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Highly Parallel Droplet Sample Preparation for Single Cell Proteomics V.3

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Human Cell Atlas Metho...

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We use this protocol and it's working

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Abstract

Protocol for preparing single cells for mass-spec analysis by nPOP as described by **Leduc et al., 2021, 2022** DOI: 10.1186/s13059-022-02817-5. nPOP uses piezo acoustic dispensing to isolate individual cells in 300 picoliter volumes and performs all subsequent preparation steps in small droplets on a fluorocarbon-coated slide. This design enables simultaneous sample preparation of thousands of single cells in a single batch. This includes lysing, digesting, and labeling individual cells in volumes below 20 nl. nPOP supports different experimental designs including label free analysis, TMT 18plex with carrier, mTRAQ, and TMT-10plex for TOF instruments (see steps).

Data, resources and additional information: https://scp.slavovlab.net/Leduc_et_al_2022 and <https://scp.slavovlab.net/nPOP>

Andrew Leduc presented a detailed workflow of nPOP at the 4th Single Cell Proteomics Conference available on YouTube: https://youtu.be/DJ1U_KpMNcY

The protocol uses the CellenONE system for liquid handling and cell sorting and assumes basic level of familiarity and training on the CellenONE. If there are any questions, please email leduc.an@northeastern.edu

Setting up system

- 1 This protocol enables highly paralleled single cell sample prep for that can be used for label free analysis but has most utility for multiplexed analysis.

We provide options for TMT 18 plex with carrier (14plex) and mTRAQ labels for multiplexed DIA analysis (2plex or 3plex).

Which option you choose depends only on the folder of droplet layout files you use.

If using TMT18 this gives you 14 single cells per set, because the carrier and reference take up two spots, and then you need to leave two spots after carrier and reference empty for isotopic impurities.

- 2 To get started with nPOP, you will need to move the the files from this [repository](#) into your CellenONE. There are three important classes of files contained:
 - Tasks (discrete actions)
 - Runs (made up of tasks)
 - Droplet Layout Files (experimental design)

To install these files on your instrument, navigate to the following base directory (this is what it is on my instrument, at least):

C: > Users > Scienion AG > CellenONE

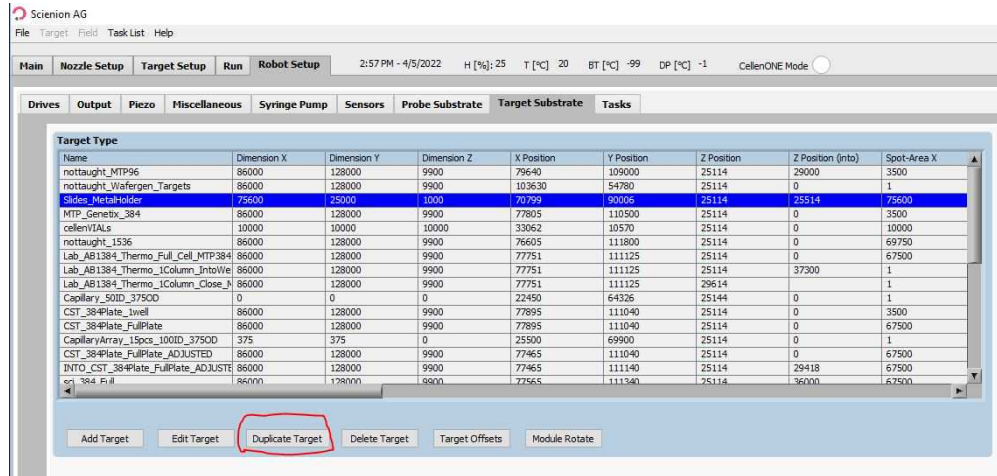
- Droplet layouts go in the "Pattern" subdirectory
- Run-associated files go in the "Settings/Run" subdirectory
- Task files go in the "Settings/Task" subdirectory

Close the CellenONE software and reopen to verify that the files have appeared in the corresponding locations (run-associated files in the "Run" panel in the Main tab, tasks in the "Do task" sub tab of the nozzle setup tab, and droplet layouts can be loaded in as field files).

