

# User manual for Cycler

Cycler is a method that constructs a trajectory of cell cycle progression (a Cell Cycle Trajectory or CCT) from fixed images of single cells growing in heterogeneous microenvironments, allowing the dissection of multiple deterministic sources of cell-to-cell variability.

## **Accompanying the publication:**

### Cycler: inferring a trajectory of cell cycle progression from fixed cell populations

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# **Material needed**

## **Step 1: Sample preparation**

### **EdU (5-ethynyl-2'-deoxyuridine)**

Click-iT® EdU Alexa Fluor® 647 Imaging Kit, Lifetechnologies

### **Paraformaldehyde**

Electron Microscopy Science

### **Triton X-100**

### **Bovine Serum Albumine (BSA)**

Sigma Aldrich

### **Phosphate Buffered Saline (PBS)**

### **DAPI (4',D-diamidino-2phenylindole)**

### **Alexa Fluor® 647 NHS Ester**

Invitrogen

### **Carbonate Buffer**

1.95 ml of 0.5M NaHCO<sub>3</sub>, 50µl of 0.5M Na<sub>2</sub>CO<sub>3</sub> in and 8ml of water for 10ml of buffer

### **1x Microscope**

## **Step 2: Image Processing & Quantification**

### **CellProfiler1**

cellcycler.org or <http://cellprofiler.org/previousReleases.shtml>

### **In-built CellProfiler modules**

cellcycler.org or <http://cellprofiler.org/previousReleases.shtml>

### **Custom CellProfiler1 modules**

cellcycler.org/Downloads

### **1x Computer**

## **Step 3: Build a Cell Cycle Trajectory**

### **MatLab**

[http://www.mathworks.com/downloads/web\\_downloads/](http://www.mathworks.com/downloads/web_downloads/)

ask your IT-support for licenses

### **Cycler**

cellcycler.org/downloads

### **1x Computer**

## Step 1: Sample Preparation

1. Grow your cells to desired confluence.
2. Add EdU at a final concentration of 200 $\mu$ M to your cells and incubate for 15min at 37°C and 5% CO<sub>2</sub>.
3. Fix cells at 4% Paraformaldehyde for 15min.
4. Thoroughly wash cells with PBS.
5. Permeabilize cells with 0.5% Triton X-100 for 15min, then wash once with 3% BSA solution.
6. We adapted the manufacturer's protocol for fluorescent labeling of incorporated EdU, to perform the click reaction with residual volume in a multi well plate (see Excel file in the Downloads section or below).
7. Mix the Click-IT Reaction Cocktail as explained on the Excel spread sheet and add it quickly (within 15min) to your cells.
8. Incubate for 30min at room temperature.
9. Thoroughly wash cells with PBS.
10. Perform immunofluorescence protocol against antigen of interest.
11. Thoroughly wash cells with PBS.
12. To stain the nuclei of cells, add DAPI at a final concentration of 400ng/ml to your cells and incubate for 10min at room temperature.
13. Thoroughly wash cells with PBS.
14. Wash cells 3 times with carbonate buffer.
15. To stain the outline of cells, add Alexa Fluor® 647 NHS Ester at a final concentration of 0.3 $\mu$ g/ml in carbonate buffer to your cells and incubate for 5min at room temperature.
16. Thoroughly wash cells with PBS.
17. Sample is ready to be imaged.

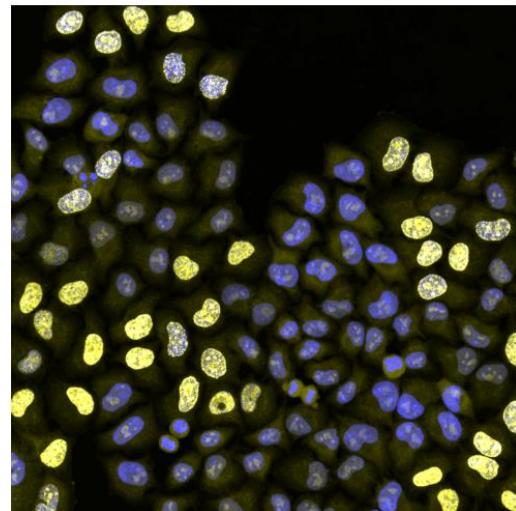


Figure 1 | Cell population stained for DNA (blue signal) and DNA replication (strong yellow nuclear signal) and cell outline (weak yellow cytoplasmic signal)

