

# ***Pheno-seq manual***

## **A- Dispensing and imaging**

**Note: For imaging of the iCELL8™ nanowell chip, we used an inverted Leica SP8 confocal microscopy system with an automated stage and the Matrix Screener software extension. In general, also other automated confocal microscopes can be used, but it is highly advantageous to be able to correct for the focus position beforehand (see ‘predictive focus’, 10-12 below).**

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### **Before starting:**

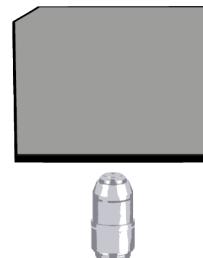
Turn on Leica SP8 and set to matrix screener (Leica Matrix Screener extension, custom-made template for 72x72 grid)

Change the experiment name (in tab “Adjust experiment”)

Prepare the chip holder as described below

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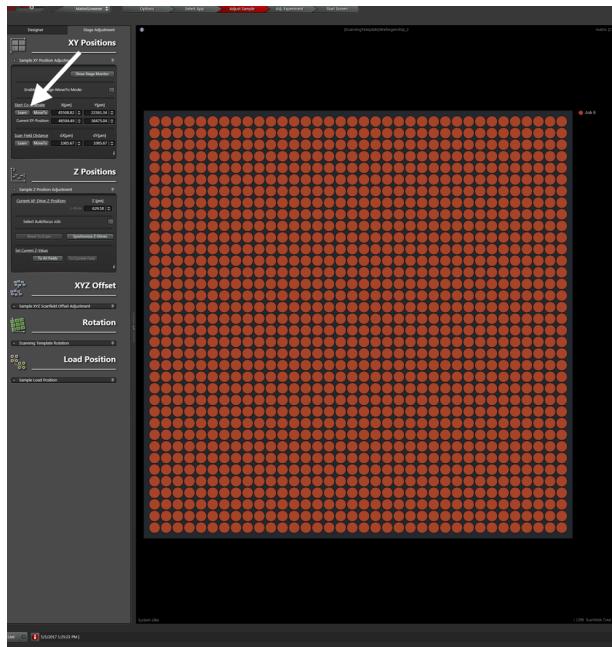
1. Dispense spheroids like single-cells (iCELL8 protocol).  
Note: to minimize the bias caused by sinking spheroids, mix wells manually with a 200 µl pipette tip after first and second round of dispensing
2. Blot chip and seal thoroughly with imaging film
3. Put the chip upside down into the chip spinner and centrifuge in cooled centrifuge (10°C) at 700g for 5 minutes with decelerated break
4. Keep chip upside down and put into the chip spinner. The chip must be fixed with tape. As shown in the picture below, the edge must be on the upper right side of the chip (orientation of chip is user-dependent but should be done consistently for image analysis workflow!)
5. Remove the protection film (not whole foil!)



6. Keep chip spinner (with chip) upside down and put into 96 well microscopy plate holder in inverted SP8 microscope. If you look from above, the edge

should on the upper left (Again: This is user dependent but should be consistent).

7. Focus manually (with ocular) on the upper left (where the edge is), so that you have the first 4 wells in one field of view.
8. Focus on computer and adjust position. Then go to tab “Adjust sample” and click on “learn” (without being in live mode).



9. Enable the stage-move-to mode and check at least the positions on the very right and at the bottom, to check if spacing is correct, if not adjust ...
10. For using the predictive focus: go to tab “Start screen”, then “autofocus” left side, most left sided tab. Then click on the last point in the list “Predictive focus”
11. Enable the stage-MoveTo Mode
  - a. Learn point 1 (upper left, optimally with spheres to focus on)
  - b. Learn point 2 (upper right, optimally with spheres to focus on)
  - c. Learn point 3 (lower left, optimally with spheres to focus on)
12. Calculate and set predictive focusmap (check with enabling the “Show focus map” button)
13. Check some positions and adapt imaging parameters in the adjust experiment tap (**not in job “matrix” but in “Job6”**)
14. Go to tab “Start screen”, click on “Run matrix” button

