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EEL FISH

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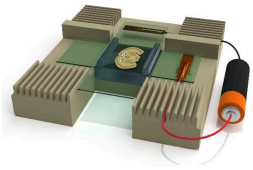

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We use this protocol and it's working

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Abstract

Protocol to perform RNA transfer to a surface using the EEL method and detection with an automated fluidics and imaging machine called ROBOFISH.

Paper title: Scalable in situ single-cell profiling by electrophoretic capture of mRNA

Website: **mousebrain.org**

Instructions to build the **ROBOFISH system**:

Code for the **ROBOFISH system**

Guidelines

Working with coverslips can be challenging because they are fragile. The experiment takes some time and it is extremely frustrating when a glas breaks late in the experiment. Therefore, we advise to practice handling the glasses. Take safety googles and break a couple of glasses while handling them, to get a feel for the limits. Sounds stupid but it is totally worth it.

Materials

EEL electrophoresis holder:

EEL_Base_v2.STL

List of Chemicals

	A	B	C	D
	Name	**Shape/size**	**Distributor**	**ID**
	ITO coverslips	24x60mm, #1.5, surface resistivity of 30-60 Ω/square	Diamond coatings	Custom size order
	2-Propanol	2.5 liter	Sigma	34863
	Aceton	1 liter	Sigma	270725
	dH2O	10 X 500 ml	Thermo	10977035
	SSC 20X	1 L	Sigma	S6639
	(3-Glycidyloxypropyl)Trimethoxysilane	100 ml	Sigma	440167
	Glass staining trough	105 mm × 85 mm × 70 mm	Sigma	BR472200-10EA
	Coverslip rack Epridia E103	25 slides, 22mm (put 24mm coverslips diagonal)	Fisher Scientific	12638436
	Hybrislip	24x24 mm	Sigma	GBL722222
	Poly-dT 60	/5AmMC6/UUUUGACTCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/iSuper-dT/TTTTTTTTTTTTTTTTTTTTTTTTTTT/iSuper-dT/TT	IDT	Custom
	Moist Mark Plus pen	Pen	VWR	MP1000
	Poly-D-lysin	5 mg	Sigma	P6407
	Europium beads 0.2um (See also item 50)	1ml	Thermo	F20881
	Propidium Iodide	10ml	Sigma	P4866
	DTT	10 ml	Sigma	43816
	Triton X-100	100 ml	Sigma	T8787
	TBE 10X	1 L	Thermo	15581-044
	Urea	1 kg (Advised to buy smaller package)	Merck	1.08487.1000
	PDMS / Sylgard		Dow Corning	Sylgard 184
	Copper foil tape with conductive adhesive	5mm x 30m	m.nu (not available anymore, replacement part: Adafruit copper tape with	

	A	B	C	D
			conductive adhesive)	
	Proteinase K (NEB)	1 ml	NEB	P8107S
	SDS	500 ml	Sigma	05030
	Tris HCl pH7.4	1 L	Sigma	T2194
	Superase RNase inhibitor	10,000 UN	Thermo	AM2696
	PBS	1 L	Thermo	AM9625
	PFA	10 x 10 ml	Thermo	28908
	Dextran Sulfate	50 g	Sigma	D8906
	E. Coli tRNA	500 mg	Roche	10109550001
	RVC	5 ml, 200mM	Sigma	R3380
	BSA	50ml, 30%	Sigma	126621
	Formamide	500 g	Thermo	AM9342
	Glycerol	500 ml	Sigma	G5516
	KCl	1 kg (Advised to buy smaller package)	Merck	1.04936.1000
	EDTA	100 ml, 0.5 M	Thermo	AM9260G
	Tris HCl pH8.0	1 L	Sigma	T3038
	PCD	25 UN	Sigma	P8279
	DBA	25 g	Sigma	37580
	NaOH	100 ml	Sigma	72068
	TROLOX	5 g	Sigma	238813
	EtoH	1 L	Histolab	UN1170
	TCEP	5 ml	Thermo	77720
	Mineral Oil	500 ml	Sigma	M5904
	Penicillin-Streptomycin	100 ml (10.000U/ml)	Thermo	15140122
	Buffer containers ROBOFISH:			
	Nalgene media bottle 2019 style	125 ml	Sigma	Z364495-48EA
	Glass bottle with septum cap	60 ml	Sigma	23229
	Glass bottle	15 ml	Sigma	27162
	Bottle septum cap for 27162	18 mm	Sigma	27020
	Europium beads 1um	10ml	Thermo	Custom order. Contact Thermo

Other materials

EEL holder:
3D print the EEL electrophoresis holder. (See file above)

In the center you should place a 20 x 20 x 5 mm piece of PDMS, this can be made by casting a 5mm thick sheet of PDMS in a petri dish and cutting it to size.

You also need two strips of 22 x 5 x 1.5 mm of PDMS to space the two glasses during electrophoresis. The 1.5 mm is the critical dimension and a convenient way of making a 1.5mm sheet of PDMS is using a 1.5 mm Western Blot gel casting cassette.

