Step-by-step SCRINSHOT protocol

Contents

1. Probe preparation

The genes of interest are selected according to the experimental aims (including optionally a positive control or a house-keeping gene). The SCRINSHOT detection of mRNA is based on a number of steps, for which two types of probes are required: (1) padlock probes to bind specific mRNA sequences and further get amplified *in situ* and (2) the fluorophore-labeled detection probes to visualize the amplified specific sequences.

1.1. Padlock probe design

Initially, the mRNA sequence of the gene of interest is identified using: http://www.ncbi.nlm.nih.gov/gene The padlock probes contain a constant backbone sequence of 53 nucleotides (nt) and the 5'- and 3'- arms, which are complementary to the corresponding

mRNA sequence (Figure 1). The gene-specific arms of padlock probes are around 20nt long each (melting Temperature [Tm] 50-60ºC), thus the total length of the gene-specific sequence of each padlock is around 40 nucleotides (nt), which is similar to the length of the Taqman qPCR probes. It is possible to use a Taqman probe design tools such as the one from Integrated DNA Technologies, Inc.

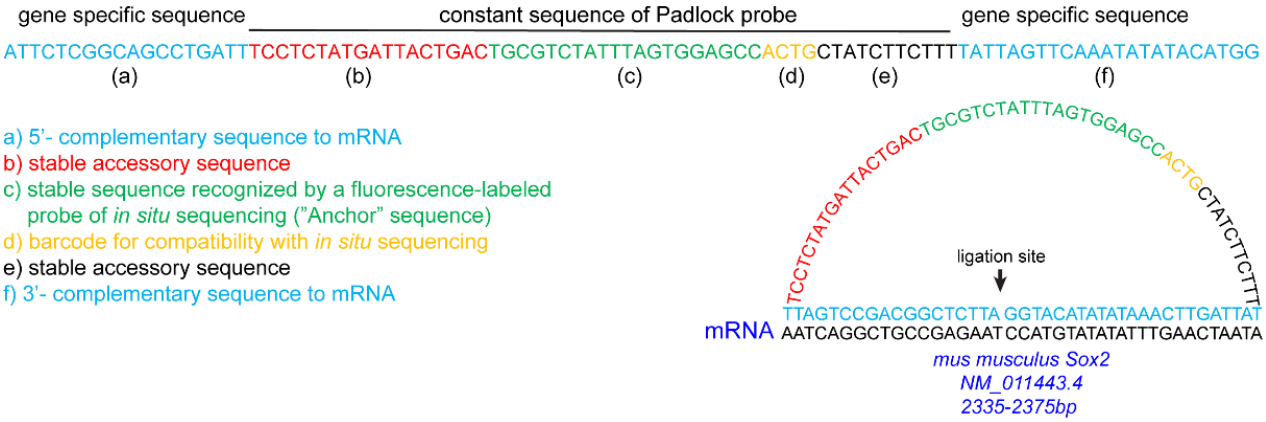


Figure 1. Description of the padlock probe structure. Padlock probes contain two variable gene-specific arms (a, f) and a stable sequence which is subdivided in four parts. Circularized padlock probe is hybridized to the complementary sequence of the corresponding mRNA (mouse Sox2). Padlock probes are designed to be compatible with *in situ* sequencing method [1].

1.1.1. Selecting specific sequence, used for the padlock probe gene-specific arms in Taqman probe design

(i) Use the https://eu.idtdna.com/PrimerQuest/Home/Index?Display=AdvancedParams
(ii) Choose "Download sequence(s)" using Genbank or Accession ID to import the sequence of the gene-transcript of interest. Add the name of the gene.
(iii) Choose the qPCR (2 Primers + Probe). For padlock design, use the sequence identified ***as probe***.
(iv) Results to return: 20
(v) Primer Criteria: Primer Tm (°C) 50-64 (the temperature should be lower than the one of the probes. In general, the parameters should not be too strict to avoid exclusion of suitable Taqman probes). Optimum: 57°C
(vi) Probe Criteria: Probe Tm (°C) 65-75 (Optimum: 70°C), Probe CG% 40-60% with optimum 50% and probe size: 40-45nt (Optimum: 45 nt)
(vii) Amplicon Criteria: Amplicon Size: 76-500bp (Optimum: 200 bp)
(viii) Press "Download Assays" and an *.xls file will be downloaded.
(ix) Open the file and choose the **probe** sequences (not the primer) for the next steps.
(x) Validate the sequences. This step is important to ensure that the probes are specific for the gene of interest and they **ONLY** bind the desired transcript or transcript variants.