15. After imaging (~30 min with our settings), put chip in normal orientation into chip spinner and spin spheroids to the bottom in cooled centrifuge (4°C) at 700g for 5 minutes.
16. Freeze chip at -80°C.
17. After selection of spheroids by PhenoSelect, use the generated filter-file to dispense reagents into selected wells and proceed as indicated in the provided protocol for single cells (TakaraBio)

## B- Image pre-processing

1. Copy the automatically created folder (depending on the name set in “Adjust experiment”) and transfer to work station. The dataset should contain 1296 images (5184/4) per channel (Hoechst and CellTracker Red).
2. Open (the latest version) of the KNIME workflow  
“PhenoSeq\_Preprocessing\_SP8.knwf”

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### Notes for KNIME workflow:

Required KNIME version and extension:

KNIME Analytics Platform	3.5.2.v201802051426
KNIME File Handling Nodes	3.5.3.v201804031047
KNIME Image Processing	1.6.0.201712051444
KNIME Image Processing - ImageJ Integration (Beta)	0.11.4.v201712041649
KNIME Interactive R Statistics Integration	3.5.1.v201712211140

Nodes where changes should be effectuated are marked in **red**.

Nodes where changes might be necessary are marked in **yellow**.

Nodes allowing for checking of correct segmentation etc. are marked in **green**.

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3. Set the two first nodes (“List files”) to the path that contains the locally saved microscopy data on your computer.
4. Run the workflow until the first node after the second metanode named “crop wells and get correct names”.
  - a. **NOTE:** If the metanode “crop wells and get correct names” throws an error, change the manual parameter for the global threshold and try again.

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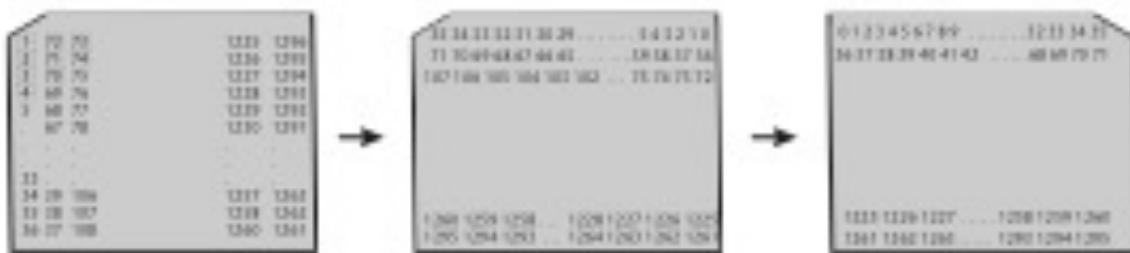
### Brief explanation of the algorithms behind the two metanodes:

#### 1. “rename rows and virtually flip chip”

orders images by their order of acquisition (given by the last number in the image name), the process is depicted by the figure below

one image still contains 4 wells

This procedure to have an output, which is comparable to the output when imaged with the default iCELL8 microscope (however 4 wells instead of 36 per field of view)



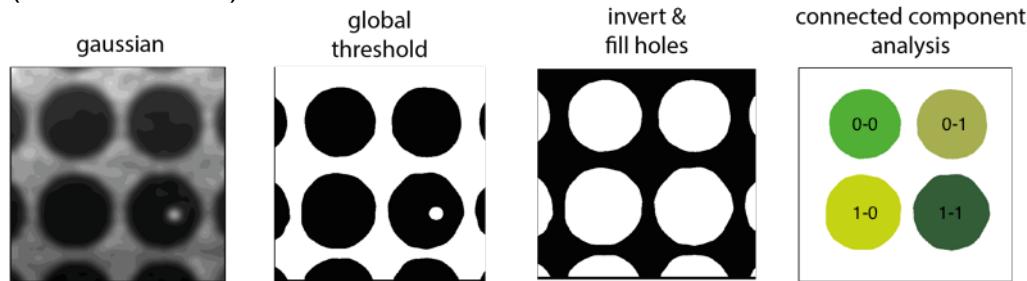
**Comment:**

The camera orientation of the used microscope should be checked beforehand. In our case, the acquired image was turned 90° compared to the “real” image. For correct well assignment, we therefore rotate all images individually 90° to the left in KNIME (ImageJ macro node). Importantly, this step is independent of the reassignment of the well positions.

