive (above a threshold) and negative (below a threshold) ones. As very large numbers of cells can be analyzed, it is possible to statistically analyze how the responses of individual cells differ from each other (for the same treatment). Also, genome-wide screens can be performed. For example, one may want to find all genes that affect expression of a certain gene. This may be accomplished by using a genome-wide RNAi library.

Instrument

Cellomics ArrayScan 4.5 high-content screening system

Software: Cellomics ArrayScan Reader, bioapplication: Target Activation

Sample

U2OS osteosarcoma cells were plated on a 96-well plate (Perkin-Elmer ViewPlate). FuGene transfection agent was used for expression of Green Fluorescent Protein (GFP) plasmid. GFP can be excited with blue light and it will emit green fluorescence. All cells were stained with Hoechst (fluorescent nuclear dye that will emit blue light when excited with UV light). GFP-transfected cells are in wells E8, E9, F8, and F9. GFP-negative cells (controls) are in wells E10, E11, F10, and F11.

Task

There is obviously not enough time for a real screening. Thus, this work will show how to **determine transfection efficiency**, which means the ratio of transfected (= GFP-positive) cells to total cell number in the sample. This is something most people need to do when they are setting up their HCS assays. The transfection efficiency is determined by using an algorithm (= bioapplication) that calculates the average fluorescence intensity for each area of interest.

Hoechst channel is used for segmentation, which in this case means determining which pixels belong to a nucleus and which ones do not. In other words, Hoechst-staining will determine the area for the cell nucleus. The number of nuclei equals the number of cells, as each cell is assumed to have just one nucleus.

GFP is expressed both in the nucleus and cytoplasm. If it is important to measure total GFP expression, then the area of interest for each cell has to be set accordingly. This is done by determining how many pixels the area have to expanded from the Hoechst-defined nucleus outwards. The area is correctly set when it matches the area where GFP is expressed (Fig. 1). However, for this work, we are only interested in the GFP expressed in the nucleus, so the area for GFP = Hoechst-stained area.

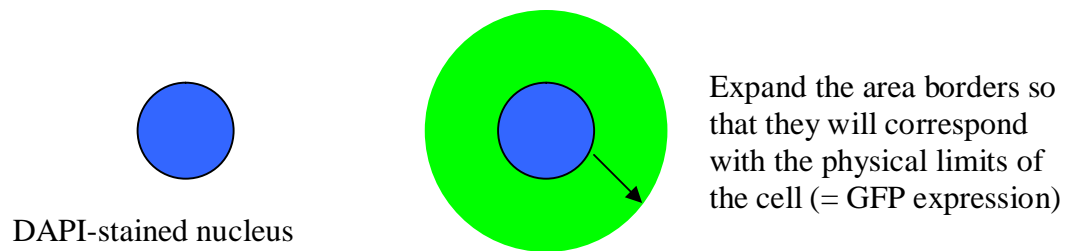


Figure 1. Setting the area borders for a typical cell.

The algorithm used (TargetActivation) will use a fluorescence intensity threshold value (set by the user) to determine which cells will be defined as GFP negative (= below the threshold value) and which ones are defined as GFP positive (= above the threshold value), see Fig. 2.

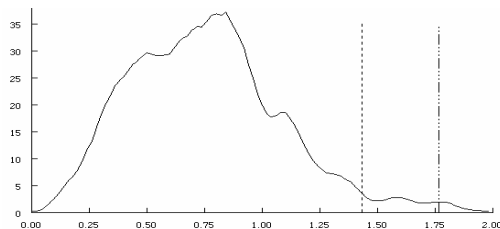


Figure 2. Two different thresholds are marked here. They will have to be tested using both negative and positive control samples.

The GFP intensity threshold should be set so high that in the negative controls, the algorithm will evaluate most of the cells as negative. However, if the threshold value is too high, some truly positive (= transfected) cells will be determined to be negative, and the threshold value should be lowered.

How the results should be presented: Percentage (%) of cells that are transfected in each well. This is determined as a ratio of GFP positive cells to the total cell number (Hoechst-stained nuclei = total cell number). The ratio will automatically be calculated by the software once the thresholds and other parameters are given.

