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EDDU Protocols

Uptake of α -Synuclein Preformed Fibrils (PFFs) in Dopaminergic Neural Progenitor Cells

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* Both authors contributed equally and are listed alphabetically

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The involved functions approve the document for its intended use:

Name	Function	Role	Date	Signature
Dr. Thomas Durcan	R&D	Associate Director, The Neuro Early Drug Discovery Unit (EDDU)		

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1 Introduction

1.1 Objectives

The uptake and accumulation of pathological α -synuclein aggregates in neuronal cells is thought to be a major underlying mechanism in the pathogenesis of Parkinson's disease (**Figure 1**). This protocol describes an assay to quantify the uptake of these aggregates by neuronal cells by monitoring the uptake of fluorescently labelled α -synuclein preformed fibrils (PFFs) in dopaminergic (DA) neural progenitor cells (NPCs) derived from induced pluripotent stem cells (iPSCs). This protocol provides the details and methods we used for other researchers to screen small molecule libraries for actives that modulate the uptake of fluorescently labelled PFFs uptake in NPCs. It describes the assay, subsequent image acquisition, and image analysis by high-content screening (HCS).

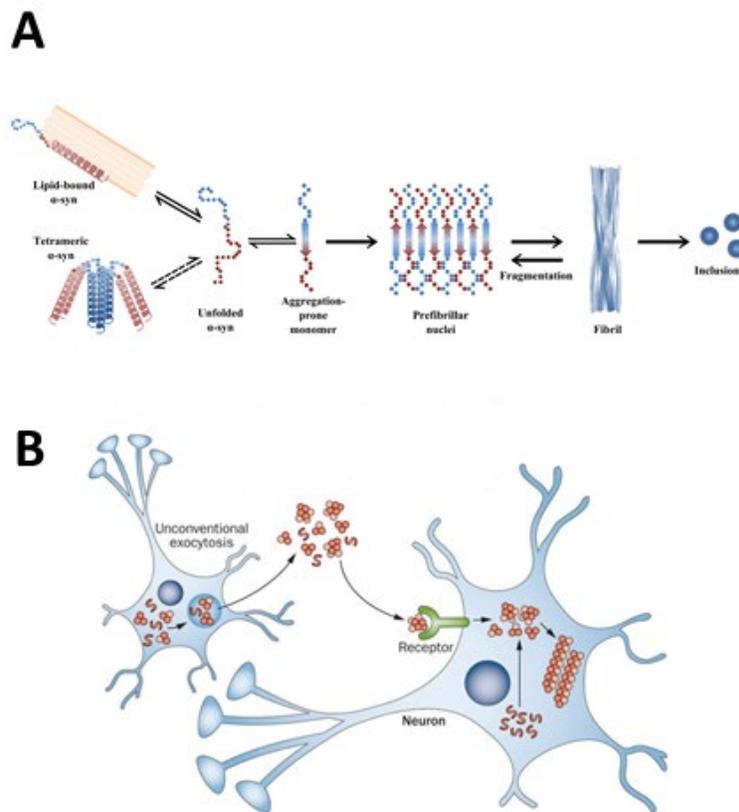


Figure 1: Formation of α -synuclein aggregates and cellular propagation. A) Endogenous α -synuclein aggregate formation: α -synuclein monomers can adopt an aggregation prone formation and subsequently accumulate in different types of aggregates, ultimately leading to cellular inclusions. B) Model of cellular propagation of α -synuclein: Different types of aggregates are exocytosed and enter surrounding cells. In part, their pathological effects are thought to be aggravated by acting as nuclei for further recruitment of α -synuclein monomers in these cells. (Cox D, Carver JA, Ecroyd H. Preventing α -synuclein aggregation: the role of the small heat-shock molecular chaperone proteins. *Biochim Biophys Acta*. 2014;1842(9):1830–1843. doi:10.1016, Lee HJ, Bae EJ, Lee SJ. Extracellular α -synuclein—a novel and crucial factor in Lewy body diseases. *Nat Rev Neurol*. 2014;10(2):92–98. doi:10.1038)

1.2 Protocol overview

NPCs are seeded in black microplates that have been coated with poly-L-ornithine (PLO)/ laminin. After preincubation with small molecules from distinct compound libraries, fluorescently labelled PFFs are added to the NPCs. After a further 24 hours of incubation, the NPCs are fixed and counterstained. Images are acquired on an automated microscope and analysed to determine the level of fluorescently labelled PFFs taken up by the cells. This protocol describes an assay developed within the EDDU and describes specifically the experimental workflow with the instruments and materials used in the facility (**Figure 2**). It has been developed to achieve a certain level of automation, to increase throughput, and to improve reproducibility; however, the workflow is adaptable to instruments and tools available in other laboratories. The assay can, for example, also be performed using a multichannel pipette for all dispensing/aspiration steps and imaged with most automated inverted light microscopes. Image analysis is also easily adaptable to other software packages.

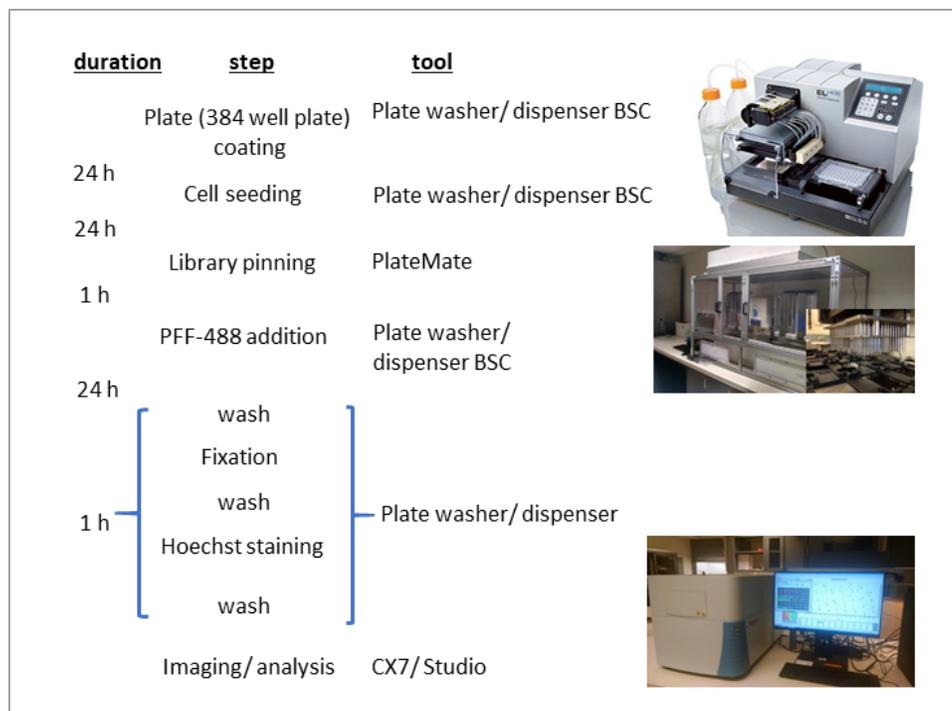


Figure 2: Outline of the protocol, instrumentation for each step and timeline, as performed at the EDDU.

1.3 Technical and safety considerations

- Refer to [EDDU-001-01](#) for information on preparation and storage conditions for reagents used in cell culture.
- Refer to [EDDU-004-01](#) for information on generation and passage of DA NPC cultures.
- Refer to [EDDU005-01](#) for information on handling and decontaminating PFFs.
- Proper training on all equipment must be completed before performing this assay.
- Instrument manuals and SOPs should be readily available for users. At the EDDU, copies of the relevant documents are stored on the desktop of each instrument PC.

- Considerations specific to the EL406 Washer Dispenser:
 - Please note that this SOP only provides the programs for washing and dispensing with the EL406 in a list form to guide users at the EDDU. It does not contain the technical details that are integral part of each program step, since these will be different for each installation and have to be developed during calibration/optimization. However, if labs choose a similar approach, this SOP provides a structure to organize and manage the washer programs throughout the protocol.
 - All programs for the EL406 Washer Dispenser are organized in folders labelled according to the assay-plate used. Each assay-plate folder contains subfolders with the steps for protocols used with that specific plate. These programs have been set with plate-specific X, Y, Z coordinates for washing and dispensing. The programs described here are calibrated for black Falcon 384-well plates (Falcon®, Cat #353962). The pathway to the programs is located in: Computer > OS(C:) > ProgramData > BioTek > LiquidHandlingControl2.21 > Protocols > 384Falcon(black). The X, Y, Z coordinates in the programs for the EL406 Washer Dispenser would need to be modified if other plate formats and types are used. The main objective is to ensure that aspiration and dispensing are optimized to avoid cell loss (i.e. by positioning dispensing tubes at the corner of the well and dispensing against the wall).
 - The EL406 Washer Dispenser and the cassettes must be sterilized before use and properly cleaned after usage as outlined in the protocol steps below.
 - The total volumes needed for reagents and solutions will vary depending on the number of plates and on the volume dispensed per well. It is important to also take into account the priming volume for the tubing (for 5 µL and 10 µL cassettes the priming volume is 7 mL and 10 mL, respectively) and the volume for pre-dispensing (~20 µL/pre-dispense)
 - The container used for holding reagents and solutions should have an opening wide enough for the cassette tubing organizer to fit through (~3 cm diameter).
- Considerations specific to the Matrix PlateMate Plus:
 - The PlateMate pinning program used in this protocol to transfer small molecules from library plates to assay plates is specific to the plates, lids and the pinning tool listed in the **Materials** section. If other plates, lids and/ or pinning tools are used, X, Y and Z coordinates for plate movements must be adjusted.
 - The working surfaces of the PlateMate must be wiped with 70% ethanol and the HEPA unit should be switched on for at least 5 minutes before running the program.
- All materials and reagents should be sterile if working with live cultures. Once the cells are ready for fixation, sterility is not required.
- Steps in this protocol are performed in either a sterile Class II biological safety (BSLII) cabinet or a HEPA enclosure.