Go to Standard Nucleotide BLAST (Blastn) tool of NCBI (National Center for Biotechnology Information, U.S. National Library of Medicine) and at the "Choose Search Set" field choose the "Mouse genomic + transcript" for **mouse** genes and "Human genomic + transcript" for **human**

genes. Also select the “Somewhat similar sequences (blastn)” in the “Program selection” field: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

Paste the probe sequence into the field “Enter Query Sequence” and run the Blastn. To be sure that the probe is specific, the results with the 100% Query Cover, should contain **only** the transcripts of interest. If the results are **plus/minus**, the Taqman probe sequence does not correspond to the mRNA but to its reverse-complement sequence and it should be transformed accordingly, as described in the next step. Ensure that no other RNA sequence, longer than 20 nt, is recognized (in plus/plus direction) (Figure 2).

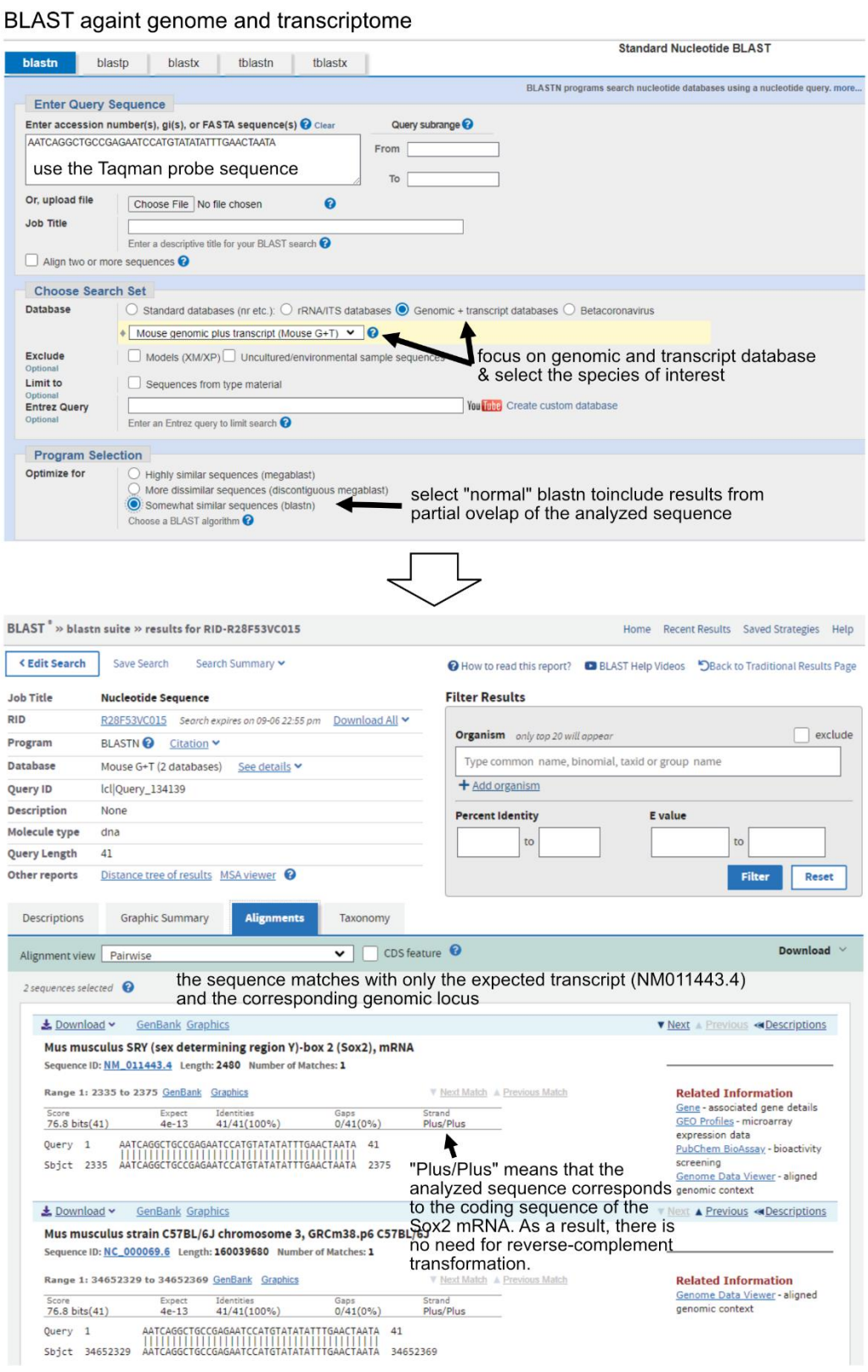


Figure 2. Blastn of the candidate probe sequence against genome and transcriptome of the corresponding organism.