General equipment:

Pipettes + tips
Sonicator bath
Beaker glas
Power source (AA battery, Lab power supply, Keithley 2450 SMU)
Electrical wires
Oven that can reach 30-37C
Petri dishes
Scalpel
Forceps
Parafilm
24x30mm coverslips or similar
Gel loading pipet tips P200

Safety warnings

Take special care when handling Formamide and PFA.

Before start

The protocol lists a number of buffer that can be prepared beforehand:
30% formamide hybridization mix. Step 33.
10% formamide hybridization mix. Step 39.
PCD stock solution. Step 39.
DBA stock solution. Step 39.
TROLOX stock solution. Step 39.

ITO glass cleaning

1 Place ITO slides in a beaker glass and submerge them in Acetone. Cover the beaker glass with aluminium foil and sonicate 00:20:00 on high power.

2 Discard the Acetone and submerge the slides in 2-Propanol. Replace the water in the sonicator if it has warmed up. Cover the beaker glass with aluminium foil and sonicate 00:20:00 on high power.

3 Discard the 2-Propanol and submerge the slides in dH_2O. Replace the water in the sonicator if it has warmed up. Cover the beaker glass with aluminium foil and sonicate 00:20:00 on high power.

4 Discard the dH2O and replace with fresh dH2O. The slides should be stored in water. Wait at least a couple of days before using. The slides can be used for up to a month, after which they might show white discoloring and should not be used.

Surface functionalization

5 Place the slides in a rack and let them dry. Optional use compressed air or nitrogen gas to remove larger droplets.

6 In a dedicated glass staining jar, prepare 300 mL of [M] 2 % volume GPTMS ((3-Glycidyloxypropyl)Trimethoxysilane) in anhydrous acetone. Put the jar in a box/bag with a steady nitrogen gas flow so that the coating is performed under an inert environment.

7 Submerge the rack with slides into the Functionalization solution for 02:00:00 under nitrogen atmosphere.

8 Pour out the acetone GPTMS mix. Submerge the slides with acetone to wash them, then discard the acetone.

9 Dry the slides with nitrogen gas.

Capture slide coating

10 Find the conductive side of the ITO glass using a multimeter set to measure resistance. Add 40 µL of Poly-T spotting solution and incubate 01:00:00 at 25 °C under a 24x24mm plastic coverslip (Hybrislip - Grace biolabs). Optionally, with a solvent prove marker (like Moist Mark Plus, Cancer Diagnostics Inc.) indicate the area of the Poly-T coating.

	Component	Final concentration	Stock concentration	Quantity for 45 ul
	Poly-T	10 uM	100 uM	4.5 ul
	Schot spotting solution	1X	2X	22.5 ul
	dH2O	-	-	18 ul

Poly-T coating solution. Make fresh. Expected usage for 1 slide: 40ul.
Sequence: /5AmMC6/UUUUGACTCGTTTTTTTTTTTTTTTTTTTTTTTTTTTT/iSuper-dT/TTTTTTTTTTTTTTTTTTTTTTTTTT/iSuper-dT/TT (ordered from IDT)

11 To remove the plastic coverslip without damaging the coating, place a couple of drops of SSC 2X on the edge of the plastic cover slip. Wait until the SSC has moved under the plastic coverslip and then lift the corner with a forceps.

Wash the slide 5 times with SSC 2X by pipetting it onto the coated area and aspirating it from one of the corners.

12 Wash the the slide twice with dH_2O.

13 Block remaining epoxy 00:30:00 with 60 µL [M] 0.1 Mass / % volume Poly-D-Lysin in dH_2O at room temperature. (The PDL mix should spread out over the oligo-dT coated area and does not need to be covered by a plastic coverslip.)

14 Wash 3 times with dH_2O.

15 Mix Europium doped beads small (200nm) and big (1um) 1:333 each in dH_2O and place ~ 100 µL on the slide, and make sure it covers the full area. Incubate 00:03:00 .

16 Pipett off the bead mixture from a corner of the coated area and let the slide dry to the air. Avoid residue in the capture area and do not touch the surface. Proceed to tissue capture.

Tissue sectioning

17 Make a 10 µm cryosection of the tissue sample and catch it on the slide onto the capture area, and let it dry for a few minutes.
Place slide in a container, like a petri dish, so that frost can not accumulate on the sample.
Store at -80 °C until further use.

Reference imaging (optional)

18 Thaw the section and on the back side make two reference crosses on either side of the tissue section using a solvent prove and non-fluorescent marker pen (like Moist Mark Plus, Cancer Diagnostics Inc.). These crosses will serve as the reference to find the sample area after the tissue has been digested.

19 Stain the nuclei for 00:05:00 with [M] 1 ug/ml Propidium Iodide in SSC 2X.

20 Wash 5 times with 200 µL SSC 2X.

21 Cut a gasket out of parafilm and place it around the tissue section and reference crosses. Close the open top with a 24x32mm glass coverslip (or any other fitting size). Make sure no air bubbles are present on the tissue or crosses by adding sufficient SSC 2X to the sample. When closed, drain the excess SSC 2X from a side with a paper towel.