2 Materials

The materials, reagents, and equipment listed in this document can be substituted for those supplied by other manufacturers; however, the performance of the assay may not be the same and may need to be optimized or redeveloped upon significant modifications to the materials and/or methods.

Refer to the product datasheet from the supplier for further details on storage and preparation instructions.

2.1 Labware

Item	Supplier	Catalogue #
Adhesive Film for Microplates, polyester, non-sterile	VWR	60941-062
Falcon® 384-well Optilux Black/Clear Flat Bottom, TC-treated Microtest Microplate, with Lid, Sterile	Corning	353962
Nunc™ 384-Well Polypropylene Storage Microplates, Square, Conical Bottom	Thermo Fisher Scientific	4309
Nunc™ Automation Friendly Storage Plate Lids, Black, Sterile	Thermo Fisher Scientific	4955
Plate sealing tape, silver, aluminum/silicone	Thermo Fisher Scientific	60180-M144

2.2 Culture reagents

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temperature
Antibiotics-Antimycotic (anti-anti)	Thermo Fisher Scientific	15240-062	100X	1X	Stock: -20°C Working: 4°C
B-27® Supplement (serum free)	Thermo Fisher Scientific	17504044	50X	1X	Stock: -20°C Working: 4°C
DMEM/F12	Thermo Fisher Scientific	10565042	1X	1X	4°C
Dimethylsulfoxide (DMSO)	Sigma-Aldrich	D8418-100ML	1X	1X	room temperature
Ethanol	Greenfield global	26456	100%	70%	room temperature

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temperature
Formaldehyde (FA)	Thermo Fisher Scientific	28908	16%	2%	room temperature
Hoechst 33342	Thermo Fisher Scientific	H3570	10 mg/mL	2.5 ug/mL	4°C
Laminin	Sigma-Aldrich	L2020-1mg	1 mg/mL	5 µg/mL	Stock: -20°C Working: 4°C
N-2 Supplement	Thermo Fisher Scientific	17502048	100X	1X	Stock: -20°C Working: 4°C
Nonessential amino acids (NEAA)	Thermo Fisher Scientific	11140050	100X	1X	4°C
PBS	BioBasic	PD8117	10X	1X	room temperature
Poly-L-Ornithine (PLO)	Sigma	P3655	1 mg/mL	1 µg/mL	Stock: -20°C Working: 4°C
Sodium Dodecyl Sulfate (SDS)	BioShop	800-100-WG	10%	1%	room temperature
Triton® X-100	Wisent Inc.	TRX506.500	10%	0.1%	room temperature
Y27632	Selleck Chemicals	S1049-50 mg	10 mM	10 µM	Stock: -20°C Working: 4°C

2.3 Equipment

Item	Supplier	Catalogue #	Model/Serial Number
5 µL Cassette	BioTek	405476	
10 µL Cassette	BioTek	406629	
EL406 Washer Dispenser	BioTek	EL406PSUB1	1804032A
Matrix PlateMate Plus	Thermo Fisher Scientific		1026A
Pinning tool 384 pins 100 nl	V&P Scientific	FP3S100	
CellInsight CX7	ThermoFisher Scientific		CX7, SN:L0716-190A-008

2.4 Software

Item	Supplier	Version
ControlMate™	Thermo Fisher Scientific	1.3.38
Liquid Handling Control	BioTek	2.21 (build 2) File version 2.19.1

Item	Supplier	Version
HCS Studio Scan	ThermoFisher Scientific	6.6.1 Build 8468
HCS Studio View	ThermoFisher Scientific	6.6.1 Build 8468
STORE database	ThermoFisher Scientific	1.6
Excel 2016	Microsoft	16.0.4266.1001
Python 2.7	Python.org	v.2.7.15

3 Protocol

3.1 Coating 384-well plates and seeding NPCs

Materials:

- 1 µg/mL PLO in PBS
- 5 µg/mL laminin in DMEM/F12
- DMEM/F12
- DA NPCs
- NPC media (DMEM/F12 + anti-anti + B27 + N2 + NEAA + Y27632)
- 0.1% Triton in PBS
- 1X PBS
- dH₂O
- ddH₂O
- 70% ethanol
- 384-well plates (black/clear flat bottom)

Equipment:

- EL406 Washer Dispenser
- 10 µL cassette
- Liquid Handling Control Software

Notes:

- Prior to seeding, NPCs are cultured and expanded in PLO/ laminin coated T25 or T75 flasks (refer to [EDDU-004-01](#)).
- Plan cell culture according to the number of plates being seeded to ensure sufficient cell numbers (4,000 cells/ well plus 10 mL priming volume for cassette).
- Low passage NPCs are preferable (2-3 passages after thawing / initial plating).
- 384-well plates should be coated with PLO the day prior to seeding cells.
- 384-well plates should be fully coated and the EL406 Washer Dispenser should be sterilized and primed before starting NPC detachment and suspension to minimize the time in suspension.

Procedure:

Day 1 (Plate coating with PLO):

1. Turn ON the EL406 Washer Dispenser and open the Liquid Handling Control Software.
2. Spray the surface of the BSLII cabinet and the EL406 Washer Dispenser with 70% ethanol.
3. Spray the cassette with 70% ethanol before placing it in the EL406 Washer Dispenser (**Figure 3 A** shows cassette position for dispensing).

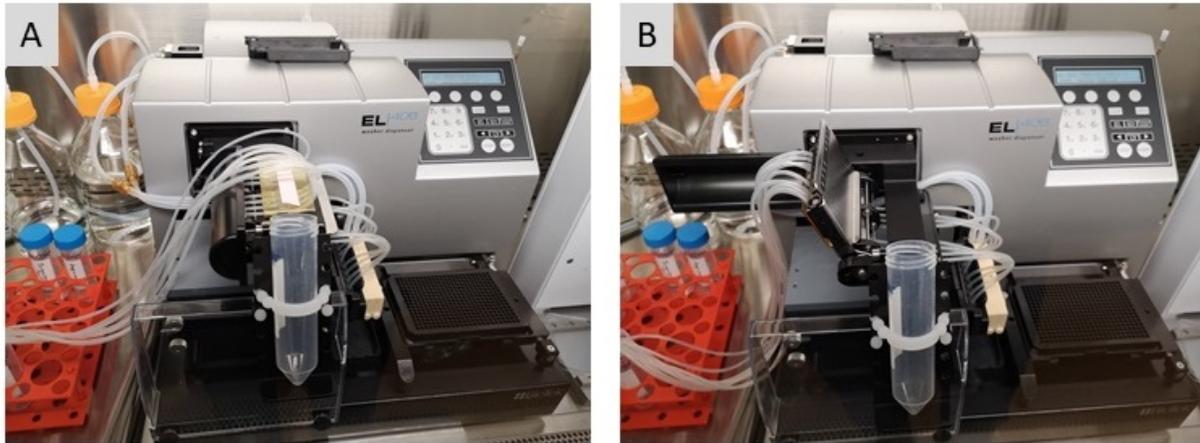


Figure 3. Cassette positions in EL406. A) Tubing under tension, ready for dispensing. B) ‘Relaxed’ position to release tube tension and allow outflow by gravity.

4. Place the cassette lines into 70% ethanol and under the folder ‘1-PLO Coating’ select the program ‘1-Sterilize 10 μ L cassette with 70% EtOH’ to sterilize the cassette by priming with 70% ethanol.
 - Ensure the washer bottles are filled with appropriate solutions before using the machine. If any bottle needs refilling it should be done before the sterilization steps.