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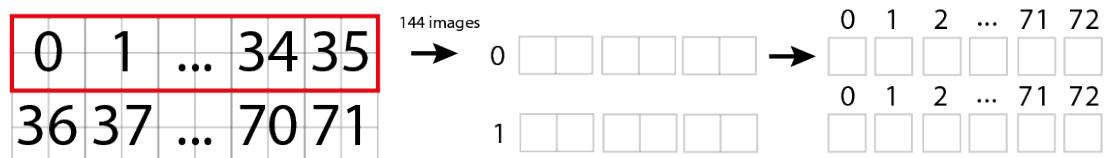
## 2. “crop wells and get correct names”

Note: This metanode will contribute the most to the final running time of the workflow (ca. 12 minutes)



A mask is built for each well based on the segmentation of the field of view containing the four most round segmentations (see workflow scheme above). This mask is applied for all wells, since we assume that their location within the field of view remains stable.

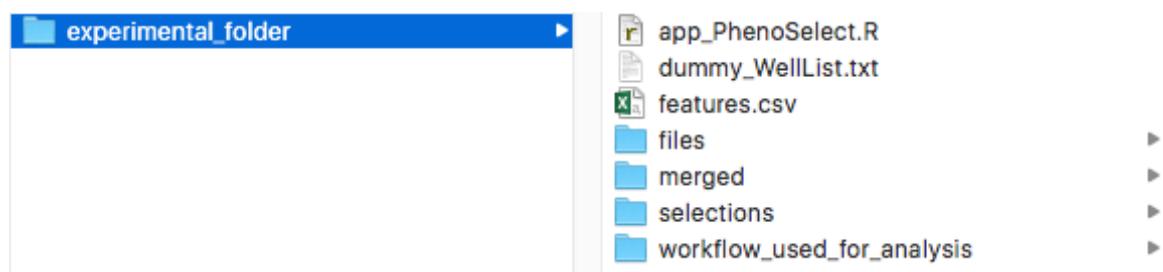
The first loop takes 144 wells ordered by position (“#Pos”, equals the field of views of the raw images), which means that it corresponds to two rows of wells (red border). The second loop takes first the upper row of the field of views, calculates the “real y” location taking into account the iteration and then adds the “real x” simply by order. Next, it takes the second row of the field of views and pursues in the same way.



Once the correct names were assigned, a joiner node adds the corresponding images in the other channel (e.g. CellTracker), to have images from both channels named correctly.

## 5. Run the rest of the workflow, but only after changing the nodes marked in **red**:

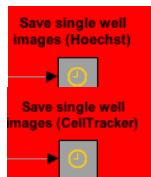
The file structure for all saved documents should be the following, since this is the structure needed for the shiny app. The shown folders should be created in a subfolder which is unique for each experiment (==chip):



- a.The .csv writer saves the 2D statistics for each segmentation (==spheroid) and gives also the respective position. This is the main input for the shiny image and corresponds to the “confocal\_spheroids\_features.csv” file shown above. The segmentation as well as the statistics are based on the CellTracker signal!



- b.These metanodes are a workaround to save the cropped images. The target file path has to be changed at the indicated position in the “Source/Target” node. Target path should be the “files” folder as displayed above.



```

1 // system imports
13 // Your custom imports:
14 import java.nio.file.Paths;
15
16 // system variables
25 // Your custom variables:
26
27 // expression start
29 // Enter your code here:
30
31
32 out_Source = Paths.get(System.getProperty("user.home"), "Desktop", "CellTracker_temp.png").toString();
33 out_Target = Paths.get(System.getProperty("user.home"), "/Documents/Uni/Master/MA/Data/FP59/files", "CellTracker_" + ROWID + ".png").toString();
34
35

```

- c. 1) Within this metanode change the location of the merged images that are merged in KNIME and then exported as .ics file. Target path should be the “merged” folder as displayed above.