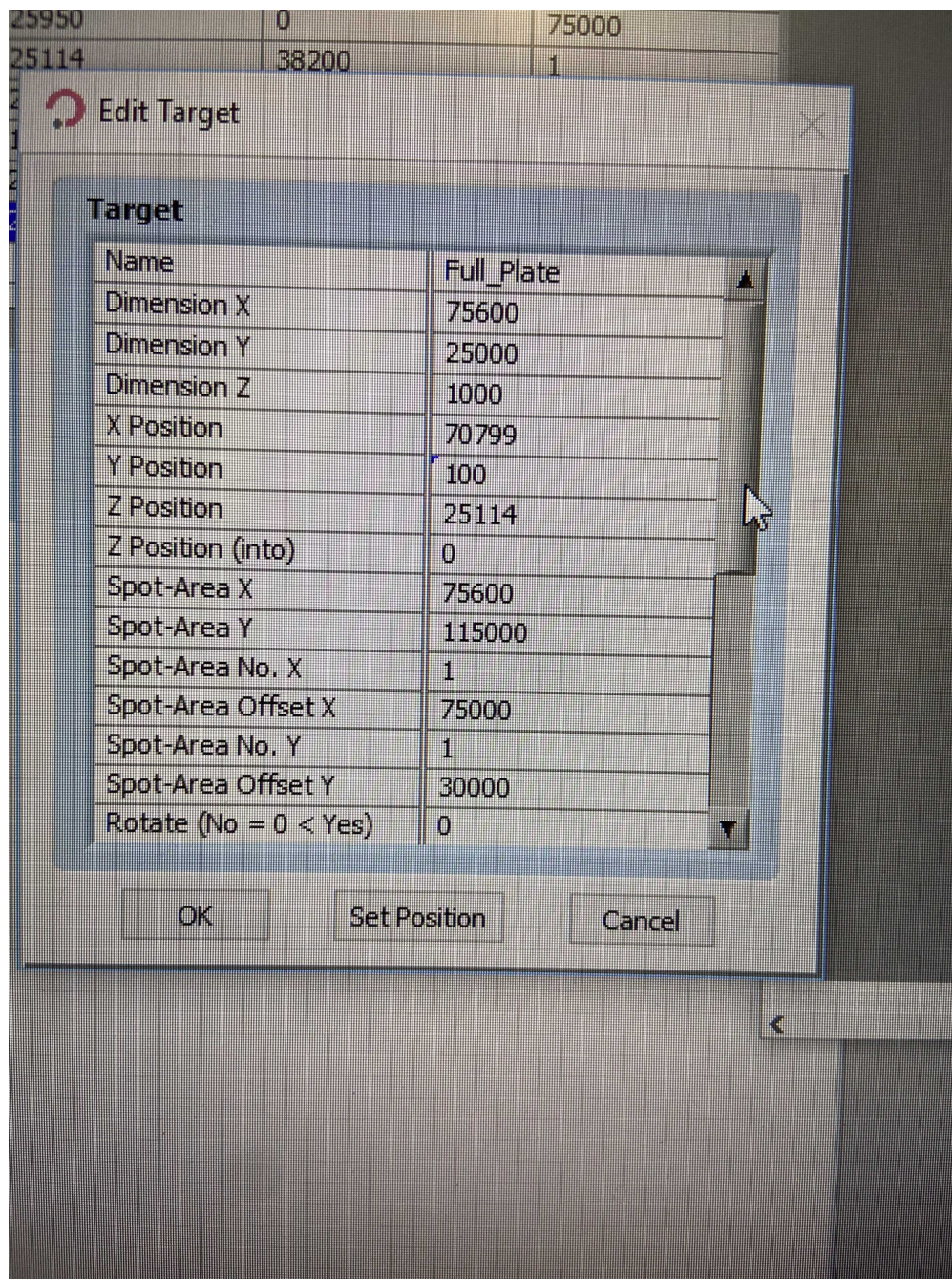
- 3 There is one aspect you will need to set up on your system that cannot be transferred over: the **Target** area, which encompasses all four slides.

To do this, go to the Robot Setup tab and navigate to the "Target Substrate" sub-tab.

Select "Slides_MetalHolder" and hit "duplicate target".



- At the bottom, you will have a new target called "Slides_MetalHolder_copy". Double click on the target and copy the settings as posted in the picture below:



- 5 Next, we need to make one more target for aspirating samples off of the surface of the glass slide.

This should be the exact same as the last one you just made (so make a new duplicate of "Full_Plate" and call it "Full_Plate_SlidePickup") but the Z Position should be ~29000.

This is different on each instrument, so what you can do is go to the Drives tab, move the nozzle to the "Slides_MetalHolder" Target area, and inch down the z height so that it is

just slightly off the surface of the slide. Once satisfied, set the new value for Z position as you did in step 3.

- 6 Time to test our new setup! In the main tab, select your Target area to be "Full_Plate" and then run to "SpotRun". Then load the following Field file: System_setup > Setup_slide_all.fld. This method should place a small droplet in all 4 corners of each slide. If you're not happy with droplet positions, you can adjust the spacing in between slides with the parameter shown below. After changing it, be sure to hit ALL to apply the spacing to for the distance between each slide. Once this is set, you will need to apply these settings to all the field files that you use (load everything and save them with the changes you made to these parameters).

What you need to get started

- 7 To get started, you will need a few things. These consumables can all be purchased from **Scienion**

Personally, I have had the most success operating with two PDC nozzles from Scienion:

- A regular glass PDC, size medium, for handling cells
- A PDC 70 with coating type 2. This is for handling organic solvents such as DMSO and protein mixtures.

If you do not have the type two coating nozzle, my advice is to still use two regular glass nozzles, leaving one for DMSO, and the other for trypsin and cells. If you only have one nozzle, it may require multiple flush steps when switching back and forth between organic and aqueous.

You can also use 2 PDC 70 nozzles, that works just fine.

For slides, we use the **H1 slides** which are regular glass slides that have a flouorocarbon coating.

In terms of the sample prep itself, you will need your cells, DMSO, 1M TEAB(triethylammonium bicarbonate), Trypsin Gold, Benzonase endonuclease, DDM (n-Dodecyl-beta-Maltoside), HEPES pH 8.5, any labeling reagents (TMT, mTRAQ, etc.), and triethylammonium bicarbonate (TEAB) if using mTRAQ.