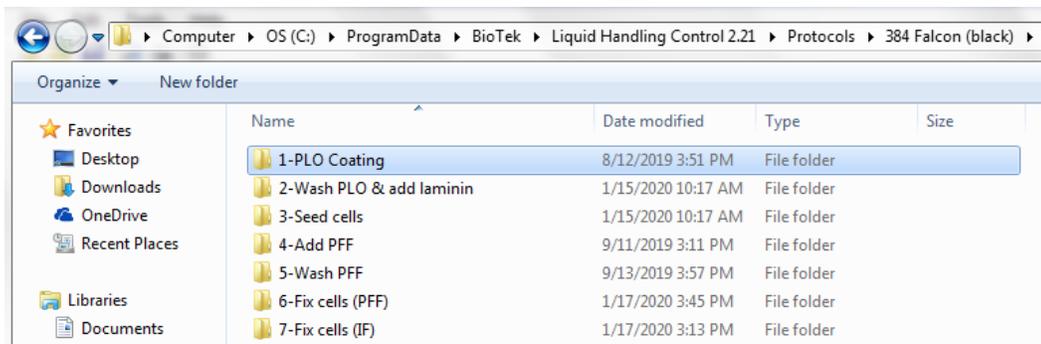


Figure 4. Screenshot of path and folder structure for PFF uptake in black 384-well plates

5. Place the cassette in a ‘relaxed’ position to empty the lines by gravity (**Figure 3 B**).
 - Allow enough time for the ethanol to evaporate before using the machine.
6. Place the cassette lines into PLO and select the program ‘2-Prime 10 μ L cassette with PLO’ to prime the cassette with PLO.
7. Place 384-well plate on plate carrier, oriented with the A1 well to the back-left corner of the carrier, and remove the lid.

8. Select the program '3-384-well plate PLO coating' to dispense 30 μ L of PLO per well.
9. Put plates in incubator for overnight incubation at 37°C.
10. Place cassette lines at the top of the PLO container and select the program '4-Purge PLO' to purge the PLO from the cassette.
 - Placing the lines at the top of the container will decrease the amount of bubbles formed in the solution.
11. Select the program '5-Clean 10 μ L cassette' to clean the cassette with 0.1% Triton, PBS, ddH₂O, and 70% ethanol, in that order.
 - The lines are rinsed with ddH₂O above a waste container before being placed into each cleaning solution.
12. Place the cassette in a 'relaxed' position.
13. Turn OFF the EL406 Washer Dispenser and close the Liquid Handling Control Software.

Day 2 (Plate coating with laminin and NPC seeding):

14. Turn ON the EL406 Washer Dispenser and open the Liquid Handling Control Software.
15. Spray the surface of the BSLII cabinet, the EL406 washer, and the cassette with 70% ethanol.
16. Place the cassette lines into 70% ethanol and under the folder '2-Wash PLO & add laminin' select the program '1-Sterilize washer and 10 μ L cassette with 70% EtOH' to sterilize the washer and the cassette by priming with 70% ethanol.
 - Ensure the washer bottles are filled with appropriate solutions before using the machine. If any bottle needs refilling it should be done before the sterilization steps.
17. Place the cassette in a 'relaxed' position to empty the lines by gravity.
 - Allow enough time for the ethanol to evaporate before using the machine.
18. Place the cassette lines into DMEM/F12 and select the program '2-Prime 10 μ L cassette with DMEM' to remove any remaining ethanol before priming with laminin.
19. Place the cassette lines into laminin and select the program '3-Prime 10 μ L cassette with laminin and Syringe B' to prime the cassette with laminin and to remove any air bubbles in the Syringe B line that might have resulted due to evaporation.
20. Select the program '4-384-well plate wash PLO + add laminin' to aspirate PLO, then wash twice with 50 μ L of ddH₂O, and dispense 30 μ L of laminin per well.
21. Incubate plates at 37°C for at least 2 h.
22. Place cassette lines at the top of the laminin container and select the program '5-Purge laminin' to purge the laminin from the cassette.
23. Place the cassette lines into DMEM/F12 and select the program '6-Prime 10 μ L cassette with DMEM'.
24. Place the cassette in a 'relaxed' position to empty the lines by gravity.
25. Wash NPC culture flasks with PBS.

26. Remove PBS and add Accutase (3mL for T25 flask). Incubate at 37°C until NPCs detach (3-4 min).
27. Transfer NPCs to 15 ml Falcon tube(s) and add 10 mL of DMEM/F12.
28. Centrifuge cells at 1200 rpm for 3 min.
29. Remove supernatant and resuspend cell pellet in 1 mL of NPC medium.
30. Determine cell number and dilute cells in NPC media in appropriate vessel (50 mL Falcon tubes or sterile glass bottles for higher volumes). Final cell concentration should be 100,000 cells/mL.
 - It is important to keep cells in suspension as short as possible and to swirl containing vessel before dispensing to prevent settling and clumping of NPCs.
31. Under the folder '3-Seed cells' select the program '1-Prime 10 µL cassette with cells' to prime the cassette with NPCs.
32. Select the program '2-384-well plate aspirate laminin and seed cells' to aspirate the laminin and dispense 40µL of NPCs per well. The cell density will be 4,000 cells/well.
33. Put plates back into the incubator.
34. Place the cassette lines at the top of the cell suspension container and select the program '8-Purge cells' to purge the cell suspension from the cassette.
35. Select the program '9-Clean 10 µL cassette' to clean the cassette with 0.1% Triton, PBS, ddH₂O, and 70% ethanol, in that order.
36. Place the cassette in a 'relaxed' position to empty the lines by gravity.
37. Select the program '10-Wash with PBS, H₂O, EtOH' to clean the washer with PBS, ddH₂O, and 70% ethanol, in that order.
38. Remove the cassette from the EL406 Washer Dispenser and store in its appropriate box.
39. Turn OFF the EL406 Washer Dispenser and close the Liquid Handling Control Software.

3.2 Pinning compounds and adding PFFs

Materials:

- Fluorescently labelled PFF
- 1% SDS in PBS
- DMEM/F12
- NPC media (DMEM/F12 + anti-anti + B27 + N2 + NEAA)
- 0.1% Triton in PBS
- 1X PBS
- dH₂O
- ddH₂O
- DMSO
- 70% ethanol
- 384-well plates (black/clear flat bottom)
- 384-well plates (conical bottom) with compounds
- Black automation lids
- Plate sealing tape (aluminum)

Equipment:

- EL406 Washer Dispenser
- 5 µL cassette
- Liquid Handler Software
- Matrix PlateMate Plus
- ControlMate™ Software
- FP3S100 Pinning Tool

Procedure:

Day 3 (Addition of PFFs and small molecules):

1. Thaw the small-molecule library plates in the dark for at least 2 hours before pinning.
2. Turn ON the EL406 Washer Dispenser and open the Liquid Handling Control Software.
3. Spray the surface of the BSLII cabinet and the EL406 washer with 70% ethanol.
4. Sterilize the black automation lids with 70% ethanol and allow them to dry in the BSLII cabinet before using.
5. Spray the 5 µL cassette with 70% ethanol before placing it in the EL406 Washer Dispenser.
6. Place the cassette lines into 70% ethanol and under the folder '4-Add PFF' select the program '1-Sterilize 5 µL cassette with 70% ethanol' to sterilize the cassette by priming with 70% ethanol.
7. Place the cassette in a 'relaxed' position to empty the lines by gravity.
 - Allow enough time for the ethanol to evaporate before using the machine.
8. Exchange regular lids for black automation lids on both the NPC and the small-molecule library plates.
9. Turn on the Matrix PlateMate Plus as well as the HEPA filter unit for the enclosure surrounding the machine.
10. On the keypad linked to the Matrix PlateMate, press 'CLEAR' to override the initial memory error.
11. Verify that the 384-well 100nl pinning tool is installed in the pipetting head (**Figure 5**). If the pinning tool has to be exchanged, the instructions can be found in the **Appendix 4.2**.
12. Place the NPC plates on the left stack chimney **A** of the Matrix PlateMate Plus and the small-molecule library plates on the right stack chimney **B** (**Figure 5**).
 - Ensure that the containers used for washing the pipetting head have enough DMSO or 70% ethanol in them before using the machine. The DMSO container is placed on the microplate adapter 2, while the 70% ethanol container is placed on the microplate adapter 3.