## 2) Once exported: Open Fiji and go to Process > Batch > Macro

<input type="button" value="Input..."/>	iedrich/Documents/Uni/Master/MA/Data/FP59/merged/
<input type="button" value="Output..."/>	/friedrich/Documents/Uni/Master/MA/Data/FP59/files/
Output format:	PNG
Add macro code:	[Select from list]
File name contains:	.ics
<pre> run("Make Composite"); run("Stack to RGB"); </pre>	

Enter the parameters shown above (input: folder “merged”, output: folder “files”). If you click “Process”, all .ics files will be converted into .png files that can be shown in shiny.

### Important note:

Before closing KNIME, save the workflow (without data) under the folder “workflows\_used\_for\_analysis” in the experiment subfolder, to keep track of the analysis workflow.

## C- Choose wells with “PhenoSelect” Shiny application

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The following packages are needed and should be installed before running the app:

“**ggplot2**”, “**shiny**”, “**shinyFiles**”, “**DT**”, “**plyr**”, “**reshape**”,

### Tested with:

R version 3.4.  
ggplot2\_2.2.1  
shiny\_1.0.5  
shinyFiles\_0.6.2  
DT\_0.4  
plyr\_1.8.4  
reshape\_0.8.7

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1. The “PhenoSelect.R” file has to be in the same experimental folder as the other data (e.g. the “files” and “merged” folder, see data structure above).
2. Open “PhenoSelect.R” in your R environment of choice. Opening the file in RStudio is recommended.
3. Adjustment of the home path:

- a. Mac and Linux users:

Your home path should be automatically set. You do not have to change anything in the code. You will be able to select the experiment in the app.

- b. Windows users:

Replace ~ in “~” by your homepath in line 293 and 299 in the code (see example picture below).

```
289 <- server <- function(input, output) {  
290   # dir  
291   shinyDirChoose(input,  
292     'dir',  
293     roots = c(home = "~~"))  
294   dir <- reactive(input$dir)  
295   output$dir <- renderPrint(dir())  
296  
297   # path  
298   path <- reactive({  
299     home <- normalizePath("~~")
```

4. Before starting, check if experimental folder contains the following objects that are needed by the app:
  - a. PhenoSelect R file
  - b. The .csv file containing the image features as generated in KNIME
  - c. The files folder containing the images (Hoechst, CellTracker and merged, all as .png format)
  - d. dummy\_WellList.txt file containing the barcodes
  - e. (if already generated: the .RData file incl. tSNE map generated by the PAGODA software)

5. Open the R-file in R (-studio). Run the app, the application should automatically be displayed.
6. Select your home path to the experimental folder. Running the app in your browser with min. resolution of 2048x1080 px is recommended.
7. Enter the respective experimental details.
  - a. Choose directory of experiment to analyze
  - b. The associated .csv file should be automatically selected
  - c. Enter iCELL8 chip ID number
  - d. Optionally select cell type (For cell # reference)
  - e. Annotate samples
  - f. Then click on “Load Dataset”.

Select experiment    [Select wells](#)    [Selected for dispense](#)    [t-sne plot](#)

## PhenoSelect

Please select input data first.

[Choose directory of your experiment](#)

Choose home path  
[1] "/Users/Stephan/"

Please choose the .csv file containing image features

Please enter the chip number

Chip number	Select cell type
<input type="text"/>	<input type="text" value="HD1495"/>

Sample in well A1	Sample in well A2
<input type="text"/>	<input type="text"/>

Sample in well B1	Sample in well B2
<input type="text"/>	<input type="text"/>

Sample in well C1	Sample in well C2
<input type="text"/>	<input type="text"/>

Sample in well D1	Sample in well D2
<input type="text"/>	<input type="text"/>

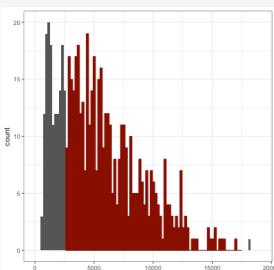
Please choose a file location!  
Please choose a .csv file!  
Please enter a chip ID!  
Please indicate all samples!