Instructions for performing the work

CREATE PROTOCOL WINDOW (SETTING THE PARAMETERS)

1. Bioapplication and plate format. The ArrayScan instrument and the computer should be on. The ArrayScan Reader software should be open. The leftmost icon on the top menu bar should be selected (pressed down); if not, click on it. Bioapplication "MBI_Course_TA.V2_10x_p5.1_1x" (you may not see all of the text) and "Packard: PackardView96" plate format are selected. If not, please contact one of the course instructors.

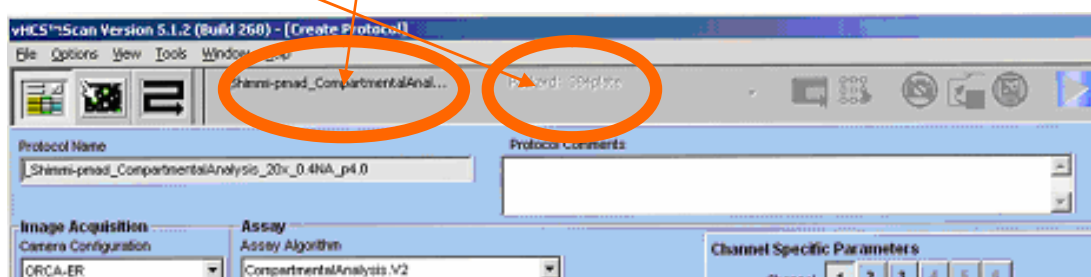


Figure 3. Create protocol window.

2. Saving your own bioapplication. Select "File" -> "Save Assay Protocol As" in the main menu. Change the bioapplication name so that instead of "Course" it will start with your name. Unmark the "Save As Read Only" box and click ok. Now you have saved your own bioapplication that you can modify.

3. Inserting the plate into ArrayScan instrument. In the main menu, select Tools/Maintenance/Move to load position. Wait until the calibration is finished. Open the blue lid on top of the ArrayScan instrument by gently pulling the small silver handle up (see Fig. 4). Take the 96-well plate that contains the samples (do not hold the plate at the bottom) and insert it without the lid into the plate holder (use the flashlight to see better). Make sure that A1 well is in the top left corner! The plate will feel loose but the instrument will secure it once you close the lid -just make sure that the plate is level.

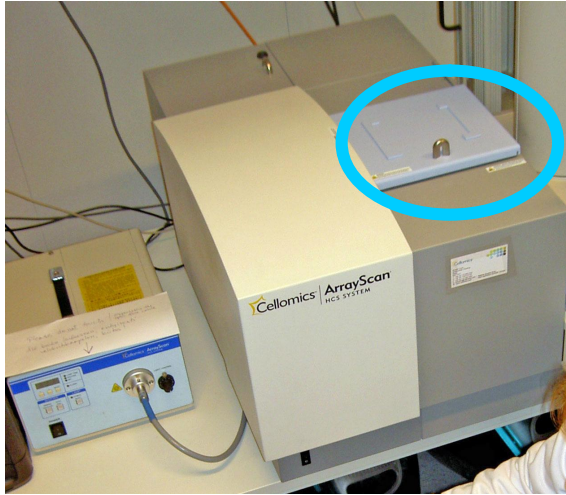


Figure 4. Lid of the scanning unit

4. Parameters in Create Protocol window. Keep all image acquisition, scan limits, and assay parameters as they are. They are listed here but there is no need to change anything.