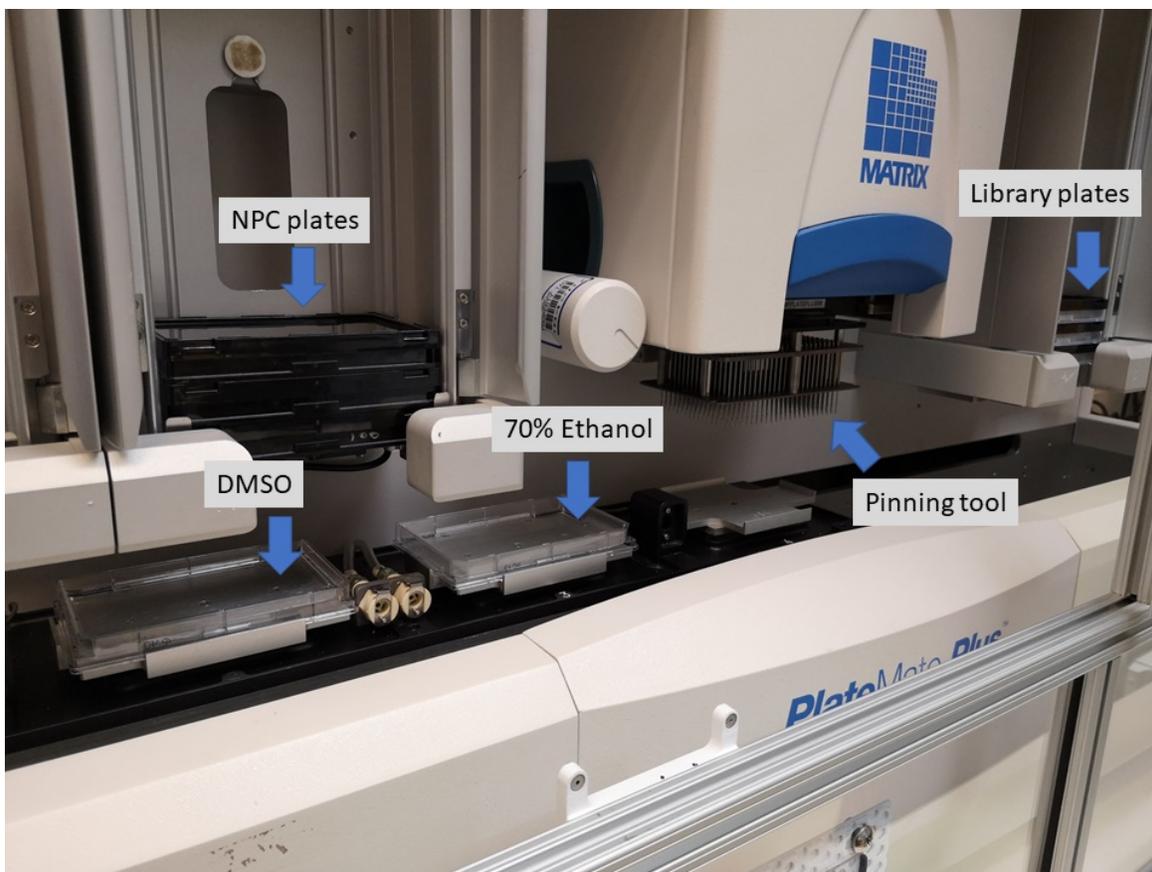


Figure 3. Overview of initial plate and tool positions in Matrix PlateMate Plus.

13. Using the ControlMate™ Software, open the file '384to384assay-100nl_Pin-WR'.
14. Under the 'Main Sequence' drop-down menu, select 'transfer' and type in the number of iterations to perform (**Figure 6 A**).
15. Select 'Run' (**Figure 6 B**), then 'Start' to transfer the small-molecule library plates into the NPC plates.
16. Remove the plates from the chimneys and exchange the black automation lids for regular lids on both the NPC and the small-molecule library plates in the BSLII cabinet.
17. Put NPC plates in the incubator for 1h.
18. Place aluminum plate sealing tape on the small-molecule library plates and store them at -20°C.
19. Turn off the Matrix PlateMate Plus as well as the HEPA filter for the enclosure surrounding the machine and close the ControlMate™ software.

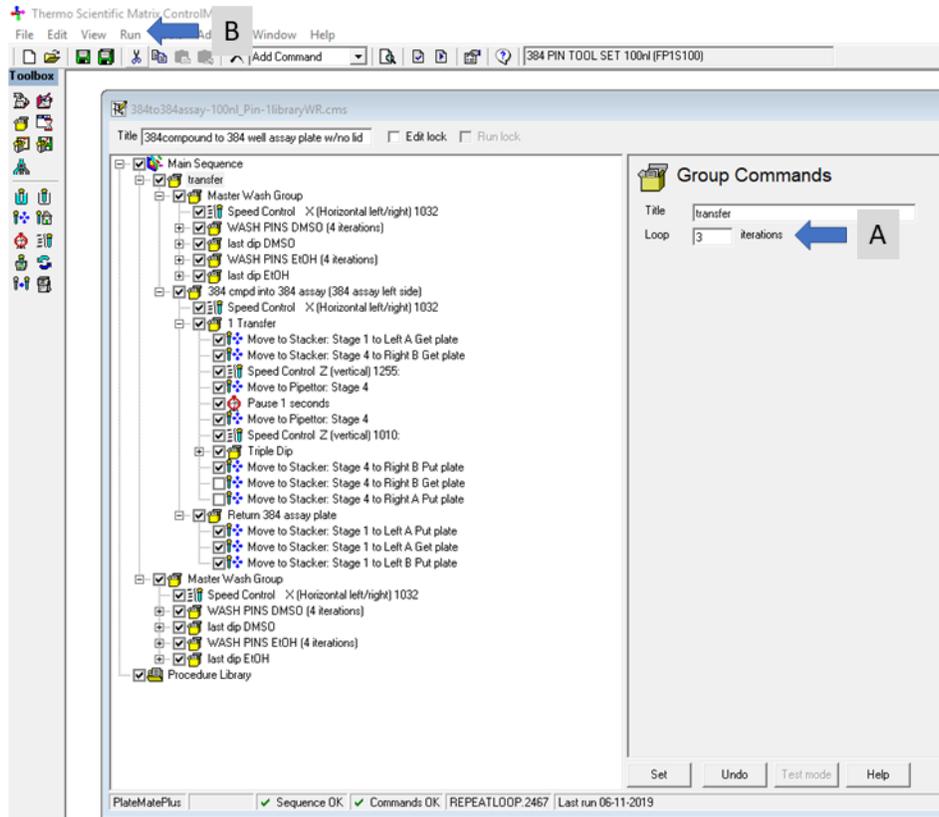


Figure 4. Screenshot of ControlMate software with opened '384to384assay-100nl_Pin-WR' file, showing the steps. A) Insert number of plates (Iterations) here, note that the transfer step has to be chosen to open the iteration command. B) Run the program.

20. Place the cassette lines into DMEM/F12 and under the folder '4-Add PFF' select the program '2-Prime 5µL cassette with DMEM'.
21. Select the program '3-384-well plate 10 µL DMEM (column 23 only)' to dispense 10 µL of DMEM/F12 into the wells in column 23.
 - Column 23 will be the 'No PFF' control.
22. Select the program '4-Prime 5 µL cassette with PFF' to prime the 5 µL cassette with NPC media containing 400 nM PFF.
 - When working with fluorescently labelled PFFs, ensure the light in the BSLII cabinet is turned OFF.
23. Select the program '5-384-well plate 10 µL PFF (minus column 23)' to dispense 10 µL of PFF media into each well, except those in column 23.
 - Final PFF concentration will be 80 nM.
24. Put NPC plates back in the incubator for a 24 h incubation.

- Plates should be covered with aluminum foil when transferring between the BSLII cabinet and the incubator to prevent photobleaching of the fluorescent dye.
25. Place the cassette lines at the top of the PFF media container and select the program '6-Purge PFF' to purge the PFF media from the cassette.
 26. Place the cassette lines in 1% SDS and select the program '7-Prime 5µL cassette with SDS'. Allow the cassette to soak in SDS for at least 1h.
 27. Select the program '8-Clean 5 µL cassette' to clean the cassette with PBS, ddH₂O, and 70% ethanol, in that order.
 28. Place the cassette in a 'relaxed' position to empty the lines by gravity.
 29. Remove the cassette from the EL406 Washer Dispenser and store in its appropriate box.
 30. Turn OFF the EL406 Washer Dispenser and close the Liquid Handling Control Software.

3.3 Cell fixation and counterstaining for analysis by HCS

Materials:

- 4% FA in PBS
- 1X PBS
- dH₂O
- ddH₂O
- 70% ethanol
- 384-well plate (black/clear flat bottom)
- 5 µg/mL Hoechst 333342 in PBS
- Clear adhesive film for microplates

Equipment:

- EL406 Washer Dispenser
- 10 µL cassette
- Liquid Handler Software

Procedure:

Day 4 (Fixation and counterstaining):

1. Turn ON the EL406 Washer Dispenser and open the Liquid Handling Control Software.
 - When working with fluorescently labelled PFFs, ensure the light in the BSLII cabinet is turned OFF. Plates should be covered with aluminum foil when transferring between the incubator and the BSLII cabinet, as well as after fixation.
2. Place the 10 µL cassette in the EL406 Washer Dispenser.
3. Place the cassette lines into 4% FA.
4. Under the folder '6-Fix cells' select the program '1-Prime washer with PBS and 10 µL cassette with FA'.
5. Empty the waste bottle.