8. The tab “Select well” allows you to select appropriate wells to be sequenced (== those wells in the final filter file that will be given back to the Wafergen dispenser).
- You can choose to display all wells that contain spheroids (also multiple) or those that only contain one spheroid. **Note that wells can appear multiple times in the list if more than one spheroid has been detected.**
  - The histogram gives you the information about the distribution of image parameters within the dataset such as spheroid size and circularity. Furthermore, you can thereby select certain thresholds, which will be automatically applied to the dataset. Spheroids not matching these parameters will not be displayed anymore.
  - By clicking on a column name, the wells in the list can be ordered accordingly.
  - You can choose between the Hoechst, CellTracker channel and composite (overlay). The CellTracker channel is generally a bit “cleaner” and not as susceptible to imaging artifacts as the Hoechst channel
  - Include wells by clicking on the “Select for dispense”.
  - It is recommended to check all selected wells/spheroids for imaging artifacts or damaged spheroids**

Name of the Experiment:  
92544\_PhenoSeq\_CA\_DSP\_processed  
Chip Number:  
92544

Spheroids or controls?  
 Spheroids  
 Controls

Included samples: Wells displayed  
 MCF10CA\_5d  All  Single



Parameters to plot  
 Size  
 Circularit

Selected size:  
 2,487  30,000  
 3,006/0,012 12,024 18,026 24,048 30,000

Selected circularity:  
 0,1 0,2 0,3 0,4 0,5 0,6 0,7 0,8 0,9 1

Number of bins:  
 21 41 61 81 101 121 141 161 181 200  
 100 200

Select experiment Select wells Selected for dispense t-sne plot

Well: Image\_1\_18

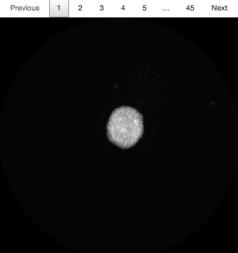
image_name	row	col	Area	Circularity	sample	estimated_cell_number	comment	
1	Image_0_12	0	12	10925	0.87	MCF10CA_5d	198	Empty
2	Image_0_17	0	17	7221	0.84	MCF10CA_5d	112	Empty
3	Image_0_2	0	2	9309	0.88	MCF10CA_5d	161	Empty
4	Image_0_43	0	43	8272	0.9	MCF10CA_5d	136	Empty
5	Image_0_48	0	48	11172	0.86	MCF10CA_5d	204	Empty
7	Image_1_11	1	11	5016	0.8	MCF10CA_5d	61	Empty
11	Image_1_18	1	18	12051	0.89	MCF10CA_5d	224	Empty
12	Image_1_19	1	19	4092	0.87	MCF10CA_5d	39	Empty
13	Image_1_20	1	20	10464	0.87	MCF10CA_5d	187	Empty
14	Image_1_22	1	22	8184	0.9	MCF10CA_5d	134	Empty

Show 10 entries Search:

Showing 1 to 10 of 441 entries Previous  2 3 4 5 ... 45 Next

Select for dispense

Displayed channel  
 Hoechst  
 CellTracker  
 Overlay



9. The “Selected for dispense” tab shows the currently selected wells. Here you can get an overview and curate selected wells

- The selected wells and their location in the chip are shown on the left
- You can save the filter file at any time (selection folder) and upload it again
- Image features (e.g. size, circularity) are plotted on the bottom right, and value for selected well/spheroid is shown as red dot (to select well click on its associated number on the left)
- On the top right, you can save the image feature file that also includes barcode information for demultiplexing (“Save selection as .csv”)