## Step 2: Image Processing & Quantification

1. Copy all images of the experiment you want to analyze with Cycler into one folder (e.g. CyclerExp01).
  2. Generate a new folder in your "CyclerExp01" folder (e.g. RESULTS), in which CellProfiler will save the measurement results.
  3. Start CellProfiler via MatLab.
  4. Parse the path of "CyclerExp01" in "Default image folder" (see Slide 1.1).
  5. Parse the path of "RESULTS" in to "Default output folder" (see Slide 1.1).
  6. Load Example\_CyclerPipeline.mat into CellProfiler (see below, Slide 1.2).
  7. Specify expression to load images with the DAPI (e.g. "C01.") and the EdU/cell outline staining (e.g. "C04.") and give the images a comprehensive name (e.g "OrigBlue" for DAPI & "OrigFarRed" for EdU/cell outline, see Slide 1.3).
  8. For optimal detection of nuclei and cell outlines, smooth both "OrigBlue" and "OrigFarRed" using median filtering ("SmoBlue" & "SmoFarRed"). We use a filter size of 5 (for 40x magnification), however this value should be adapted to your individual data set (Slide 1.4).
  9. To detect nuclei in your images, select the smoothed DAPI image ("SmoBlue"), specify the name of the detected objects as "Nuclei". We use [100, 100] as Min and Max values for the typical diameter. Our favorite thresholding method is "OtsuGlobal" with a threshold correction factor of 0.8, however these settings might need to be optimized for your personal image set (see Slide 1.5).
  10. Specify "Nuclei" as primary objects from which to detect cells in your images. The detected secondary objects should then be called "Cells" using the smoothed EdU/ cell outline channel ("SmoFarRed") using "OtsuGlobal". You can add/delete threshold correction factors, for our images these are however the optimal settings (see Slide 1.6).
- Importantly** you need to provide the intensity value of the "Background" in your "SmoFarRed" image. For this you measure the intensity of a reagion in your "SmoFarRed" image where there is no cell. This value must then be divided by 65535 for 16bit image, 4096 for 12bit images and 256 for 8bit images.

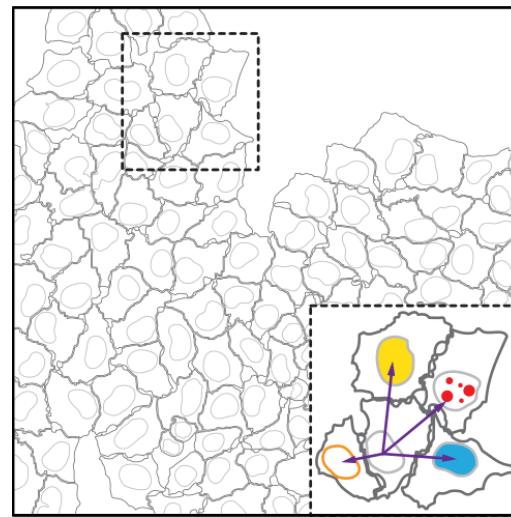
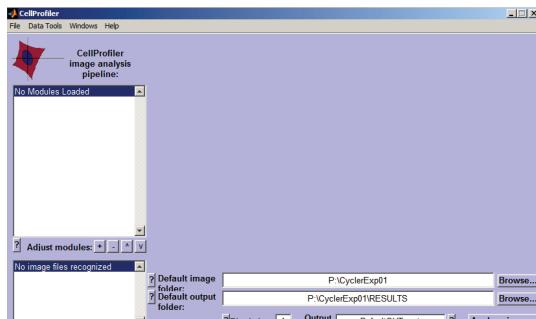


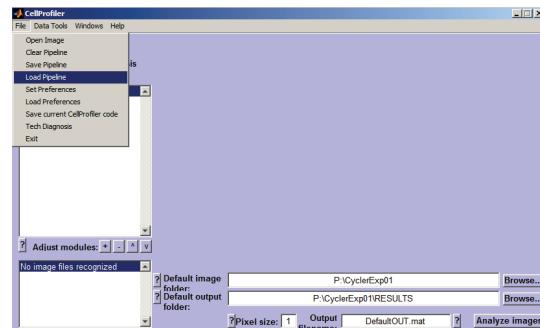
Figure 2 | Image processing and Quantification with CellProfiler 1