As an additional control, the mRNA sequence, which has been used to create the probes, is aligned with the Taqman probe. In case of plus/minus result, the “Query” sequence is used for padlock probe design (NOT the “sbjct”). Use the function “align two or more sequences” of the Blastn for this step:
http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome It is important to use the accession number of the analyzed mRNA as “Query Sequence” and the Taqman probe sequence, as “Subject sequence”. Run the blast and then save the obtained “Query” sequence and the corresponding numbers of the first and the last nucleotides in order to know the domain of the transcript, which is recognized by the probe.

Notes: 1) Any other freeware or commercial program for probe-primer design can be used (e.g. Beacon design).

2) The following webpage can be used to obtain the reverse and/or complementary sequences: http://www.bioinformatics.org/sms/rev_comp.html*)*

1.1.2. Design of the Padlock Probes and Detection oligos

(i) Open the “padlock_probe_design_v6_empty_stable_backbone.R” file in R-Studio and follow the instructions to install and load the necessary packages. Also, set the working directory. In the initial script, it is "C:/Users/alex/Desktop/" (Figure 4). (https://github.com/AlexSount/SCRINSHOT_scripts_for_EMBO_course)

(ii) Complete the information of target gene name (line-39), NCBI mRNA accession number (line-41) and species (line-43).

(iii) Add the Taqman probe sequences in the lines 48-51, as they have been obtained after blast (be careful to use the coding sequence and not its reverse-complement).

(iv) Select the number of the probe (based on the given setup, it has to be between 1 and 4) (line-54).

(v) Select the anchor primer sequence, using 1 (for low and medium abundant genes) or 2 (for highly abundant) (line-57).

(vi) Select the fluorophore of the detection oligo, between [FAM], [Cy3], [TxRed], [Cy5], [AF750] (line-60).

(vii) Run all the steps up to the line-111.

(viii) Set the number in line-112, according to the results of the “arm_list” output of the previous steps in order to use melting temperature (Tm) close to 60°C. Next, set the number of line-115, in order the 2 arms of the sequence to have equal Tm (not more than two degrees apart), close to 45°C. In the given example, line-7 of the “arm_list” output is the first with 60°C Tm and the column-4 the one with similar arm temperatures, close to 45°C.

(ix) Run all the steps of the lines 112-177 and set the number of line-178, based on the results of “barcode_list” output. This should be the number of the line with Tm close to 36°C. In the given example, that number is 22, that corresponds to 35.189°C.

(x) Run the remaining lines of the script (178-273). If all parameters have been set properly, a report will be created in the working directory.

BLAST of the used trascript sequence for Taqman probe design and the obtained probe

