Imaging Protocol for NIS-Seq Optical Pooled Screens

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http://jsb-lab.bio/opticalscreening/

Place plate on Microscope:

→ Place a microscopy plate on the microscope stage, ensure that the plate is firmly aligned in the plate holder by positioning on the opposite site of the plate clamp

Switch on Instruments and start Software Interfaces:

→ In the following order switch on the Nikon microscope box, the microscope, the Lumencor Celesta, the Yokogawa W1 and the Hamamatsu OrcaFlash camera.
→ Open http://jsb-lab.bio/opticalscreening/jsMicroscope/

1. Startup - Connecting the microscope, laser unit and spinning disc unit:

→ Start the local camera webserver by double-clicking "Start camera.bat"
→ Connect the Microscope by clicking “Connect Microscope (choose Ti2)”
→ Connect the Lasers by clicking “Connect Lasers (choose *COM4*)”
→ Connect the W1 confocal unit by clicking “Connect W1 (choose *COM3*)”
→ After successful connection, the note “connected” appears below each button

2. Move to well - Control the microscope:

→ Choose your plate format
  - Certain plate types are preset, including
    - Ibidi μ-Plate 96-well (#89626)
    - PerkinElmer CellCarrier-96 Ultra microplates (#6055302)
    - Greiner Sensoplate 24-well glass bottom (#662892)

→ Navigate through the microplate by clicking the well, which is highlighted in red
  - Next to the plate layout displayed, you can navigate within the selected well by clicking on the corresponding position
  - A red crosshair displays the physical well position reported by the microscope

3. Focus – set the right focus position:

→ Select the desired objective
  - Certain objectives are already preset, including a Nikon 10X CFI P-Apo 10x Lambda (#MRD00105) and a Nikon 20x CFI P-Apo Lambda (#MRD00205) objective.
  - The microscope setup also contains a 1.5x tube lens; the software automatically identifies whether the lens has been inserted into the light path in microscope and displays the selected configuration.

→ Use the button “Auto-Start PFS” to automatically start Nikon’s “perfect focus system”

- → PFS focus position can be set manually under “PFS position”; press “snap image” to move to the corresponding position (for different wells distinct focus positions can be set for automatic imaging, separated by comma)
- → Manually adjust the focus position by choosing the buttons “-100”, “-30”, “-10” etc. until cells in displayed frame are in focus

4. Take a test image

- → Changing the channels in the drop-down menu, take a look at all channels you want to image.
- → If the brightness is too low, adjust the exposure time (must be multiples of 15 ms due to physical properties of the CSU-W1 and the software)
- → **IMPORTANT:** Before imaging the first cycle of NIS-Seq navigate to the center of your first well of imaging by clicking on the well on the plate layout
- → Set channel to “DAPI”, focus on the nuclei and press “save image” to take a reference image
    - o This image will be loaded as reference for perfect stage alignment before imaging consecutive NIS-Seq cycles in order to minimize the shift of stage positions
    - o Move the saved reference picture from the download folder to your experiment folder on a hard drive and name it “reference cycle 1”

5. (optional) Load a reference image from the same position and channel to fine-align the stage:

- → For NIS-Seq cycles 2-x, load the reference image of the nuclei taken before imaging cycle 1
- → Navigate to the center of the well where the reference was imaged
- → Click “fine-align stage" and wait until a pop-up alert appears, which takes around 30 seconds
- → The stage shift in pixels is displayed in the pop-up alert
- → Repeat alignment until shift in x and y position is max ~+/-10px, and the alignment looks nearly perfect when switching back and forth between the a new snap and the reference.

6. Image multiple positions and channels:

- → Enter the path of the folder where images will be saved
- → Enter the plate name
- → Enter wells to be imaged in “List of wells”
- → Adjust how many images will be acquired per well at maximum (80 by default = complete well)
- → Edge margin can be set to cut off tiles at the edge of the well to avoid positions that are partially outside of the well
- → Set the exposure times for each channel that will be imaged (typically 90 ms for each channel)
- → If required, set timepoints, otherwise leave default setting of "1 timepoint"

→ If required, set the number and distance of z-stacks and enable “wait for PFS to stabilize at each position”; otherwise leave default setting of "1 z-stack"
→ Press “start” and acknowledge pop-up indicating the number of tiles and exposures
→ A tile position file will be saved in the download folder, which contains information about all stage positions specific for the parameters, plate type, objective and lens settings. Move this file to the experiment folder, since it will be used for downstream analysis

Example settings:

- Plate name = 20240701-HeLa-ILb
- List of wells = B2
- Channels imaged = DAPI, GFP
- Timepoints = 1
- Z-stacks = 2 every 0.5 μm

resulting images:
20240701-HeLa-ILb_B2_time001_tile0001_z01_channel02
20240701-HeLa-ILb_B2_time001_tile0001_z02_channel02
20240701-HeLa-ILb_B2_time001_tile0001_z01_channel03
20240701-HeLa-ILb_B2_time001_tile0001_z02_channel03

Phenotype imaging settings used in Fandrey et al.:

a) HeLa cell screens:
   - 20x objective, 1x tube lens
   - DAPI 90 ms
   - GFP 150 ms
   - Cy5 90 ms
   - 80x80 grid
   - Edge = 0
   - No stacks
b) THP-1 cell screens:
   - 10x objective, 1x tube lens
   - GFP 90 ms
   - Cy5 90 ms
   - 80x80 grid
   - Edge = 0
   - 3 stacks, every 10 μm → enable “wait for PFS to stabilize at every position”

NIS-Seq imaging settings used in Fandrey et al.:

→ Append the plate name with cycle numbers (cycle1, cycle2, …)
→ Go through the wells that will be imaged in the DAPI channel and check if the focus position changes a lot
   - If yes, use specific, comma separated focus positions for each well in step 3. Focus
→ For every cycle starting from cycle 2, fine-align stage to reference image from cycle 1 as described above
→ Use the following settings for imaging:

- 20x objective, 1x tube lens
- DAPI, GFP, Cy3 and Cy5 all 90 ms
  - **NOTE**: some cell types have brighter NIS-Seq signals than other cells lines. Therefore, take some test images with 90 ms exposure, open in an image analysis tool (e.g., ImageJ), and check whether spot signals exceed the maximum intensity. In case of saturated pixels, reduce exposure to 60 or 30 ms for GFP, Cy3, and Cy5
- 80x80 grid
- Edge = 0
- No stacks