6. Select the program '2-384-well plate 2x wash + add 20 μ L FA' to rinse each well twice with 60 μ L of PBS and then dispense 20 μ L of 4% FA.
 - Final FA concentration will be 2%.
7. Incubate plates for 10 min at room temperature in the dark.
8. Place the cassette lines at the top of the FA container and select the program '3-Purge FA' to purge the FA from the cassette.
9. Place the cassette lines into 5 μ g/mL of Hoechst 333342 in PBS and select the program '4-Prime 10 μ L cassette with Hoechst'.
10. Select the program '5-384-well plate 2x wash + add 20 μ L Hoechst' to rinse each well twice with 60 μ L of PBS and then dispense 20 μ L of Hoechst.
 - Final Hoechst concentration will be 2.5 μ g/mL.
11. Incubate plates for 10 min at room temperature in the dark.
12. Empty waste bottle in the fume hood into an FA waste container.
13. Place the cassette lines at the top of the Hoechst container and select the program '6-Purge Hoechst' to purge the Hoechst from the cassette.
14. Select the program '7-Clean cassette' to clean the cassette with PBS, ddH₂O, and 70% ethanol, in that order.
15. Place the cassette in a 'relaxed' position to empty the lines by gravity.
16. Remove the cassette from the EL406 Washer Dispenser and store in its appropriate box.
17. Select the program '8-Wash 2x PBS' to rinse each well three times with 60 μ L of PBS.
18. Seal plates with clear adhesive film, then wrap them in aluminum foil.
 - Plates can be scanned directly on an HCS microscope or stored at 4°C for later analysis; however, it is recommended that plates be scanned within a month after fixation.
19. Select the program '9-Wash with H₂O and EtOH' to clean the washer with ddH₂O and 70% ethanol, in that order.
20. Turn OFF the EL406 Washer Dispenser and close the Liquid Handling Control Software.

3.4 HCS: Image acquisition and analysis

- A more general protocol for this section is also available: [EDDU011-01](#)

3.4.1 Overview:

The following sections describe the steps to acquire and analyze images. The aim is to detect compounds that reduce cellular uptake of fluorescently labeled PFF (PFF-Alexa488). Images are acquired in 2 channels to detect the nuclear counterstain (**Figure 7 A**) and the intracellular PFF (**Figure 7 B**). In the analysis protocol, a mask is created to delineate the nuclei of each cell (**Figure 7 A'**). To quantify the fluorescent signal of PFF, the nuclear mask is expanded by a fixed number of pixels to create a larger circle encompassing the perinuclear space. Pixels above background values within the expanded mask are detected (**Figure 7 B'**) and the intensity of the (PFF) pixels is summed for each cell. The mean of the total PFF intensity per cell in each well is used to provide a measurement of cellular PFF uptake. Wells with strongly reduced PFF uptake (**Figure 7 E**) relative to wells with carrier only (**Figure 7 C**) are considered actives. In addition, cell count, mean nuclear area, and mean nuclear intensity values are collected to detect potential toxic compounds.

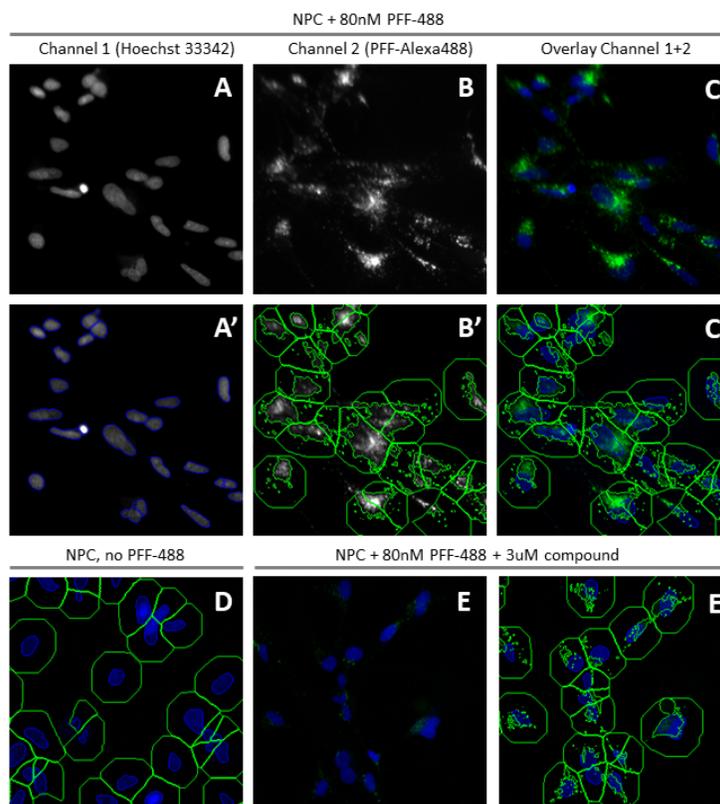


Figure 5: Image analysis of PFF-Alexa488 uptake in NPCs. A-C) NPCs + PFF + carrier (DMSO). A) Channel 1, nuclei. A') Channel 1, nuclei with object mask (blue outline). B) Channel 2, PFF. B') Channel 2, PFF + expanded object mask (green boundary) and identified intracellular PFF (green areas within the outer boundary). C) Overlay nuclei and PFF. C') Channel 1+2 with overlay from B'. D) NPC + DMSO without PFF in the expanded mask (green ring). No signal is detected in Channel 2. E) Channel 1+2, NPC + PFF + active compound. E') Channel 1+2 + masks + identified PFF areas. Intracellular PFF has lower intensity and occupies smaller areas per cell compared to DMSO control A-C.

Equipment:

- ThermoFisher Scientific CellInsight CX7
- Studio software
- Liquid Handler Software

Procedure:

3.4.2 Setting acquisition parameters:

1. Switch on the CX7 before opening the **HCS Studio Navigator**. Choose **Scan** and enter your username and password to access the software.
2. In the Scan software, click **Open** to open the protocol search interface and select an existing PFF-uptake protocol under the CellHealthProfiling tab (a template protocol is available: YYYYMMDD-template_PFFup).
3. Click **Create** to open the chosen template. This will open the **Configure Acquisition** tab.
4. Click on the “insert plate” symbol (**Figure 8 A**) to move the microplate tray out of the instrument. Place the plate in the microplate tray with well A1 pointing towards you. Press on the “Ctrl” key and click “Okay” simultaneously to move the tray back into the instrument.

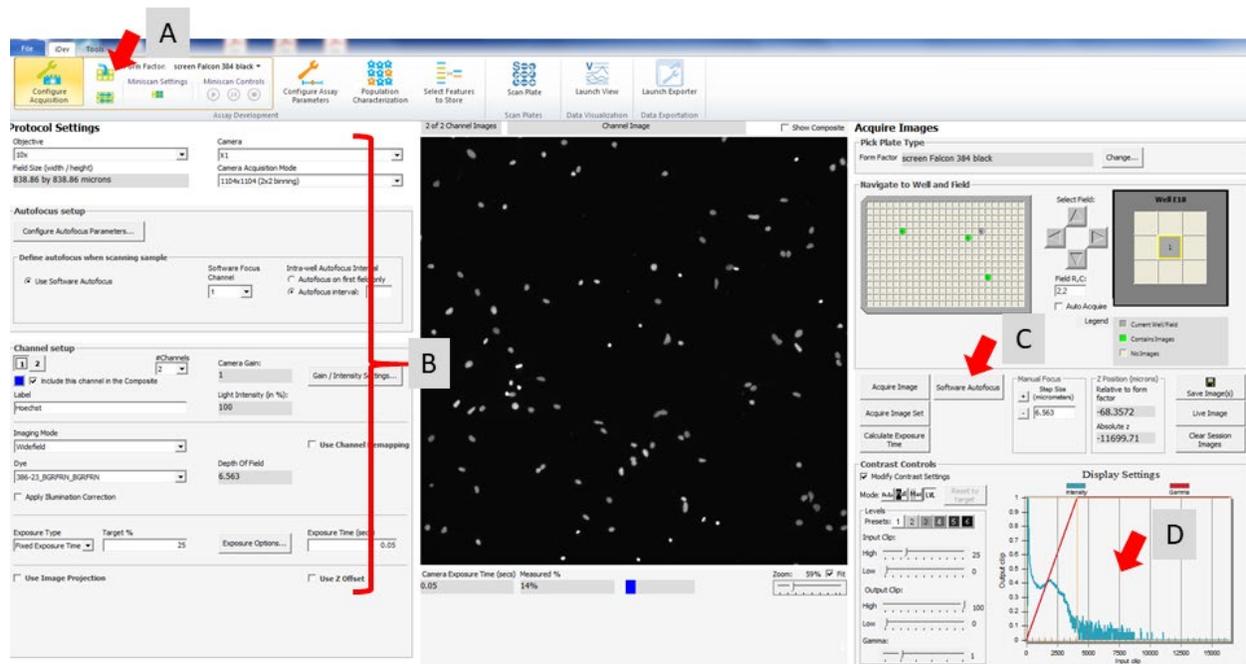


Figure 6. Screenshot CX7 Studio Scan Acquisition tab. A) Microplate tray open/close. B) Image acquisition settings. C) Software autofocus and image acquisition field. D) Pixel intensity histogram.