Choose filter file to upload

338 MCF10CA\_5d wells

sample  
● MCF10CA\_5d

Select experiment
Select wells
Selected for dispense
t-sne plot

Add comments here

Select a dataset

 Spheroids
  Controls

Save selection

Show: 10

image_name	row	col	Area	Circularity	sample	estimated_cell_number	comment	
1	Image_0_12	0	12	10925	0.87	MCF10CA_5d	198	Empty
2	Image_0_17	0	17	7221	0.84	MCF10CA_5d	112	Empty
3	Image_0_2	0	2	9309	0.88	MCF10CA_5d	161	Empty
4	Image_0_43	0	43	8272	0.9	MCF10CA_5d	136	Empty
5	Image_0_48	0	48	11172	0.86	MCF10CA_5d	204	Empty
7	Image_1_11	1	11	5016	0.8	MCF10CA_5d	61	Empty
11	Image_1_18	1	18	12051	0.89	MCF10CA_5d	224	Empty
12	Image_1_19	1	19	4092	0.87	MCF10CA_5d	39	Empty
13	Image_1_20	1	20	10464	0.87	MCF10CA_5d	187	Empty
14	Image_1_22	1	22	8184	0.9	MCF10CA_5d	134	Empty

Showing 1 to 10 of 338 entries

Previous 1 2 3 4 5 ... 34 Next

Well: Image\_1\_18

Unselect well

Displayed channel

 Hoechst
  CellTracker
  Overlay

Parameters to plot

 Size
  Cell number
  Circularity

Dataset to plot

 Selected wells
  Single spheroid wells
  All spheroids

Image\_1\_18

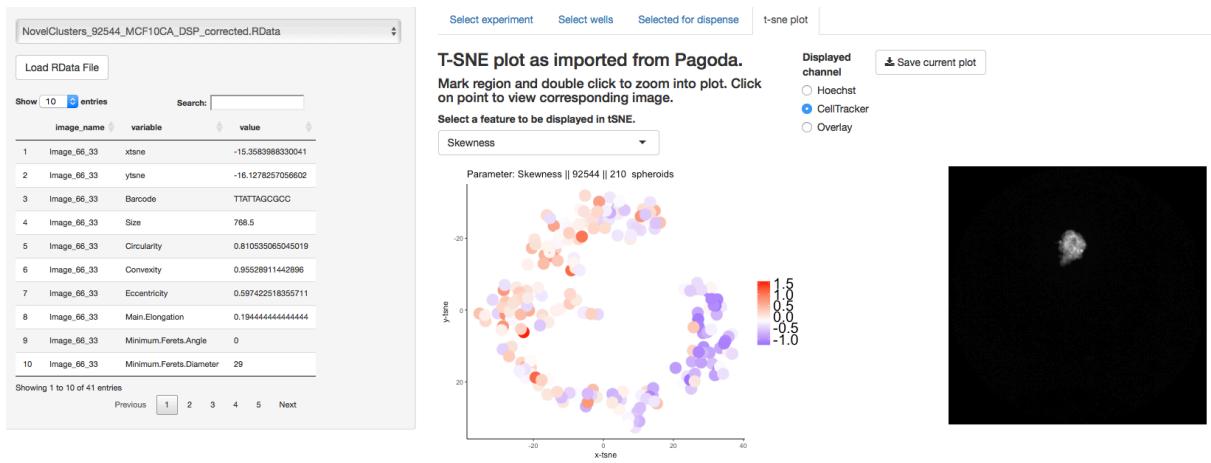
Circularity

MCF10CA\_5d sample

11

10. The “t-sne plot” tab enables the mapping of image features on an externally generated t-SNE map (in our case generated by PAGODA)

- a. Load PAGODA .RData file (Needs to be located in experimental subfolder)
- b. The tSNE map will appear after a few seconds
- c. You can select all measured image features and color the tSNE map accordingly
- d. Click on data points to visualize the respective well/spheroid
- e. Plots can be exported as .png.



11. Enjoy!