- 8 Once you have everything you need to get started, it's worth investing some time to get your workflow ironed out prior to attempting a full prep. In the following two sub-points



you will find some preparatory steps that I take alongside some troubleshooting suggestions.

8.1 **Getting the nozzles in the exact same condition each time you start a prep.**

My routine is to use the syringe attachment to clean and flush the nozzle and lines within the PDC. I place the tip of the PDC in water that is sonicating and pull water in and out of the PDC for ~5 minutes, followed by a minute of 100% ethanol and then another minute of water. Be careful not to get the black ring of the PDC wet, as this can cause electronics to short if you use directly after.



8.2 Practice with the reagents you will be using before starting a prep.

Several factors such as humidity, room temperature and the specific nozzle can have large effects on dispensing accuracy.

As an example, for dispensing mTRAQ labels, I use DMSO as I found it easiest to work with, however, we have also used Ethanol and IPA for dispensing mTRAQ in the past. This can work well, but, requires you to aspirate air and then ~20 uL of organic solvent prior to aspirating label and dispensing.

Some tips that have worked for me:

1) the trypsin is highly viscous, so be sure to run "dip wash" after aspirating, and reduce frequency, voltage, and pulse considerably

2) if a droplet seems to be shooting off to the side, dabbing the bottom of the tip twice with a kimwipe (damp with ethanol) can really help. If the issue persists, move nozzle to home position, turn on continuous dispensing, and try cleaning tip with an ethanol-dampened kimwipe for ~60 seconds.

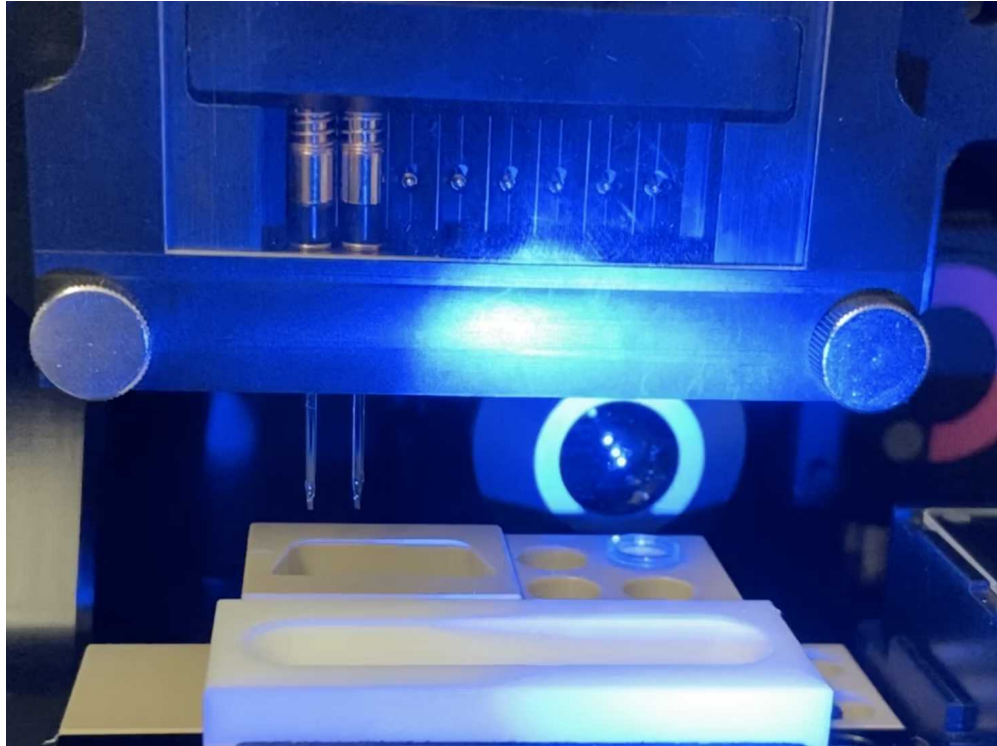
3) Generally higher voltage has helped with DMSO dispensing. Also make sure humidity is not too high when dispensing DMSO, this can cause droplets to stick to the end of nozzle.

Carrier and Reference Preparation (**Only if Carrier is used**)

- 9 Prepare cell pellets of at least 500,000 cells for all relevant cell types . Add 100% DMSO to cells to a cellular concentration of 6000 cells/uL. Incubate cells in DMSO for 20 minutes to lyse cells. Add mass spectrometry grade water to bring solution to 2000 cells/uL. More information on carrier and reference preparation is available at Petelski, A.A., Emmott, E., Leduc, A. *et al.* Multiplexed single-cell proteomics using SCoPE2. *Nat Protoc* **16**,5398–5425 (2021). <https://doi.org/10.1038/s41596-021-00616-z>
- 10 Add 1x benzonase, 100mM TEAB and 20 ng/uL of trypsin. Digest sample at 37 degrees C overnight.
- 11 Label carrier and reference samples with desired labels. Dilute samples before combining such that the composite sample is at 200 cells/uL of carrier and 5 cells/uL of reference.