22 When using the provided microscope Job for Nikon microscopes running NIS Elements software, follow the steps in the Job. Select the New Experiment option.

When using other software:

- Make a quick overview image with a low power objective (like 10X) to generate points for Nuclei imaging and RNA signal imaging later on.
- Generate points for imaging the RNA signal of the sample with a high power objective (like 60X). This is Point_set_1

- Generate points to image the nuclei with a medium power objective (like 40X). This is Point_set_2.
- Image the nuclei with the medium power objective using Point_set_2. These images will be used for segmentation. It is important to also image the beads in this step so that the images can be aligned.
- With the high power objective add the locations of the crosses to the previously generated Point_set_1 and save. Typically we make bright-field images of the 4 armpits of the crosses. Because the relative location between the sample and the crosses is now known, they can be used to find the sample area later on.

Electrophoresis

23 Remove the top coverslip, gasket and SSC 2X.

24 Permeabilize the sample for 00:05:00 in permeabilization buffer:

	Component	Final concentration	Stock concentration	Quantity for 1ml
	Dithiothreitol (DTT)	10 mM	100 mM	100 ul
	Triton X-100	0.1 %	-	1 ul
	TBE	1X	10X	100 ul
	dH2O	-	-	799 ul

Permeabilization buffer. Make fresh. Expected usage for 1 experiment: 200ul.

25 Wash 5 time with TBE 1X.

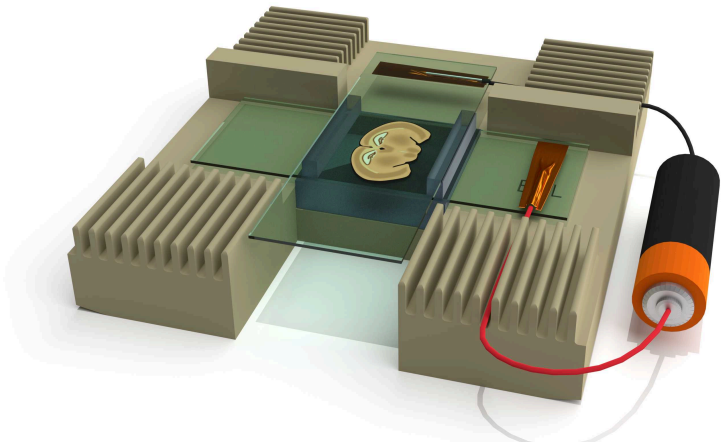
26 Assemble the electrophoresis setup:

- Stick a short wire to the sample ITO coverslip using copper tape with conductive adhesive (Anode).
- Do the same on a clean uncoaded ITO slide to make the other electrode (Cathode).
- Place the coverslip with the sample in the EEL holder and slide the wire in one of the slits to keep the slide in place.
- Place two 1.5 mm thick PDMS strips on either side of the sample.
- Cover with the top electrode perpendicular to the sample slide, and slide the wire in one of the slits. Make sure the conductive side is facing the sample.

- Optionally place a weight on top of the sample to keep the slides neatly stacked.
- Connect the power source to the two wires. The capture slide with the sample should be connected to the positive pole making the Anode. The top electrode should be connected to the negative pole, making the Cathode.
- Add the electrophoresis buffer:

	Component	Final concentration	Stock concentration	Quantity for 1ml
	Dithiothreitol (DTT)	10 mM	100 mM	100 ul
	Urea	1 M	10 M	100 ul
	TBE	1X	10X	100 ul
	dH2O	-	-	700 ul

Electrophoresis buffer. Make fresh. Expected usage for 1 experiment: 400 - 600 ul.



The bottom glass contains the tissue and is placed on top of the EEL holder. This is the capture slide and is connected to the positive pole of the power source (here depected as a battery) with the red wire and the coper tape. Two PDMS spacer separate the sample with the top electrode, which is connected to the negative pole with the black wire and coper tape. Pipett the electrophoresis buffer intbetween the glasses.

27 Turn on the power source set to 10 Volts/cm and electrophorese for 00:20:00 . When using 1.5 mm PDMS spacer set to 1.5V. We recomend to use a laboratory power supply but a 1.5V battery will work.

Tissue digestion

28 Turn off the power source, disconnect the wires and remove the electrophoresis buffer with a paper tissue. Now, remove the electrophoresis sandwich from the EEL holder and place on a flat surface. Carefully remove the top electrode and the PDMS spacers, clean these later for re-use. While pushing the capture slide onto a flat surface, carefully remove the wire and copper tape. Keep forces horizontal in order to not break the glass. Place glass in a petri dish.