Image acquisition

Camera configuration: Orca-ER

Objective: 10x

Acquisition Camera Mode: Standard

Autofocus Camera Mode: Autofocus

Intrawell Autofocus Interval: not selected

Scan limits

Max Fields per Well: 10 (maximum number of imaging fields that will be imaged per well; for a 10x objective, max 49 fields can be selected)

Min Objects per Well: 500 (how many Hoechst positive nuclei the algorithm should find per well before it will move on to the next well)

Min Sparse Fields per Well: 10 fields (how many sparse fields the algorithm accepts per well until it will move to the next well)

Min Objects for Field: 10 (if there are 10 or less nuclei in a field, it will be considered as a sparse field)

Intraplate Stopping Criteria

Not selected (unmark)

Assay

Assay Algorithm: TargetActivation.V2

No of Channels: 2 (Hoechst and GFP)

Focus Channel: 1 (Hoechst)

Assay Parameters: do not change the default settings at this stage

Default Well Feature: do not change

Channel Specific Parameters

Next to text "Channel", click on the box marked with 1 (for channel 1). For the "label" text box, write Hoechst or Nuclei. For dye, select XF100 - Hoechst. This is the filter needed for Hoechst. Mark the "Apply Illumination Correction" box (select Yes in the pop-up window) and leave the rest of the settings as they are. Check that Exposure Type is AutoExposure. This means that a well and field selected later is to be used for determining exposure time. That is fine for the Hoechst channel, as it is only used for object segmentation.

Now click on the box marked with 2 (next to text "Channel") and do the settings for GFP channel: write GFP for label and select XF100 - FITC(GFP) filter in the "dye" text box. For Exposure Type, select Fixed Exposure because now we want to keep the exposure time the same for all images. Only if the exposure time is fixed, we are able to compare fluorescence in different images. For Object Identification Method, select "None". This is because the objects (= nuclei) will be identified solely based on Hoechst staining.

Select "File" -> "Save Assay Protocol" on the main menu to save your settings. Click the second leftmost icon on the tool bar. This will open the Protocol Interactive window (Fig. 5).

PROTOCOL INTERACTIVE (SETTING THE THRESHOLDS)

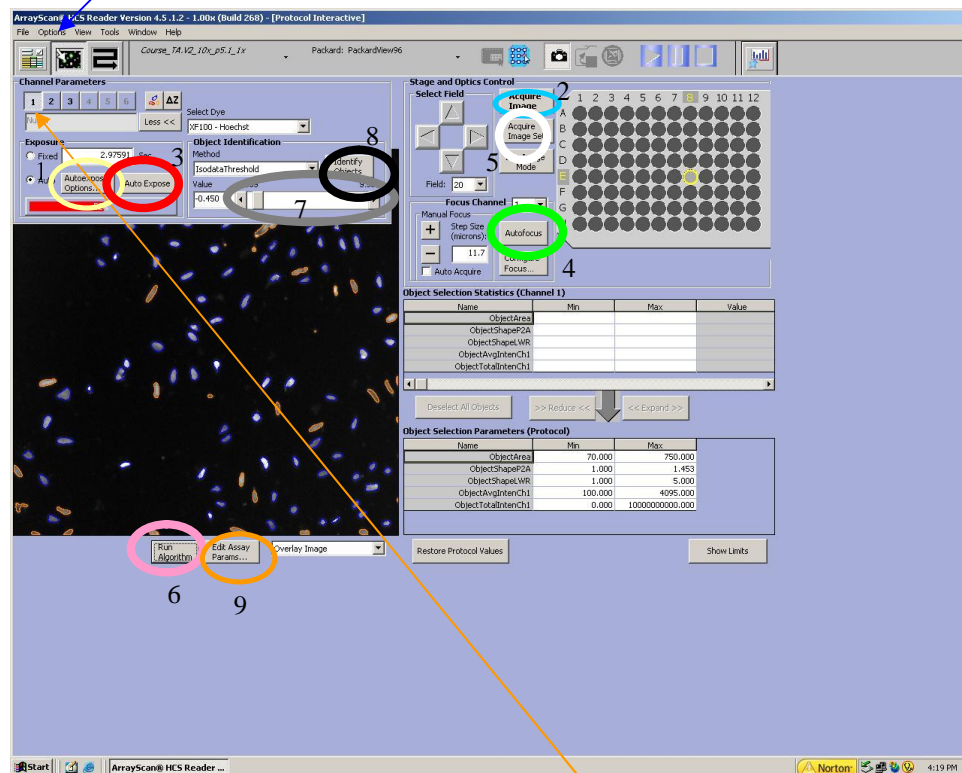


Figure 5. Protocol Interactive window.