Preparing CellenONE

- 12 Follow start-up procedure for CellenONE with two nozzles attached. In the far left position, attach a size medium glass PDC nozzle. In the adjacent slot to the right, attach a size medium PDC 70 nozzle with type 2 coating.



- 13 In the target spotting location, place the glass slide holder (make sure it fits well) and then place your fluorocarbon-coated glass slides (CellenONE H1 Slides) in their designated slots (make sure they are all level).

In the probe location, place any desired 384 well plate for storing and pickup of cell suspensions and reagents used in sample preparation. On the CellenONE "Main" tab, set the probe location to the relevant 384 well plate and set the target location to our new "Full_plate".



Preparing cell suspension

- 14 Suspend cells in 1X PBS at a concentration of 200-300 cells/uL. If cells are prone to clumping, first filter with a 40 micron filter to ensure a fast pace of cell sorting.

DMSO for Cell Lysis

15

Make sure the target location is "Full_plate". Load the DMSO_L field.

On the main page, set the Run to "Sample_pickup_and_dispense".

Make sure the second nozzle (the type-2 PDC is selected).

16

Load 20 uL of 100% mass-spectrometry-grade DMSO into all relevant wells in the 384-well plate. The number of wells you need to use depends on the sample prep you are doing (wells used at each step can be found in the field file). For simplicity, you can also just load wells D1-D8 as this will work for any layout.

Make sure the plate is secure, and run the "Sample_pickup_and_dispense" method!

17

DMSO droplets for lysis should be automatically dispensed over the relevant slides. After run is over, check images to make sure droplets did not deviate (these can be found in the Run folder you created/selected when starting prep).

If deviations are noticed, wipe droplets on the relevant slide with a KimWipe, flip slide over, and re-dispense for required slide. When removing slides, be careful not to move or touch adjacent slides as this will cause misalignment of droplets for subsequent steps and likely failed cells/preps for those drops.

18

Turn the humidifier on and set it to maintain 70% relative humidity and set the plate temperature to maintain 19C.

The DMSO droplets will need to persist for entire time of cell dispensing. If you're dispensing thousands of cells, the dispensing can take almost 2 hours.

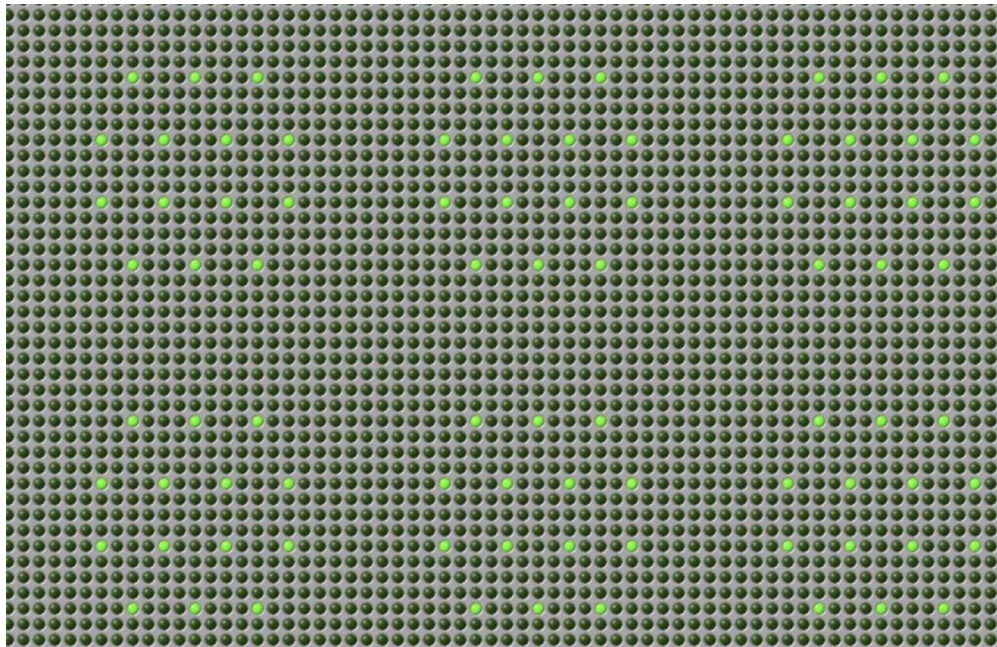
You may need to adjust humidity and cooling settings to see what works best for you, maybe go to 70% relative humidity and 18.5C if you find it necessary.