29 Incubate the sample 00:05:00 in 6X SSC. 5m

30 Wash the sample two times with 2X SSC.

31 Digest the tissue 3 times for 00:10:00 with 200 µL of digestion buffer at 30 °C.
The tissue should visibly digest over the incubation steps.
Make sure no buffers with SDS come under the glass slide, they will dissolve the ink of the reference crosses.

	A	B	C	D
	Component	**Final concentration**	**Stock concentration**	**Quantity for 1ml**
	Proteinase K (NEB)	1 U/ml		2 ul
	SDS	1%	20%	50 ul
	Tris HCl pH 7.4	20 mM	1 M	20 ul
	Superase			5 ul
	dH2O	-	-	923.50 ul

Digestion buffer for mouse adult brain tissue. Make fresh. Expected usage for 1 experiment: 600 ul.

	Component	**Final concentration**	**Stock concentration**	**Quantity for 1ml**
	Proteinase K (NEB)	3.2 U/ml		4 ul
	SDS	1%	20%	50 ul
	Tris HCl pH 7.4	20 mM	1 M	20 ul
	Superase			5 ul
	dH2O	-	-	921 ul

Digestion buffer for human adult brain tissue. Make fresh. Expected usage for 1 experiment: 600 ul.

Adjust the digestion protocol for each type of tissue. The concentration of Protinase K, the incubation time or number of washes can be adjusted. Under-digestion, will leave tissue on the capture slide, which can be seen as increased background signal and signal distributed in the Z-axis during imaging. Over digestion can lead to reduced RNA capture (Presumably because the PDL gets degraded).

32 Wash 3 times 00:05:00 with 5% SDS in SSC 2X at 30C. Place the 5% SDS in SSC at 30C beforehand and during these steps.

33 Wash 5 times with SSC 2X.

34 Fix the RNA for 00:10:00 with 4% PFA in 1X PBS.

35 Wash 5 times with SSC 2X.

Encoding probe hybridization

36 Using a Speed Vac, dry the probe stock solution in a low binding Eppendorf tube and afterwards dissolve in 20 µL of 30% formamide hybridization mix. Probes should be hybridized at [M] 1 nanomolar (nM) per individual probe. (If you have 1000 individual probes, the total concentration should be 1000 nM to have 1nM per individual probe)

	Component	Final concentration	Stock concentration	Quantity for 10ml
	dH2O	-	-	5.58 ml
	SSC	2X	20X	1 ml
	Dextran sulfate	0.1 g/ml	-	1 gram
	Mix the above and vortex untill Dextran is dissolved			
	E. Coli tRNA	1 mg/ml	40 mg/ml	250 ul
	RVC	2 mM	200 mM	100 ul

	BSA	200 ug/ml	30 % (w/v)	66.75
	Formamide	30% (v/v)	-	3 ml

30% Formamide Hybridization mix. Can be stored at -20C. Expected usage for 1 experiment: 20 ul

37 Add a paper tissue to a petri dish and wet with some SSC 2X. Cover with a piece of parafilm and place two spacers on top. Place the slide on the spacers. The spacers are there to ensure that the liquid can not drain out during the incubation.
Remove as much of the liquid from the slide as possible. Add the Hybridizaion mix, be careful not to cause bubbles. Cover with a plastic coverslip (Hybrislip, Grace Biolabs). Close the petridish and place a few paper towels on top to prevent condensation on the lid during the incubation.

38 Incubate at least 40:00:00 at 38.5 °C in the humidified petridish.

Automatic system

39 Take the slide out of the incubator. Place some SSC 2X on the edge of the plastic coverslip and wait until it is adsorbed, to safely remove the plastic coverslip.

40 Mount the slide in the Flow cell.

41 Place the flow cell on the microscope stage and connect to the fluidic system. Connect outlet to the waste bottle and connect the temperature control cable and hoses. Using the manual prime port, prime the chamber with SSC 2X using a syringe.

42 Make buffers for 16 rounds of readout imaging and place them in the system.

Hybridization mixes with fluorescent probes.

	Component	**Final concentration**	**Stock concentration**	**Quantity for 40ml**
	dH2O	-	-	30.33 ml
	SSC	2X	20X	4 ml
	Dextran sulfate	0.1 g/ml	-	4 gram
	Mix the above and vortex untill Dextran is dissolved			

	E. Coli tRNA	1 mg/ml	40 mg/ml	1 ml
	RVC	2 mM	200 mM	400 ul
	BSA	200 ug/ml	30 % (w/v)	267 ul
	Formamide	30% (v/v)	-	4 ml

10% Formamide Hybridization mix. Can be stored at -20C. Aliquot 700 ul. Expected usage for 1 experiment: 16 aliquots or ~12 ml

For all 16 rounds add the fluorescent probes tot the 10% formamide hybridiation mix.

	A	B	C	D
	Component	**Final concentration**	**Stock concentration**	**Quantity for 700 ul**
	10% Hybridization mix	-	-	700 ul
	Fluorescent probe	50 nM	100 uM	0.35 ul

Probe mix. Make 700 ul mix for each round and put in system.

Wash buffer. 20% formamide.