5. Protocol Interactive window. Make sure that channel 1 is selected in Channel Parameters. Click "Autoexpose Options" Button (yellow circle [1] in Fig. 5) and

select E7 for well, and 1 for field, click ok. These settings will determine which well and field the software uses when it determines the exposure time for Hoechst channel.

6. Channel 1 acquisition settings. On the plate image, select E8 well by clicking on that well. Again, make sure that channel 1 is selected. Open the shutter in the light box (Fig. 6) and click "Acquire Image" button (blue circle [2] on the right in Fig. 5) for getting an image. You will probably not see much because the exposure time is not set and the image is not focused. To fix that, click "AutoExpose" button (red circle [3] in Fig. 5) on the left. Wait until the software is done or it may crash (if the software is busy, you will see an hourglass when trying to use the mouse). Then click the "AutoFocus" button (green circle [4] in Fig. 5) and again wait till the software has performed the autofocus. If the bar below AutoExpose is blue or red (= under/overexposed), click on the AutoExpose button again until the bar is green (exposure time ok). You should now see the Hoechst-stained nuclei.

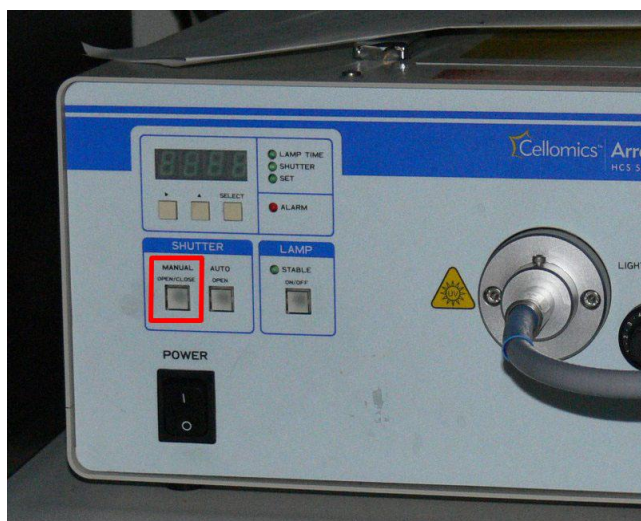


Figure 6. The light box and the shutter button.

7. Channel 2 acquisition settings. As fixed exposure time is used for GFP, it has to be determined. First, click the "2" button to select channel 2. Again, click "Acquire Image" to see the cells on green channel. How does the image look like? If too dim, increase the exposure time (write longer exposure time in the Exposure - Fixed text box). Click "Acquire Image" again. If the image is too bright, shorten the exposure time. The bar below AutoExpose should be green (not too close to saturation).

8. Acquisition of an image set and setting the threshold for channel 1. Once both Hoechst and GFP channels look ok, click "Acquire Image Set" button (white circle [5] in Fig. 5) to acquire images of both channels. Then select channel "1" and click "Run Algorithm" button (pink circle [6] in Fig. 5). The algorithm (= bioapplication that you are using) will show selected objects in blue and rejected objects in orange. Selected objects are nuclei whose average fluorescence intensity (in Hoechst channel) is above the threshold, and rejected objects are nuclei whose average intensity is below the current threshold. Also, nuclei that touch the borders of the imaging area are rejected.

The aim is to have all the nuclei selected (= blue). If that is not the case, you need to lower the threshold by dragging the slide bar above the image (grey circle [7] in Fig. 5) more to the left. Every time you want to see the effect of changing the threshold value, you must click "Run Algorithm" button again. If the blue line seems to be outside the true border of the Hoechst fluorescence, the threshold is too low, and have to be increased by dragging the slide bar to the right. If there are nuclei that do not separate well or some other unsatisfactory segmentation, it can be corrected using more advanced features of the software (which will not be covered here).