11. To measure signal intensities of your segmented objects, select first the channel you want to measure (e.g. "OrigBlue") and then the objects for which you want to measure the intensities ("Nuclei" & "Cells").  
If you want to measure multiple channels (e.g. "OrigBlue" & "OrigFarRed"), you need to add for each channel an additional "MeasureObjectIntensity"-module (see Slide 1.7).  
**Importantly** always measure object intensities in non-smoothed images!
12. Measuring texture is important for the construction of a CCT and the classification of the cell cycle phases. Specify the channel (e.g."OrigBlue" & "OrigFarRed") for which you want to measure the texture within objects (e.g. "Nuclei"). We use 5 as a scale of texture (at 40x magnification), this value might need to be adjusted to your personal image set (see Slide 1.8).  
**Importantly** always measure object intensity textures in non-smoothed images!
13. Specify objects (e.g. "Nuclei" & "Cells") for which you want to measure a set of morphological features. To build a CCT you are **not required** to calculate the Zernike features (see Slide 1.9).
14. Use the ExportToCycler- module at the end of your pipeline to export your single-cell measurements from CellProfiler1 to Cypler. Specify how you want to call the export file (e.g. "Exp01") and for which objects the measurements should be exported (e.g. "Nuclei" & "Cells", see Slide 1.10).
15. If wished, measure local cell crowding and correct nuclear area for effects caused by local cell crowding by calling the MatLab function „CalculateAndCorrectForLCC“. The only input argumnt required is the path to your data set which has been generated by the CellProfiler module „ExportToCycler“, which you will find in the „RESULTS“ – folder if you followed the instructions.

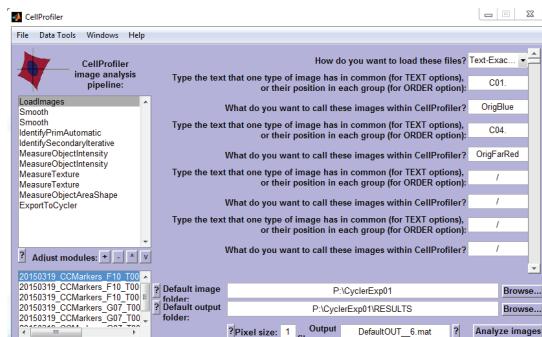
# Slide overview Step 2: Image Processing & Quantification



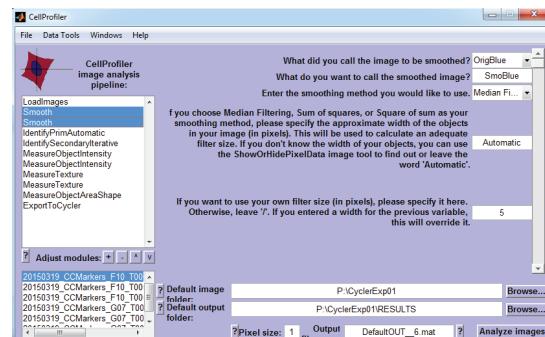
Slide 1.1 | Parse project path into CellProfiler1



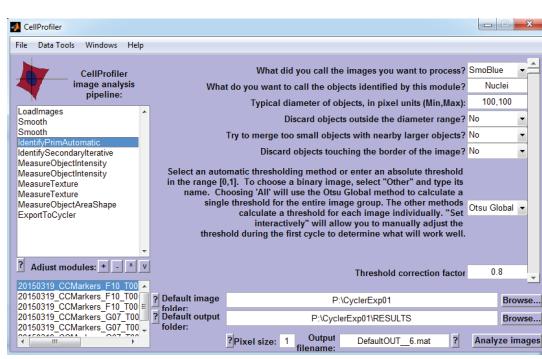
Slide 1.2 | Load pipeline



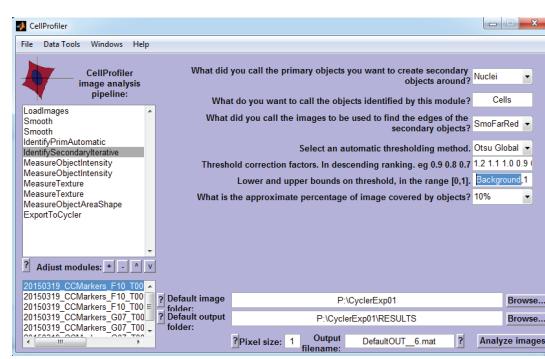
Slide 1.3 | Load images in to CellProfiler1