Cell Dispensing

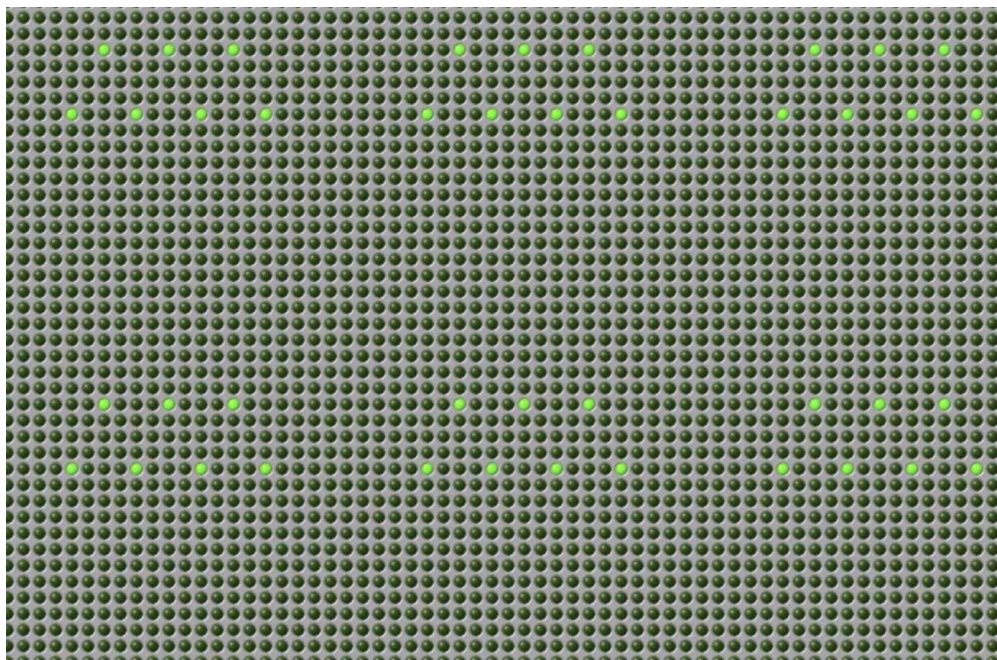
19

Load the "Cells" field file.

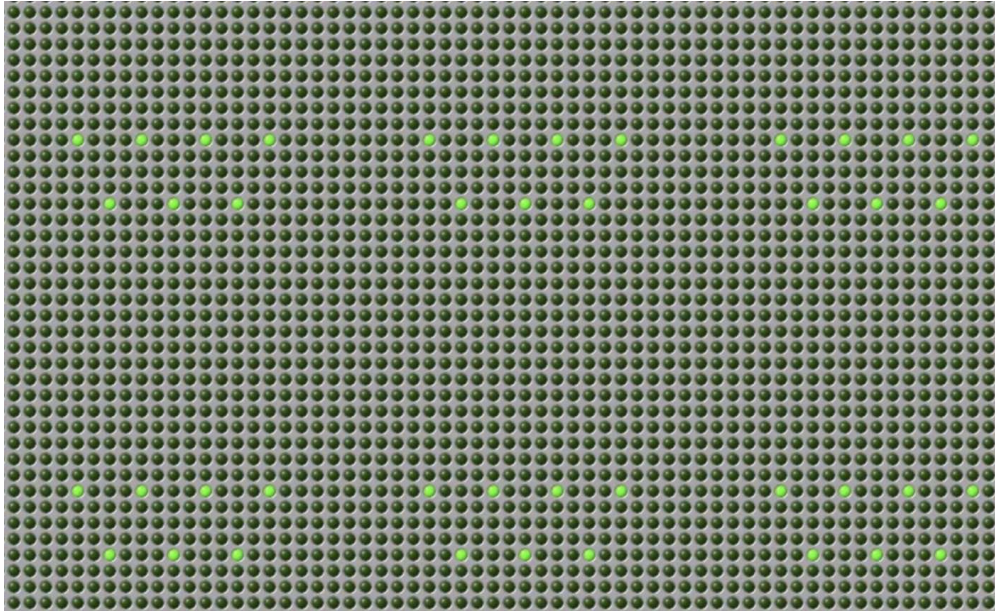
If you are dispensing multiple separate cell suspensions, remove a portion of spots, (remove some from each cluster if using labels) to ensure analysis of all relevant cell types. See picture for example.



No deleted cells



Adjusted field file for cell type 1 (top cells)



Adjusted field file for cell type 2 (bottom cells)

I recommend making the field files for all required cell types before hand.

We also suggest leaving some droplets without any cell to serve as negative controls.

- 20 On main tab, set run program to "CellenONE_Basic".
Aspirate 10-20 uL of your cell suspension from the 384-well plate using the leftmost PDC (the glass one).

- 21 Dispense first cell type.

Repeat steps 17 and 18 for additional cell types

Evaporation Control

- 22 After cell dispensing is finished, set humidity control to 75 % relative humidity and set cooling control to "dew point chase" with a deviation of -1 degrees.

These settings vary based off the room temperature of your lab, they may need to be adjusted to maintain stable drops. I find that colder lab spaces ~19 C require lower value near -1.2 while warmer lab spaces ~24/25 C require closer to -0.75

- 23 Change to "SpotRun" in the Run menu.
Load the perimeter field file.
Dispense a perimeter of system water to control local evaporation.



Start preparing digest.

Digestion

24

To prepare the digest you will need, Trypsin Gold, DDM, Benzonase, and HEPES pH 8.5.

- 24.1 First prepare 60 uL of a solution that contains Trypsin gold at 100 ng/uL, 5 mM HEPES, 0.1 mM DDM, and .5 x Benzonase.

Place in a 1.5 mL tube.