	Component	**Final concentration**	**Stock concentration**	**Quantity for 70 ml**
	SSC	2X	20 X	7 ml
	Formamide	20% (v/v)	-	14 ml
	dH2O	-	-	49 ml

20% formamide wash buffer. Prepare fresh in 250 ml Nalgene bottle. Expected usage ~60 ml

Wash buffer. 30% formamide.

	A	B	C	D
	Component	**Final concentration**	**Stock concentration**	**Quantity for 20 ml**
	SSC	2X	20 X	2 ml
	Formamide	30% (v/v)	-	6 ml
	dH2O	-	-	12 ml

30% formamide wash buffer. Prepare fresh in 250 ml Nalgene bottle. Expected usage ~10 ml

Imaging buffer stock solutions:

Protocatechuate 3,4-Dioxygenase (PCD) enzyme stock solution 2 uM

	Component	Final concentration	Stock concentration	Quantity for 1.5ml
	Glycerol	50%	-	750 ul
	KCl	50 mM	1M	75 ul
	EDTA	1 mM	0.5 M	3 ul
	Tris-Hcl pH8.0	100 mM	1 M	150 ul
	dH2O	-	-	522 ul
	Add 1.42 ml of above mix to the 2mg PCD enzyme bottle.			

Enzyme storage buffer. Add 1.42 ml to the dry PCD enzyme, gently mix and aliquot into 120 ul aliquots. Store at -20C.

3,4-Dihydroxybenzoic acid (DBA) stock solution 50mM

	Component	Final concentration	Stock concentration	Quantity for 20 ml
	3,4-Dihydroxybenzoic acid	50 mM	-	154 mg
	dH2O	-	-	19 ml
	NaOH	50 mM	1 M	1 ml

DBA stock solution. Aliquot to 1ml and store at -20C.

6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX) stock solution 200 mM

	Component	Final concentration	Stock concentration	Quantity for 5 ml
	TROLOX			250 mg
	EtOH	-	Absolute	5 ml

TROLOX stock solution. Aliquot to 120ul aliquotes and store at -20C. Flush TROLOX bottle with Nitrogen gas before closing.

Imaging buffer.

	A	B	C	D
	Component	**Final concentration**	**Stock concentration**	**Quantity for 12 ml**
	SSC	2X	20X	1.2 ml
	Trolox	2 mM	200 mM	120 ul
	3,4-Dihydroxybenzoic acid (DBA)	5 mM	50 mM	1.2 ml
	dH2O	-	-	9.36 ml
	Degas the buffer in a vacuum chamber.			
	Protocatechuate 3,4-Dioxygenase (PCD)	20 nM	2 uM	120 ul
	Degas the buffer in a vacuum chamber.			
	Add 700 ul mineral oil to cover the imaging buffer			

Imaging buffer. Prepare fresh in 15ml glass vial with septum cap. Stick a thin needle through the septum to bleed air. Expected usage ~10ml.

Stripping buffer

	A	B	C	D
	Component	**Final concentration**	**Stock concentration**	**Quantity for 50 ml**
	SSC	2X	20X	5ml
	TCEP	100mM	0.5M	5ml
	dH2O	-	-	40ml
	Degass the buffer in a vacuum cahmber.			
	Add 3ml of mineral oil to cover the stripping buffer			

Stripping buffer. Prepare fresh in 60ml glass vial with septum cap. Stick a thin needle through the septum to bleed air. Expected usage ~ 45ml.

43 Once buffers are placed in the system, Start the ROBOFISH_user_program.py program by pasting the below command in an Anaconda command prompt:

```
python ROBOFISH_user_program.py
```

Enter "all" to update the experimental information. This will open Notepad++ wih the system info file.
Edit the information with all experimental metadata under Parameters.

```
# Automatic FISH system 2 info
# Make sure to always put a space between the colon and the value!
FISHSystem:
    Parameters:
        #   Operator running the experiment. Same as name linked to Pushbulet code, lower case
        Operator: operator5
        #   Machine the experiment is run on: ROBOFISH1, ROBOFISH2 or NOT-DEFINED
        Machine: ROBOFISH2
        ########## EXPERIMENT 1 ##########
        #   Experiment name: Initials with date, like: LBEXP20181220_1
        EXP_name_1: EXPLB20220111_example_experiment
        #   Short description of the Experiment
        Description_1: Example experiment
        #   Link to the protocol file on Protocols.io
        Protocols_io_1: https://www.protocols.io/edit/eel-t92er8e
        #   YYYYMMDD
        Start_date_1: 2021210
        #   Chamber1 or Chamber2
        Chamber_EXP_1: Chamber1
        #   First Hybridization time in hours (encoding probes)
        Hyb_time_1_A: 0.16
        #   Second Hybridization time in hours (amplifier probes)
        Hyb_time_1_B:
        #   Third Hybridization time in hours (detection probes)
        Hyb_time_1_C:
        #   Experiment_type: smfish-serial, smfish-barcoded, eel-barcoded
        Experiment_type_1: eel-barcoded
        #   Total number of rounds
        Target_cycles_1: 16
        #   True or False if a barcode is used
        Barcode_1: true
        #   Name of the detection chemisty, like: EELV2_core, EELV2_all, indirect_64
        Chemistry_1: EELV2_all
        #   Name of the codebooks
        Codebook_DAPI_1:
        Codebook_Atto425_1:
        Codebook_FITC_1:
        Codebook_Cy3_1: codebookHG2_20210508.parquet
        Codebook_TxRed_1:
        Codebook_Cy5_1: gene_hGBM20201124.parquet
        Codebook_Cy7_1:
        Codebook_Europium_1:
        #   True if there are multicolor barcodes
        Multicolor_barcode_1: false
        #   Name of the FASTA file with the probe sequences
        Probes_FASTA_DAPI_1:
        Probes_FASTA_Atto425_1:
        Probes_FASTA_FITC_1:
        Probes_FASTA_Cy3_1: HG2.fasta
        Probes_FASTA_TxRed_1:
        Probes_FASTA_Cy5_1: HG.fasta
        Probes_FASTA_Cy7_1:
        Probes_FASTA_Europium_1:
        #   Number of bits in the barcode
        Barcode_length_1: 16
        #   Species:
        Species_1: Homo sapiens
```