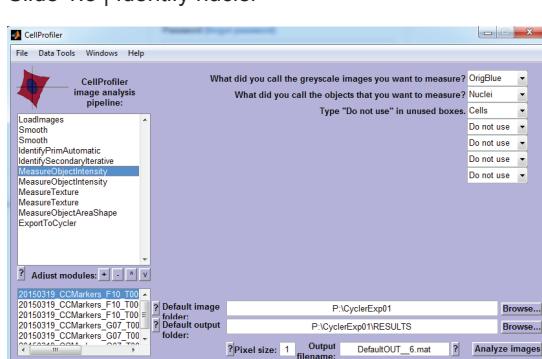
Slide 1.4 | Smooth images



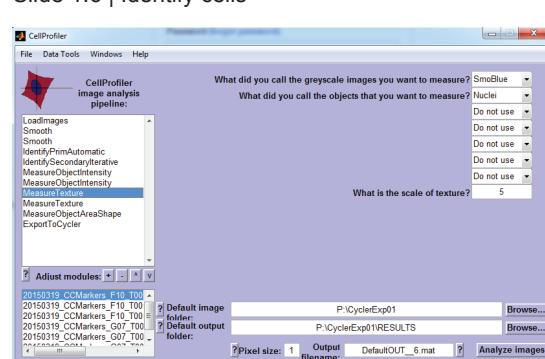
Slide 1.5 | Identify nuclei



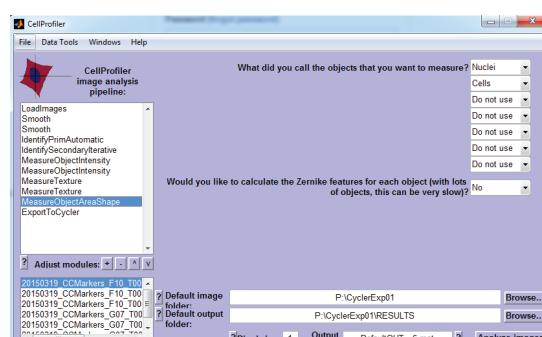
Slide 1.6 | Identify cells



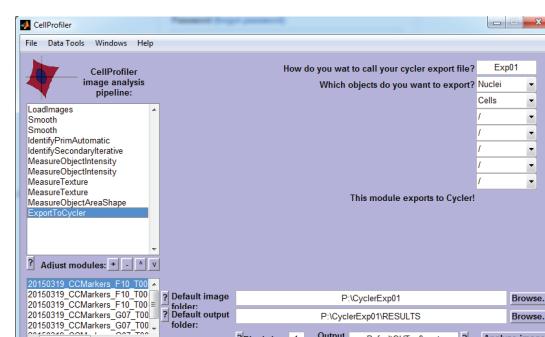
Slide 1.7 | Measure channel intensity in nuclei and cells



Slide 1.8 | Measure texture of intensities in objects



Slide 1.9 | Measure morphology of nuclei and cellist



Slide 1.10 | Export CellProfiler1 measurements to Cycler

## Step 3: Construction of a Cell Cycle Trajectory

1. Download Cybler (see Download section or below) and add it to your MatLab path.
2. Type "cybler" in to your MatLab command line to open the Cybler GUI.
3. To import your data set in to Cybler, click on the "Plus" button. Specify the path to the folder that contains your data set. Then double-click on your data set. Your data set will appear in the right section of the window. Hit "Done" to import your data set, which might take some time depending on the size of the file (see Slide 2.1).
4. Once loaded, you will see the name of your data set appearing in the "Gates" section of the Cybler GUI (see Slide 2.2).
5. Select the tricolored circle to classify the cells into cell cycle phases and to discard outliers (see Slide 2.3).
6. Once finished, seven new entries will appear in the "Gates" section: G1, S, G2 comprise each the cells which have been classified as G1, S and G2 respectively. M1 consists of mitotic cells in prophase and metaphase. M0 includes anaphase and telophase cells, as well as cells in cytokinesis. Interphase is a combination of the G1, S and G2 cells and is the gate you will use to construct a Cell Cycle Trajectory (CCT). „Start“ is a small subpopulation of G1 cells, from which you will start the Cybler algorithm (see Slide 2.4). Additionally, you will find 5 entries added to the "Channel" section called G1, S, G2, M0 and M1. You can later overlay the CCT with the cell cycle classification, to aid the biological interpretation of cell activities plotted along the CCT (see Slide 2.5).
7. To construct your CCT: First, select the "Interphase" gate. Then, keep the "CTRL"-key for Windows, "Cmd"-key for MacOS pressed, while you select the features in the "Channels" section, to construct a CCT. The features are:  
Intensity\_OrigBlue\_Nuclei\_1\_IntegratedIntensity  
Intensity\_OrigFarRed\_Nuclei\_1\_IntegratedIntensity  
Texture\_OrigFarRed\_Nuclei\_5\_InverseDifferenceMoment