5. The following conditions are used for image acquisition (see also **Figure 8 B**):

- **Objective:** 10x magnification
- **Camera Acquisition Mode:** default is 1104x1104 (2x2 binning)

- **Autofocus setup:** default is software autofocus, each field; Standard
- Number of **Channels:** 2. Channel 1 filter set: 386-23_BGRFRN_BGRFRN. Channel 2 filter set: 485-20_Y_Y
- **Plate form factor:** screen Falcon 384 black (if a different plate type is used, its dimensions have to be added to the form factor list).

3.4.3 Acquisition optimization and collection of test images:

6. Select a well where the highest fluorescence intensity values are expected (DMSO + PFF control well).
7. Acquire image in channel 1 (Hoechst) using **Software Autofocus** (**Figure 8 C**). Verify in pixel intensity histogram (**Contrast Controls, Modify Contrast Settings, Figure 8 D**) that signal is well within the dynamic range of the camera.
8. If the pixel intensity histogram is out of range, increase or lower **Exposure Time** accordingly and collect image again using **Acquire Image**.
9. Collect image in channel 2 (PFF) using **Acquire Image** or **Acquire Image Set** (**Figure 8 C**) and adjust exposure time to achieve a good dynamic range as above.
10. Collect image sets from at least three DMSO + PFF control wells from different areas of the plate, always using **Software autofocus** in channel 1 first, and then **Acquire Image Set** for a complete set of channels 1 and 2. Confirm that the pixel intensity histograms are within the dynamic range of the camera. If necessary, adjust exposure time until all wells are within range. Only the final exposure times will be used to collect image sets for the analysis template in the next step.
 - The image sets for the analysis template should represent the negative control (DMSO+PFF) and the positive control (DMSO only). In addition, other positive controls (known PFF uptake inhibitors + PFF) can be included. At least 3 image sets for each condition should be used.

3.4.4 Setting of analysis parameters:

11. Select the Configure Assay Parameters tab. Under Layout (**Figure 9 A**), select Configure groups to open the plate map with the already acquired image sets. Double-click on the fields from a selected well to load the image sets into the assay template (**Figure 9 B**). Assign positive and negative controls into two separate groups. To view the images from the positive and negative controls side by side, check group 1 and 2 in **Groups to Display in Layout**.
12. The settings in the PFF uptake protocol can be reused but will usually need some adjustments to compensate inter-experimental variance. Optimize the parameter settings for the image analysis by going through the **Protocol Optimization Task List (Figure 9 C)** from top to bottom. **Table 1** indicates the parameters to be optimized. During the optimization, toggle between the image sets (wells and fields) in both groups to verify the settings (**Figure 9 D**).
 - To visualize the parameter values measured in an image, select **Data** and/or **Statistics** in **Layout (Figure 9 E)**.

- To modify the displayed image (in each channel), use the **Image Settings** block (Figure 9 F)

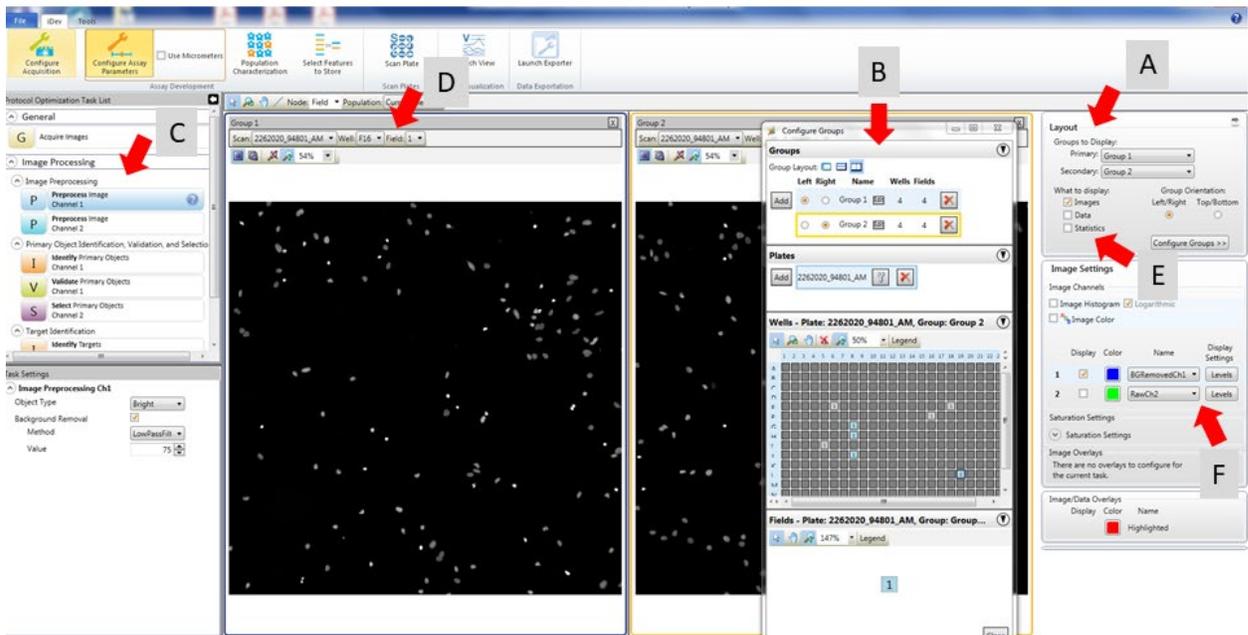


Figure 7. Configure Assay parameters tab. A) Group layout. B) Loading image sets. C) Image processing list. D) Image toggle. E) Data/ statistics visualization. F) Image display adjustment.

Table 1. Optimization parameters in image processing list

	Step	Description	Comments	Channel
1	Preprocess Image	Background removal	No changes necessary.	Channels 1 and 2
2	Identify Primary Objects	Find objects (e.g. nuclei, cells) and generate mask	Adjust threshold value to adjust nuclear mask for best fit.	Channel 1
3	Validate Primary Objects	Filter objects by morphological and intensity-based features	Examine filtered objects and adjust filter settings to optimize object validation.	Channel 1
4	Select Primary Objects	Filter objects in channels (ROI-based)	No changes necessary.	Channel 1 and 2
5	Identify Targets	Secondary objects identification	Adjust intensity threshold to settings above background (in the no PFF control the ROI should be mostly below threshold).	Channel 2
6	Reference Levels	Population identification	Set High level threshold in Target Total Intensity Channel 2 to the average of the median in the intensity histogram of the DMSO+ PFF control images.	Channel 2

3.4.5 Scan set-up

13. In the **Population Characterization** tab, **Scan Limits** should be set to define the number of fields scanned per well. The maximum fields for 384-well plates is 9 (**Figure 10, arrow**). With 4,000 cells seeded per well, 6 fields are sufficient to image more than 1,000 cells/well.

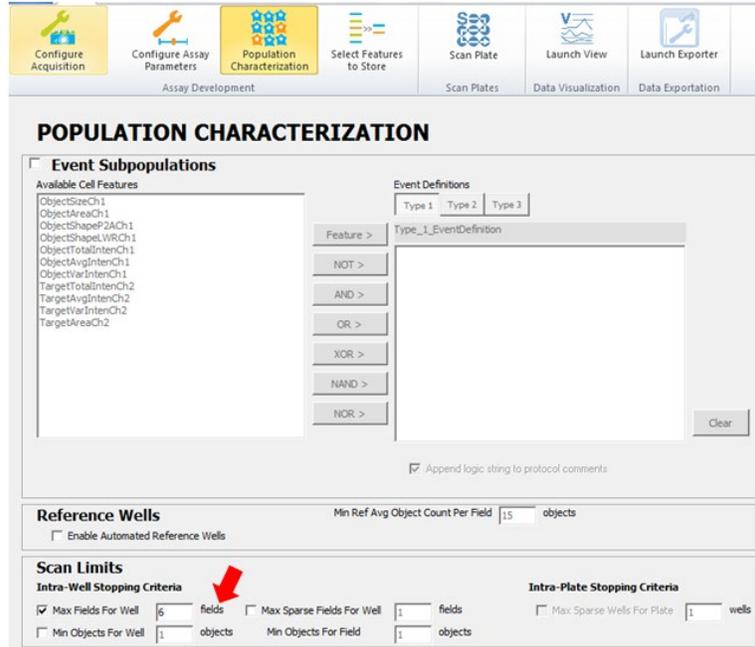


Figure 8. Population Characterization tab. Arrow: Set limit for fields to acquire per well.

14. Under the **Select Features to Store** tab, the full list of well features should be selected and placed into the left field. (**Figure 11, arrow**).