- 24.2 This step is **optional** based off how easy dispensing trypsin solution is for user. In this step we degas the trypsin solution on ice. Find a bottle with the same opening as the cellenONE bottle where the system water is stored. Fill with ice to brim and place in a styrofoam container filled with ice.

Then place trypsin in ice (cap open), attach vacuum attachment used for degassing cellenONE water, turn on vacuum, and wait for 10 minutes.



- 25 How much master mix you aspirate depends on how many cells you are preparing. If preparing 3,000 cells using the 14 cell per cluster method, we advise:
- Fill 3 wells of the 384-well plate each with 30 uL of your master mix
 - Manually use the Take_20uL_sample task from nozzle setup to aspirate 20 uL of master mix **three times** with the PDC type-2 out of each, one after the other so the PDC contains 60uL of master mix in total. You can just run the Take 20 uL task back to back to back.



- Make sure to run dip wash immediately after aspirating master mix to ensure stable droplet.

** if you dont see the Take_20uL_sample task in Nozzle Setup → Do Task, go to Robot Setup → Tasks and make sure there is no X next to the task on the Task Macro List

26 Set Run file to "SpotRun_ImageField" and load the "Digest" field file.

Dispense master mix.

Let single cell digestion proceed for 4 hours.

(If you set up remote access, you can monitor this from home / leave for day)

27 To control evaporation, you can reload the "Perimeter" field file and refresh perimeter field with system water 1-2 times throughout digestion depending on how much the perimeter seems to be evaporating.

Either PDC should work fine for this.

28 After digestion turn off humidity and cooling and wait ~15 minutes and let single-cell peptides dry out on the slide.

This is a pause point. Labeling can proceed the next day as peptides rest dried out on the slide.

If doing label free, proceed to the "Prepare 384 well plate for Storing Sample" section. The pickup of all 384 samples will take nearly a full day, so you can leave this until the following day if desired.



Labeling

29 The labeling routine depends a bit on the labels that you are using. We only have experience with TMT and mTRAQ.

For TMT we have found the labeling proceeds best in pure organic phase. For mTRAQ, sufficient and well buffered aqueous phase is needed.

If using mTRAQ, Load the field file for "TEAB" and dispense 200 mM TEAB to each spot. First pick up 20 uL of TEAB 3 times to get 60 uL of TEAB in the nozzle and then use spot



run to dispense.

NO humidity or cooling is needed for TMT, but be sure to set cooling and humidity settings back to as previously set during digest for mTRAQ (75% humidity, -1 degree dew point chase).

- 30 If multiplexing reagents are stored in acetonitrile, evaporate off acetonitrile completely in speed vacuum on low heat or lyophilizer.

Re-suspend TMT labels in 100% DMSO at a concentration of 1/3 maximum strength.

You will need a volume of 25 uL of TMT label (this volume is post 1/3 dilution). This gives enough volume for a retry if there is a failure with the label.

Critical: Pipette mix labels vigorously to prevent TMT crystals from forming as these can interfere with label dispensing.

- 31 For TMT, load all labels into wells G1 → G14 if using all 14 labels.

For mTRAQ 2 plex, load labels D0 in G1, D4 in G2 and repeat 3 more times to well G8 (so then again D0 in G3, D4 in G4, D0 G5 ...).

For mTRAQ 3 plex, load labels D0 in G1, D4 in G2, D8 in G3 and repeat 3 more times to well G12 (so then again D0 in G4, D4 in G5, D8 G6 ...).

- 32 Load the field file for the first Label. Set the run method to "SamplePickup_And_Dispense" and run labeling.

I suggest leaving the light on (clicking in the lightbulb button on the nozzle setup page) so you can make sure there is no residual DMSO on the tip of nozzle when it comes back in front of camera to test droplet.

If residual DMSO is present, you can pause the run with the "nozzle setup" button and dab tip with a Kimwipe to remove any hanging droplets.

Critical: Check droplet stream before and after dispense to ensure the label was accurately dispensed as there will be no imaging validation until end. Imaging each field after each label will significantly increase time for dispensing labels.

- 33 If using mTRAQ, Load the field file for "TEAB" and dispense 200 mM TEAB to each spot. First pick up 20 uL of TEAB 3 times to get 60 uL of TEAB in the nozzle and then use spot run to dispense.



- 34 Let cells label for 1 hour after last label is dispensed.

Quenching Labeling Reaction

- 35 Turn on humidity and cooling to previous settings used during digest.
- 36 Prepare a solution of 5% hydroxylamine (HA) diluted with mass spectrometry grade water.
- Load the HA field file and set the run method to "Spot_Run".
- Load 30 uL into two wells of the 384 well plate. Aspirate 40 uL of HA by taking 20 uL from each well.
- Dispense HA and incubate for 20 minutes.
- 37 Repeat step 34 for two quenching steps.

Prepare 384 well-plate for Storing Sample

- 38 Pipette desired carrier amount diluted in 2 uL cells worth of combined carrier and reference into all the wells of your 384-well plate. For 2/3 plex analysis with plexDIA, you will need 2 384 well plates to hold all the samples.
- If not using carrier, fill every well with 2 ul of mass-spec-grade water.
- If you are using carrier but dont yet have it made, you can resuspend samples in carrier later prior to injection.
- 39 We recommend using the 384 well PCR plate from thermofisher. In order to use this plate to pick up the samples with an ultimate 3000, you will need to purchase the attachment below which raises the plate up a bit.