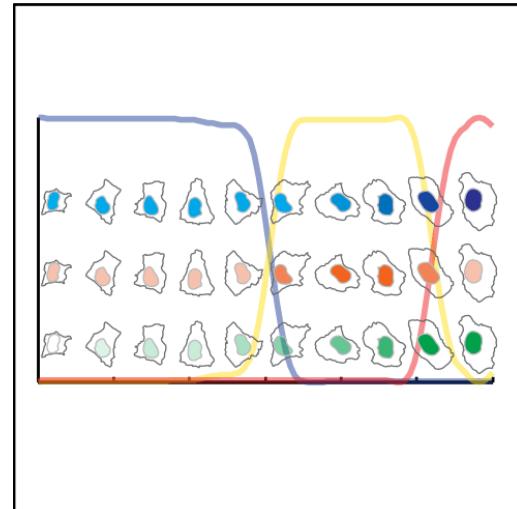


Figure 3| Cybler constructs a Cell Cycle Trajectory (CCT), which orders single cells to represent the cell cycle. The lines represent the cell cycle phases (G1 in blue, S in yellow, and G2 in red). The nuclei of the cells plotted along the CCT show the dynamics

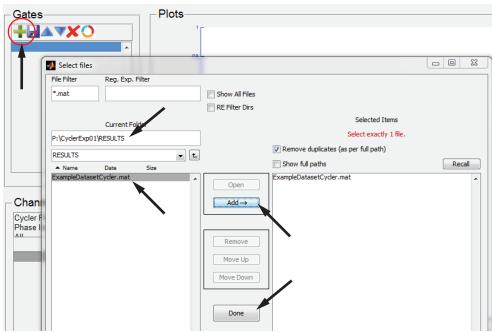
Texture\_OrigFarRed\_Nuclei\_12\_InfoMeas1

NucAreaCorrectedForLCC

Then, right-click on any of the above mentioned features and a small pop-up menu will appear, from which you select "Cycler" (see Slide 2.6).

8. The appeared window will allow you to specify the run parameters of the Cycler algorithm. First, select the "Start" gate as your starting population in the section called "Select Gate of Starting points" in the left part of the window. You can now also tweak the Cycler parameters in the right part of the window (see Slide 2.7). However, we encourage you to use the one provided, since we have experienced them to be the most robust over several experiments in different cell lines.
9. Once you are ready, hit the "Run" - button and Cycler will construct your CCT (see Slide 2.8).
10. Be patient, as Cycler is working.  
The bigger your data set, the more landmarks you select (default is 100), or the more runs you ask Cycler to perform on your data (default is 10), the more time the construction of the CCT will take.
11. Once Cycler is done, a new entry called "cct" will appear at the bottom of the "Channels" section of the "Interphase" gate, which is your new Cell Cycle Trajectory (see Slide 2.9).
12. You can now plot any single-cell feature you want along the CCT by simply selecting your feature of interest in the "Channels" section, selecting the "Plot along CCT" option in the drop-down table and hitting the "Plot" button (see Slide 2.10).
13. You can combine multiple features along the CCT by simply pressing down "CTRL"/"Cmd"-key and selecting multiple entries in the "Channels" section, such as "Intensity\_OrigBlue\_Nuclei\_IntegratedIntensity", "G1", "S" and "G2".
14. To save your plotted cell activity along the CCT as a pdf or png file, hit the "save figure" button and specify the name and the location to save your figure (see Slide 2.11).
15. Don't forget to save your session, in order not to lose your progress. Click on the "Session" tab and select "Save Session", specify name and location to save your session (see Slide 2.12).
16. Once saved, you can load your old session by simply clicking on the "Session" tab, select "Load Session" (see Slide 2.13\).
17. Enjoy using Cycler!