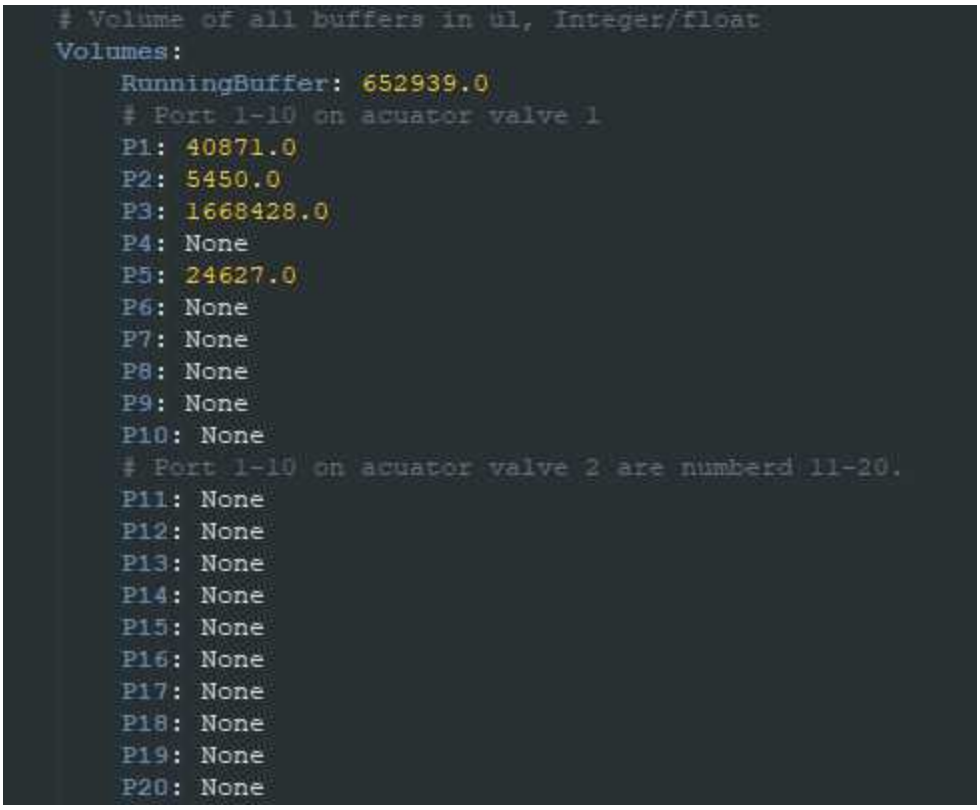
Screenshot example of the info file with experimental metadata.

Give the names of the buffers connected to the various ports.

```
Ports:
    # Buffer connected to the Syringe pump
    RunningBuffer: SSC2X
    # Port 1-10 on acuator valve 1
    P1: WB
    P2: IB
    P3: Waste
    P4: Chamber1
    P5: SB
    P6: HYB11
    P7: HYB12
    P8: Valve2
    P9: HYB13
    P10: HYB14
    # Port 1-10 on acuator valve 2 are numberd 11-20
    P11: HYB01
    P12: HYB02
    P13: HYB03
    P14: HYB04
    P15: HYB05
    P16: HYB06
    P17: HYB07
    P18: HYB08
    P19: HYB09
    P20: HYB10
```

Port numbers and buffer names

Then fill in the volumes of the buffers that were placed in the system.



Screenshot example of the info file with buffer volumes.

Fill in the codes of the hybridization mixes. The code is C<chamber number>_<round number>. So chamber one hybridization round 6 is "C1_06".