<https://www.thermofisher.com/order/catalog/product/6820.4088#/6820.4088>

Sample collection

- 40 **DISCLAIMER:** The pickup can take a long time, its about ~2 hours per 256 sets. Good news is that it is very hands off and robust. With 2/3 plex, you will have closer to 700

sets and this can take ~6 hours.

Samples should be fine on the slide for extended periods of time, we have not noticed any significant batch effects.

- 41 Change the target substrate in the main tab to "FullPlate_SamplePickup" that we set up in beginning. Change Run to "Pickup_off_Slide". (for LF or 2/3plex, use the "Pickup_off_Slide_smallVol" run).

Load the field file "pickup". (if using 2/3 plex, 2 plates are required. You will have to load a second field file "pickup2" and change out 384 well plate half way through.

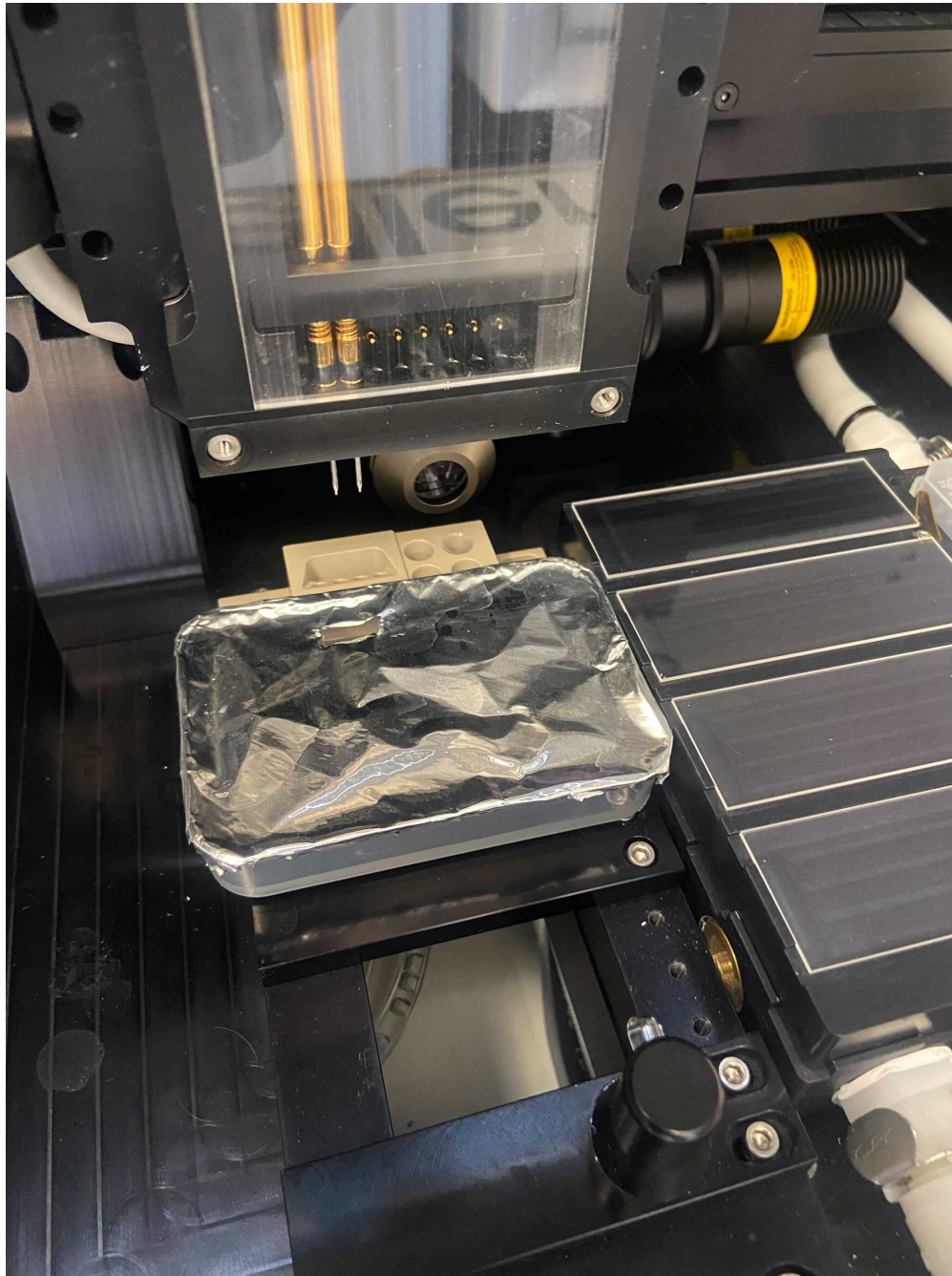
- 42 For the pickup, a wash tray is needed to hold liquid for pooling droplets on slide. Unfortunately the CellenONE only comes with a small container that can hold ~3 mL. This will need to be filled many times throughout pickup.

To get around this I ordered this polypropylene tray off amazon and made a container myself for holding more liquid.