# Slide overview Step 3: Build a Cell Cycle Trajectory



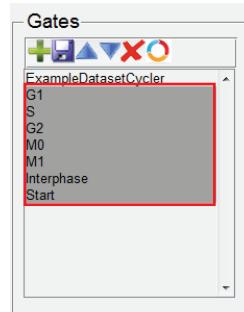
Slide 2.1 | Import data set into Cybler



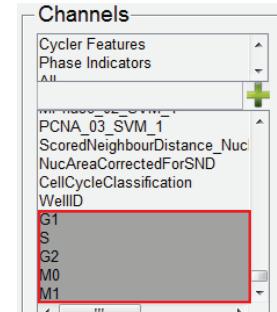
Slide 2.2 | Gate list with imported data set



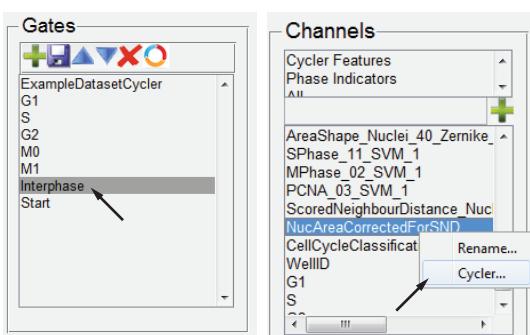
Slide 2.3 | Unsupervised cell cycle phase classification



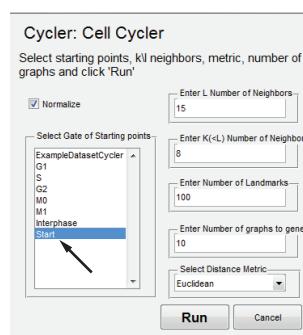
Slide 2.4 | new CCP



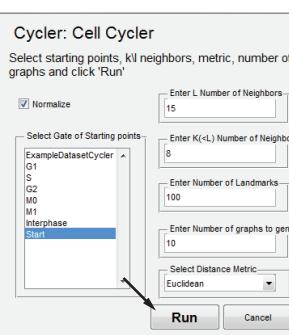
Slide 2.5 | new features



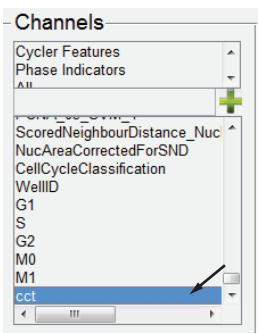
Slide 2.6 | Feature selection for Cycler algorithm



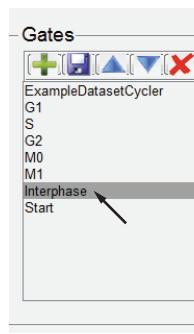
Slide 2.7 | Input parameters



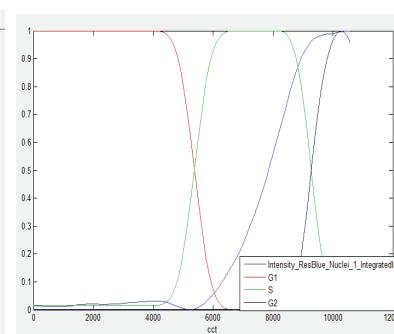
Slide 2.8 | Run Cycler



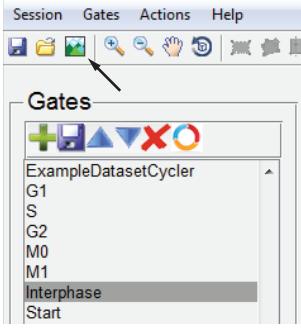
Slide 2.9 | New CCT



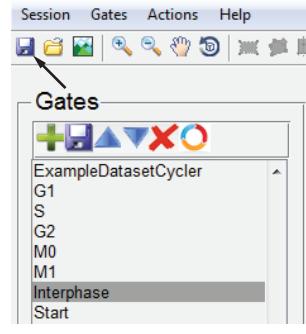
Slide 2.10 | Plot cell activities along the CCT



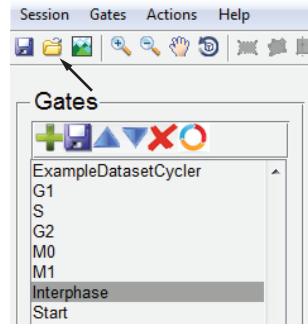
Slide 2.10 | Plot cell activities along the CCT



Slide 2.11 | Save figure



Slide 2.12 | Save session



Slide 2.13 | Load session