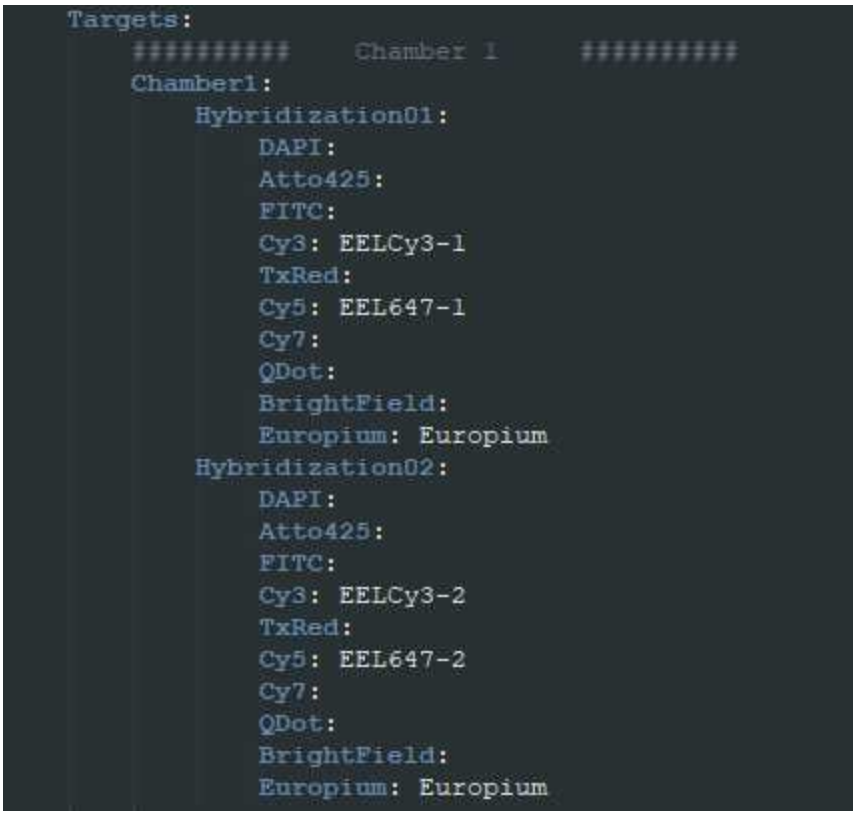
```
Hybmix:
    # Port 1-10 on acuator valve 1
    P1: #WB
    P2: #IB
    P3: #Waste
    P4: #Chamber1
    P5: #SB
    P6:
    P7:
    P8: #PORT TO VALVE 2
    P9:
    P10:
    # Port 1-10 on acuator valve 2 are numberd 11-20.
    P11: C1_01
    P12: C1_02
    P13:
    P14:
    P15:
    P16:
    P17:
    P18:
    P19:
    P20:
```

Screenshot example of the info file with hybridization numbers.

Optionally add the names of the genes or detection probe code to the Targets. The System will take this information and put it in a round specific info file that will be placed with the images, so that downstream the targets and images can be matched.

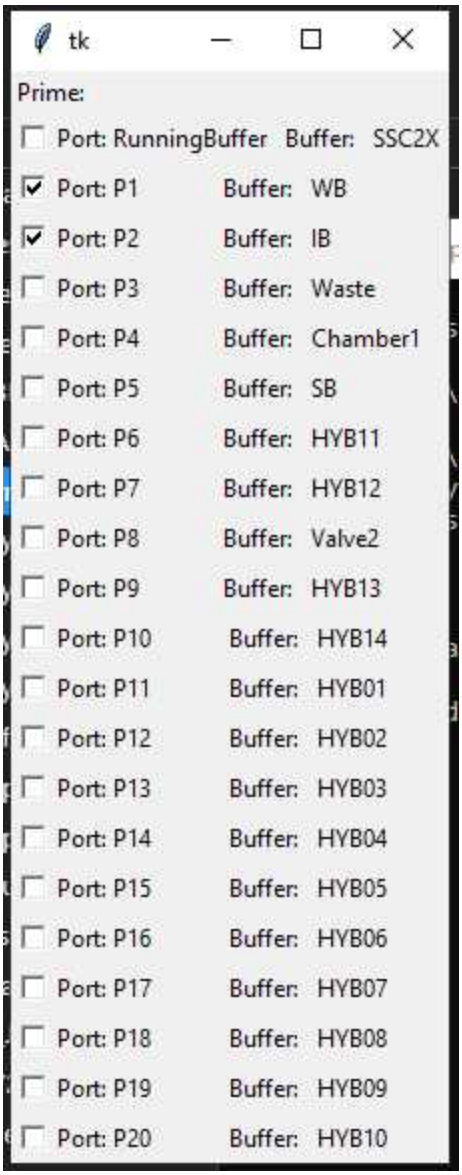
```
135     # Targets of each round
136     # Gene name, Background, polyA, Beads or Nuclei
137     Targets:
138       ##########   Chamber 1   ##########
139       Chamber1:
140         Hybridization01:
141           DAPI:
142           Atto425:
143           FITC: Cux2
144           Cy3: Rorb
145           TxRed: Foxp2
146           Cy5: Syt6
147           Cy7: Fam84b
148           QDot:
149           BrightField:
150           Europium: Europium
```

Screenshot example of the info file with the round targets. In this example Round 1 will contain 5 genes in different channels of the microscope. "Cux2" is labeled with a green probe and is imaged with the FITC channel.