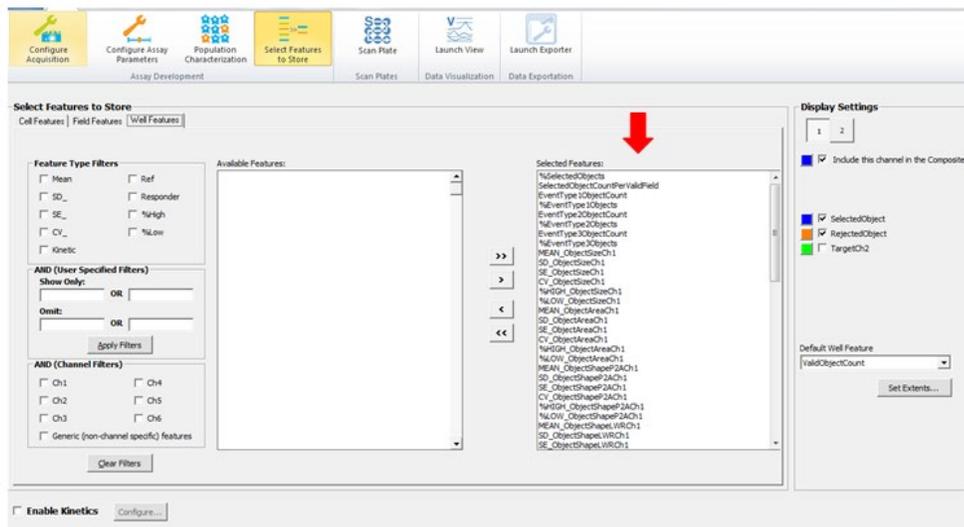


Figure 9. Select Features to Store tab. Arrow: Selected features.

15. In the **Scan plate** tab, select the wells to be scanned by clicking on the plate icon under **Scan Settings** (**Figure 12 A**).

16. Under **Plate Name** and **Plate ID (Figure 12 B)** enter the plate name (*date (yyyymmdd)-experiment*).

- Each plate scan will also be assigned a machine-generated **Unique Plate Description**, following the nomenclature: *233-drucker-pc1(ComputerName)_YYMMDDHH(date and hour) xxxx(running number)*.

17. Save the protocol under **File → Save as** and start the scan by clicking the arrow under **Scan Controls (Figure 12 C)**.

- An estimate of the total scanning time will be posted as soon as the first well is completed and readjusted as the scan progresses.
- Extracted features of already scanned wells can be visualized in the **Plate/ Data** tabs using the **Well Feature** drop down menu (**Figure 12 D**).
- The Scan can be paused or stopped at any time under **Scan Controls**.

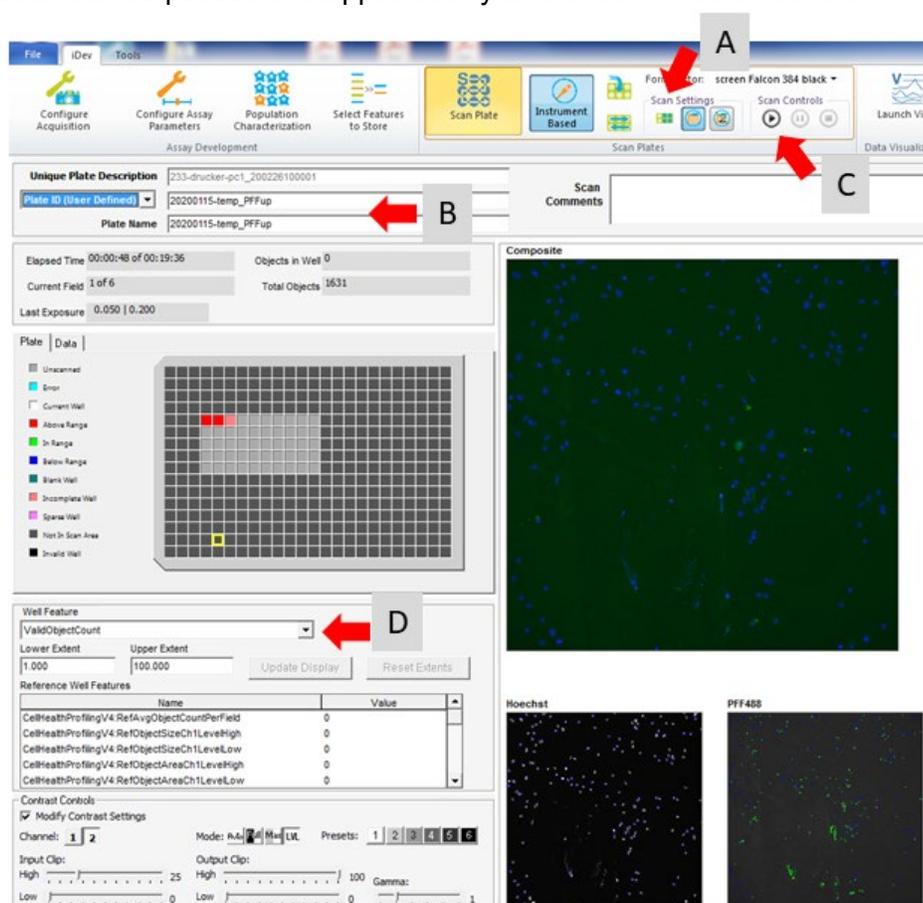


Figure 10. Scan Plate tab. A) Well selection. B) Insert plate name. C) Start scan. D) Well feature selection.

3.5 Retrieval of data and data processing

18. To access the data once the scan is finished, use **Launch View** on the **Scan** tab to open Studio View. Alternatively, open **View** from the **HCS Studio** navigator, click on **Change Filters (Figure 13 A)** and search the database (e.g. Plate ID or date of scan, **Figure 13 B**).

In the list of search results, double click on the scan of choice and select all well features to be loaded.

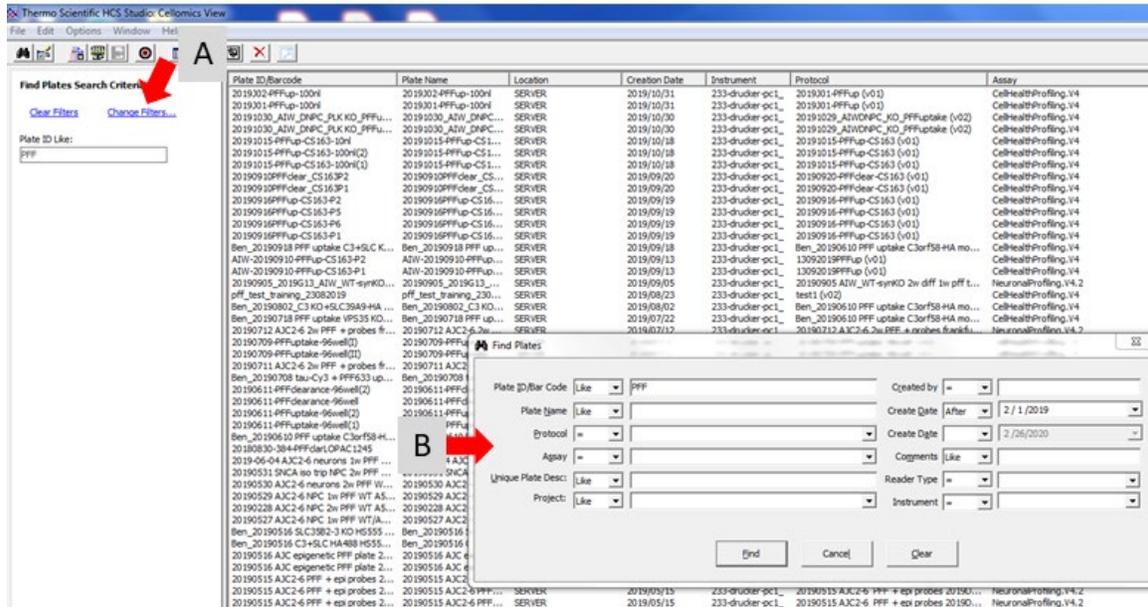


Figure 11. View database search. A) Click to open search window. B) Search window. Set features to select scan results.

19. Choose any feature of interest (Figure 14 A) to visualize as a graphical (Figure 14 B) or numerical representation (Figure 14 C).