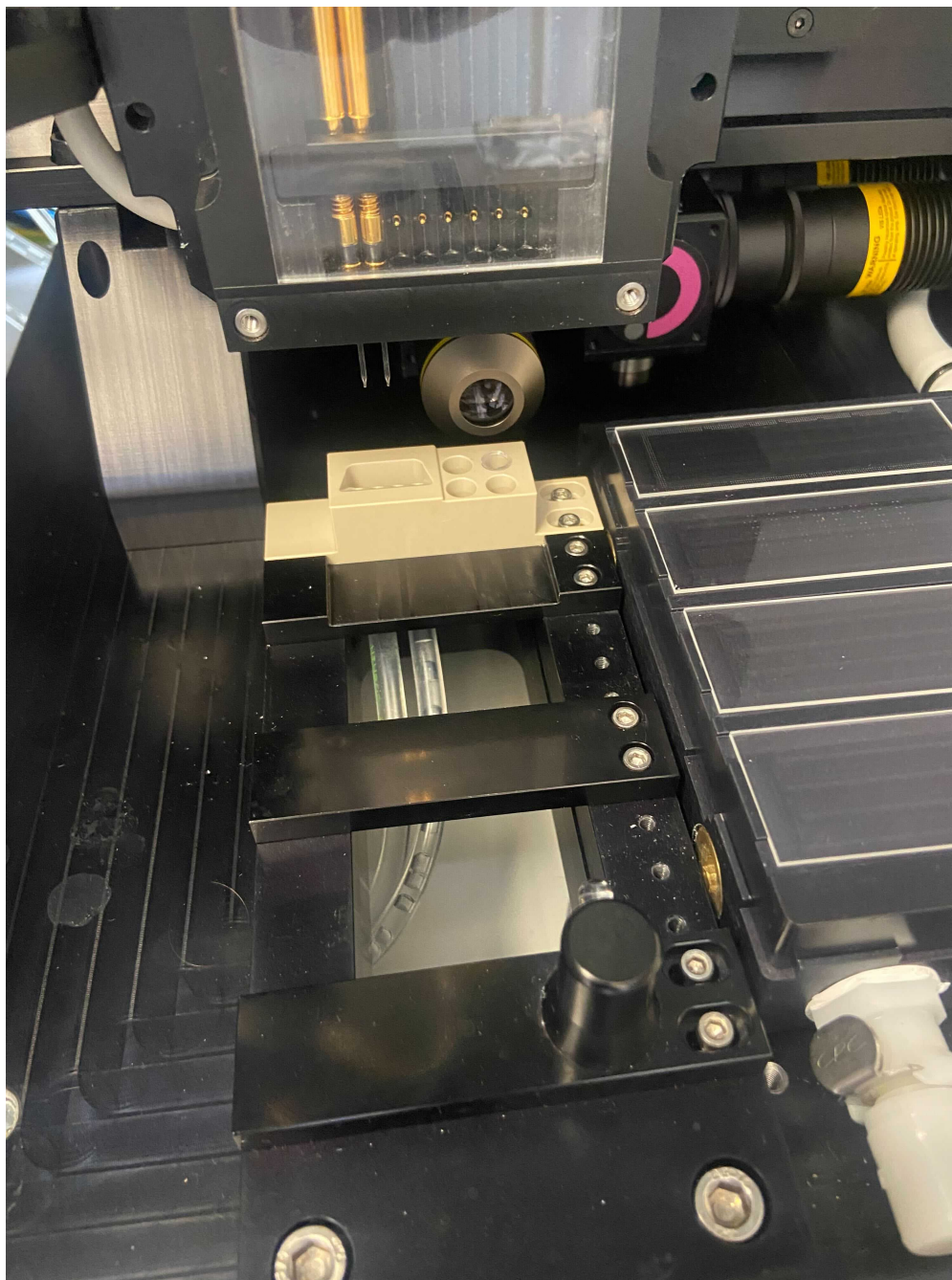
https://www.amazon.com/AKOAK-Polypropylene-Rectangle-Containers-Accessories/dp/B07JZP7CG2/ref=sr_1_6?keywords=Polypropylene%2Bstorage%2Bbox&qid=1666642150&qu=eyJxc2MiOi0LjY5liwicXNhIjojNC4xMSIsInFzcCI6IjMuMzcifQ%3D%3D&sr=8-6&th=1

Size:

3.26" x 2.12" x 0.7"



Sticky foil is used to cover top and slit is cut to allow nozzles to pass down into well



Without tray

- 43 Fill your wash tray with 40 mL of 50% acetonitrile/ 50% LC/MS grade water. Run the Sample pickup method. Watch to make sure the first few go well. If doing 2/3 plex analysis you will need to change out plate and run method again in 2 hours.
- 44 Dry down each sample in a vacuum evaporator (we use a SpeedVac) for 30 minutes to remove any residual DMSO in the samples and store in -80 until samples are ready to be run on LC/MS.



- 45 When samples are ready to be run for LC/MS analysis, resuspend in desired volume of 0.1% Formic acid.

Data analysis

- 46 For analyzing samples we have provided code at Github. This allows mapping single cells from the analysis. See [Github](#).