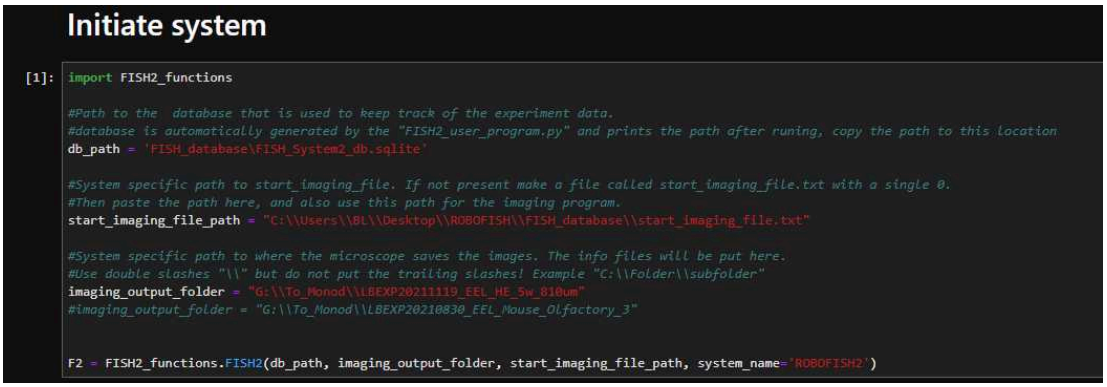
Screenshot example of the info file with the round targets. This is the codes we use for barcoded EEL experiments. The 1 or two indicates the bit location in the brcode.

After saving and closing the info file, and pressing enter in the ROBOFISH_user_program, a pop-up will open where you can tick the boxes of new buffers that need to be primed.



Screenshot example of the priming tickbox. In this case the Washbuffer (WB) and Imaging buffer (IB) will be primed. (Only prime newly connected buffers and never prime hybridization mixes!)

44 Open a new terminal and start the Jupyter lab. In the first cell make sure that all the paths to the different folders and files are correct. Then, edit the fluidics program to your needs and start the scheduler to start the detection cycles. The program will notify the microscope when to start imaging.



See the full documentation of the ROBOFISH programs on the Github page:
ROBOFISH code and instructions.

Labeling first round

45 **The system will perform the labeling. These are the steps it will perform.**

Wash 4 times 15 minutes with 30% formamide wash buffer at 47C.

46 Wash 4 times with SSC 2X.

47 Manually Inject 1ml of 4% PFA in 1X PBS into the chamber and fix for 10 minutes at 22C.

48 Manually wash with >5ml of SSC 2X at 22C.

49 Hybridize 10 minutes with the fluorescent probes at 37C.

50 Wash 3 times 3 minutes with 20% formamide washing buffer at 37C.

51 Wash 4 times with SSC 2X at 37C.

52 Set temperature to 22C and inject imaging buffer.

Imaging

53 The fluidics system should now have performed the first labeling and injected the imaging buffer into the flow cell.

Correct Point_set_1 using the reference crosses.

- Find one of the reference crosses and go to exactly the same part of the cross that was imaged before. Use the previously made picture to go to the same spot.
- Now correct the Point_set_1 positions and save the points.

When using the provided microscope Job for Nikon microscopes running NIS Elements software, follow the steps in the Job. Select the Readout option and load the corrected Point_set_1 for the imaging. Make sure the path to the Start Imaging file is correct. This can be found in the Job, in the N LOOP under the "MacroReadValueInFile" and in the "Reset_start_file". Both need to be correct.

Set up the perfect focus to focus on the surface.

Then start the imaging.

When using other software:
Make a program that can read the Start_imaging_file located in the "ROBOFISH/FISH_databse" folder and start imaging the corrected Point_set_1 when the value in the file is changed to 1 by the ROBOFISH program. When it is done imaging it should reset it to 0, so that the ROBOFISH program knows it can start the next round of labeling.

Labeling repeat round

54 **This section can be repeated for as many rounds as needed. Followed by imaging.**

Wash 4 times with SSC 2X at 22C.

55 Strip the fluorophores 2 times 10 minutes with the stripping buffer at 22C

56 Wash 6 times with SSC 2X.

57 Hybridize 10 minutes with the fluorescent detection probes at 37C.

58 Wash 3 times 3 minutes with 20% formamide washing buffer at 37C.

59 Wash 4 times with SSC 2X at 37C.

60 Set temperature to 22C and inject imaging buffer.

61 Image the signal.
Then: go to step #54 for the desired number of repeats.