9. Setting the threshold for channel 2. This is a bit counter-intuitive; do NOT set the threshold value in the table "Object Selection Parameters" but follow these instructions:

- select channel 2
- choose a cell that is very weakly positive (= low intensity), point with your finger at that cell while clicking "Identify Objects" (black circle [8] in Fig. 5)
- click on the selected cell: you'll see its average intensity (= ObjectAvgIntenCh2) on the right in the "Object Selection Statistics" table. Now you know that your threshold value should be less than the intensity of your selected cell. Write down the value
- next, select a cell that is negative and repeat what you just did for the previous cell
- now, you know that your intensity threshold value for GFP should be higher than the value you got for the negative cell
- if you want to, you can select other weakly positive and/or negative cells to tighten and verify that your threshold value is set correctly
- once you are satisfied with the threshold value, click "Edit Assay Parameters" (orange circle [9] in Fig. 5)
- scroll down the list until you find "AvgIntenLevelHighCh2" and write the threshold value there. **Also write down the threshold value for yourself, you will need it in work 8**

Now you have set the threshold values for Hoechst and GFP and you are ready for scanning: click the third icon from the left on the icon bar to get to the Scan Plate window. Select "Yes" in the pop-up window.

SCAN PLATE WINDOW

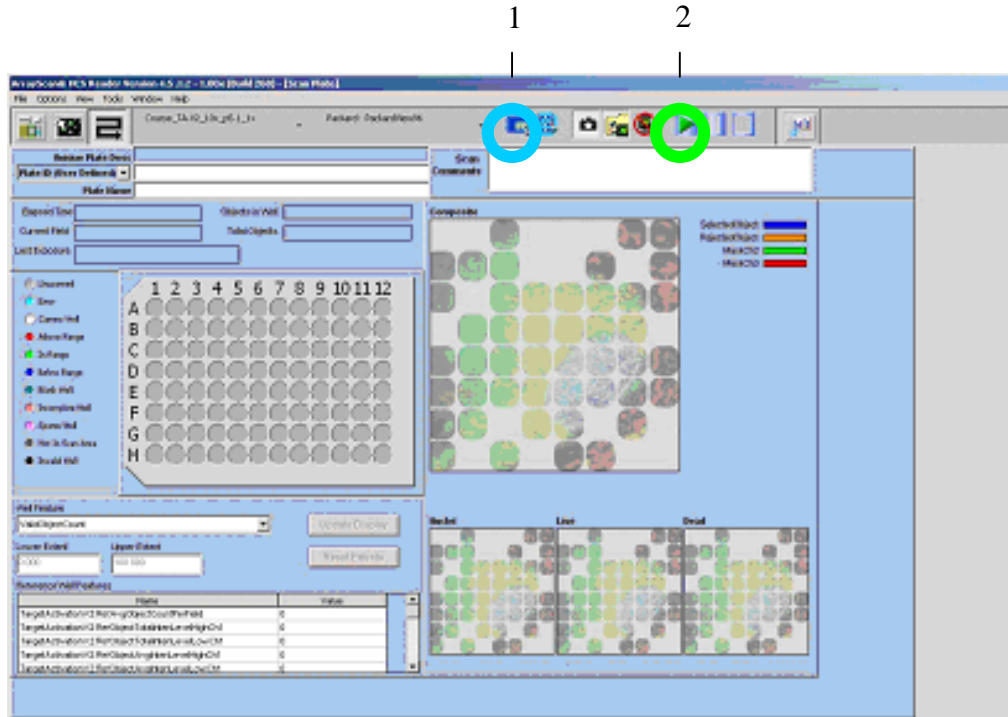


Figure 7. Scan Plate window

11. Selection of the scan area. Give a name for your scan by writing the name in the text box next to Plate ID. **Write down the name, you will need it for work 8.** Click on the blue icon to set the scan area (blue circle [1] in Fig. 7). Select wells E8, E9, F8, F9 for the scan area by dragging the mouse over selection. Selected wells are shown in gray (the yellow highlight just indicates the current cursor position, not selection). Click ok.

12. Scanning your sample. Click the Scan button (green circle [2] in Fig. 7). The scanning should start (click ok for the two warnings that will pop-up). The results will be automatically saved, and they will be analyzed in work 8.

Further information:

<http://www.miu.helsinki.fi/instruments/index.htm>

If you follow the ArrayScan link on that page, you will find more links related to HCS.