20. Right-click on the graph or plate map to access the features below:

- Image Browser (**Browse Images in iView**) to view and configure acquired images, tiles, overlays and masks, and to export sample images.
- Single cell data (**Cell Details**) displays per feature basis data, images, and overlays for each cell in a well, including a histogram of the feature for all cells.
- Export single cell data per well (**Export to Spotfire, csv or txt**)

21. To export data, select a feature and go to **Spreadsheet > Export Current Spreadsheet > to File (Figure 14 D)**. This will export the feature chosen as an .xls file in plate map format. Every feature is exported separately. Exported assay features are:

- Cell count
- Mean Average Intensity Channel 1
- Mean Average Area Channel 1
- Mean Total Intensity Channel 2
- Mean Total Intensity Level High Channel 2

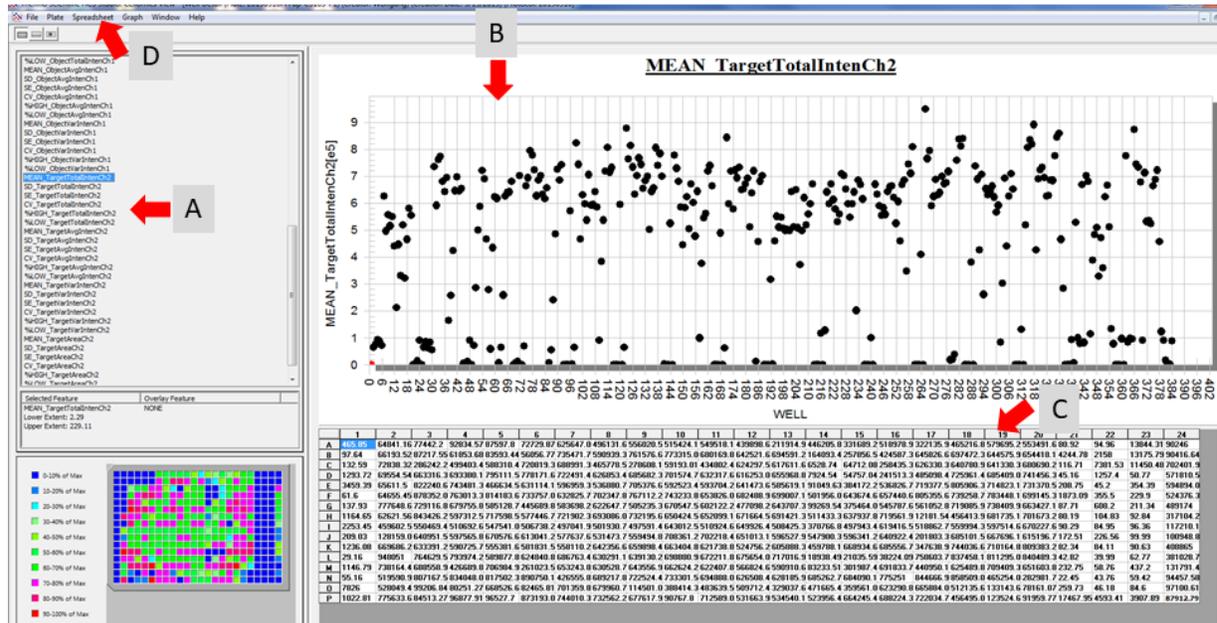


Figure 12. View Scan results. A) Feature selection. B) Scatterplot of selected feature values. C) Feature values in plate map format. D) Spreadsheet selection for data export.

22. Compile plate data sets into a list format: While there is an application in the Studio software to export features in list format (see [EDDU-011-01](#)), we use a simple Python script to compile the 5 features already exported in plate format (XLS_Transposer.py). The Python script can be installed following the instructions in [Appendix 4.1](#)

4 Appendix

4.1 XLS_Transposer script

This script transposes a folder of per feature files exported from **Studio View** into one per well feature list. The code below can be copied into notepad and saved as an XLS_Transposer.py file to run. This script requires Python2.7. To run the script:

1. Make sure that all feature spreadsheets from one plate (.xls format) are stored in the same folder
2. Open the windows command prompt (*C:\Windows\system32\cmd.exe*)
3. Enter the location of the Python executable and the script, e.g.: *C:\Python27\python.exe C:\Python27\python.exe\Scripts\XLS_Transposer.py"*
4. When prompted, choose folder containing the spreadsheets. A single file with the transposed data (*output.csv*) will be added to the folder.

```
import csv
import os
import Tkinter, tkFileDialog

num_columns = 24      #change the number to number of columns
num_rows = 16         #change the number to number of rows

root = Tkinter.Tk()
root.withdraw()

directory = tkFileDialog.askdirectory()

dataset = {}
fieldnames = []
fieldnames.append('Column')
fieldnames.append('Row')
for filename in os.listdir(directory):
    if filename.endswith('.xls'):
        with open(directory + '/' + filename) as tsv:                #these are specific to this xls file
            format
                field = []
                name = ""
                for line in csv.reader(tsv, delimiter="\t"):
                    if len(line) > 2:
                        field.append(line)
                dataset[filename] = field
            fieldnames.append(filename)
```

```

output = []
for i in range(1, num_columns+1):
    for j in range(1, num_rows+1):
        line = {}
        line['Column'] = i
        line['Row'] = chr(j+64)
        for field in dataset:
            if field != ":":
                line[field] = dataset[field][j][i]
        output.append(line)

```

```

with open(directory + "/output.csv", 'w') as csvfile:
    writer = csv.DictWriter(csvfile, fieldnames=fieldnames, extrasaction='ignore', lineterminator = '\n')
    if os.path.getsize(directory + "/output.csv") < 1:
        writer.writeheader()
    for item in output:
        writer.writerow(item)

```

4.2 Changing pinning tool on the Matrix PlateMate Plus

1. To change the pinning tool for the 384-well 100nl tool, open the ControlMate software and go to Add-Ins in the toolbar.
2. In the 'Select Pipettor or Tips' tab choose '384 PIN TOOL SET 100nl' from the drop-down menu (**Figure 15, arrow**).

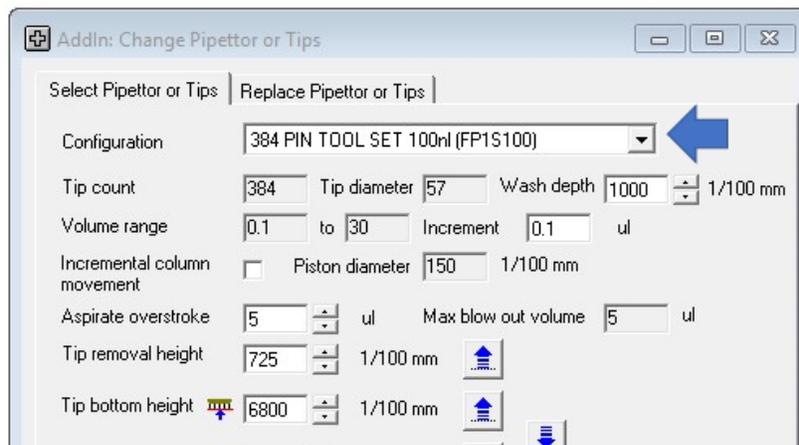


Figure 13. Screenshot for selecting pinning tool. Arrow: drop down menu with pipetting head configurations.

3. In the 'Replace Pipettor or Tips' tab select 'Tips only' (**Figure 16 arrow**) and follow the instructions in blue under Action.

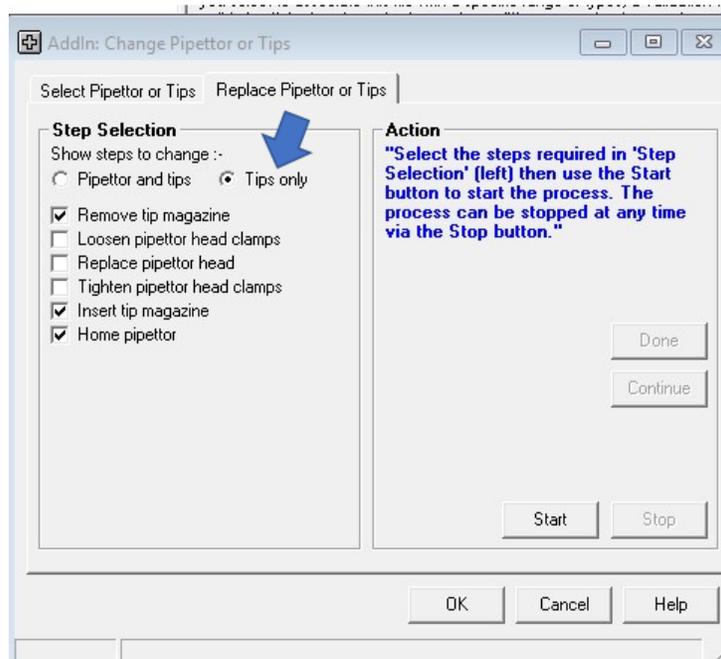


Figure 14. Screenshot of interface for changing pipetting head.

4. Click 'Start' in the Action window to release the clamps holding the pinning tool.
5. Lift the front plastic cover of the pinning tool and remove the pinning tool by lifting it up slightly and sliding it out.
6. Click 'Done' in the Action window.
7. Insert the 384-well 100nl pinning tool into the slot and click 'Done'. The clamps will close.
8. Lower the front plastic cover of the pinning tool.

NOTE: It is very important that the actual pinning tool and the chosen pinning tool in the drop-down menu correspond. The measurements of each pinning tool differ and improper settings might result in damage of the pins from crashing into the plates.