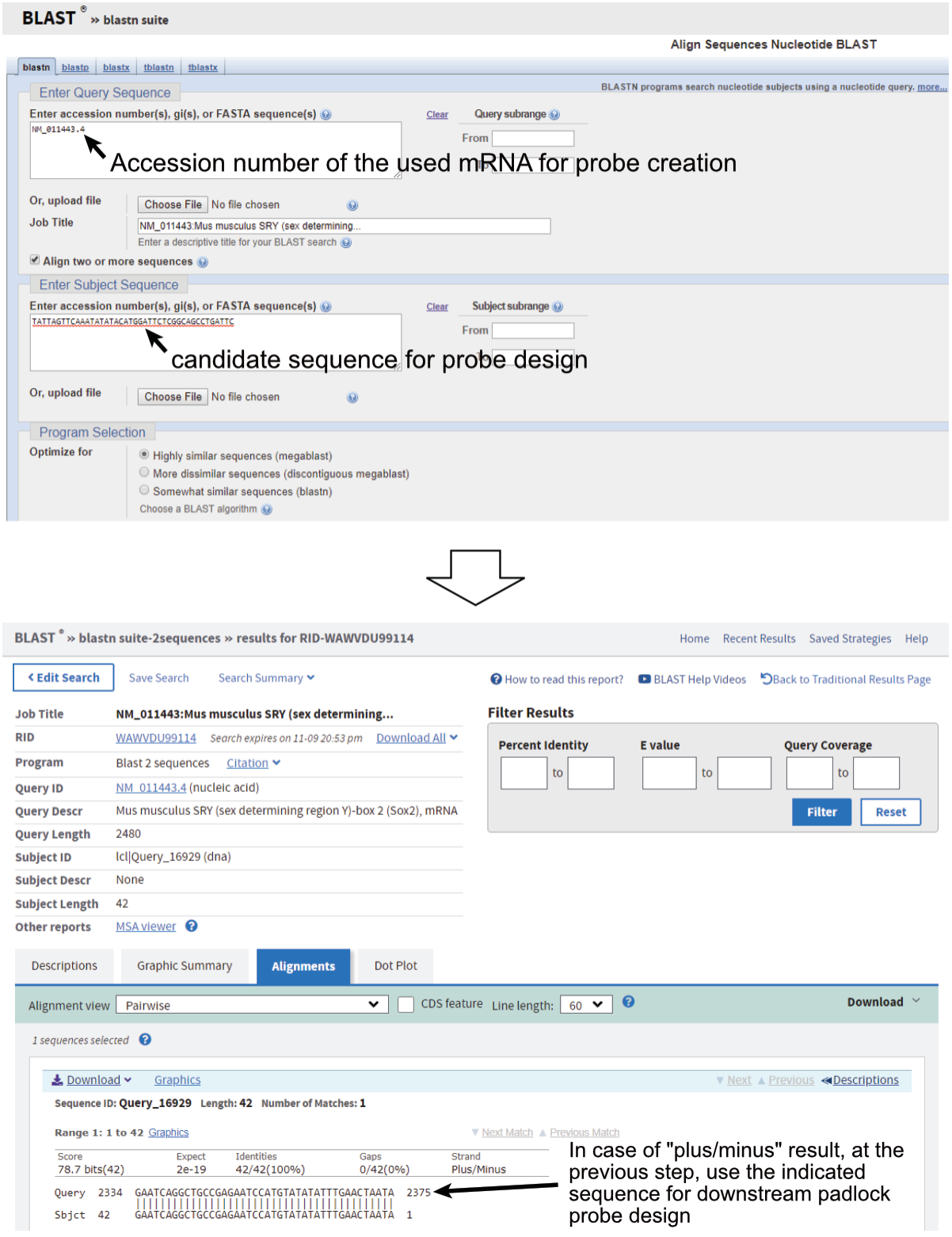


Figure 3. Blastn of candidate sequence against the used transcript for probe design (control step).

(xi) For sequential detection of padlock-probe RCA-products, it is necessary to remove the already hybridized and imaged detection oligos. For that reason, we should destabilize them. Exchange of 2-3 Thymidine nucleotides (T) along the detection probe sequence with uracil nucleotides (U) facilitates the recognition of “U” by the uracil DNA glycosylase (UNG) enzyme, which cuts the probe at these positions, producing DNA fragments shorter than 10 nucleotides. Aim to position one of the uracils as close as possible to the fluorophore. The degraded detection oligos are removed with stringent formamide washes. In the given example, the GCGAGTCCTCTTTGCTG [Cy3] should be modified to **GCGAGUCCTCUTTGCUG [Cy3]**

1.1.3. Ordering of padlock probes

The padlock probes for SCRINSHOT are ordered as 5’-phosphorylated (to facilitate ligation), 4 nmole Ultramer® DNA Oligos, from Integrated DNA Technologies, Inc. Both tube and plate options work fine, but plates are more economical option. Probes are shipped lyophilized and diluted in 100 µl RNA Free H_2O, for 40 µM final concentration and stored at -20^{o}C.

1.1.4. Ordering of detection oligos

We order the detection oligos from Eurofins Genomics or IDT as custom DNA oligos. The synthesis scale is 0.01 µmol and the oligos are HPLC purified. FITC-, Cy3- and Cy5-fluorophores are conjugated to the 3’-arm of the oligos. Oligos are ordered in tubes. Oligos are shipped lyophilized and diluted in RNA Free H_2O, for 100 µM stock concentration and they are stored in -20^{o}C.

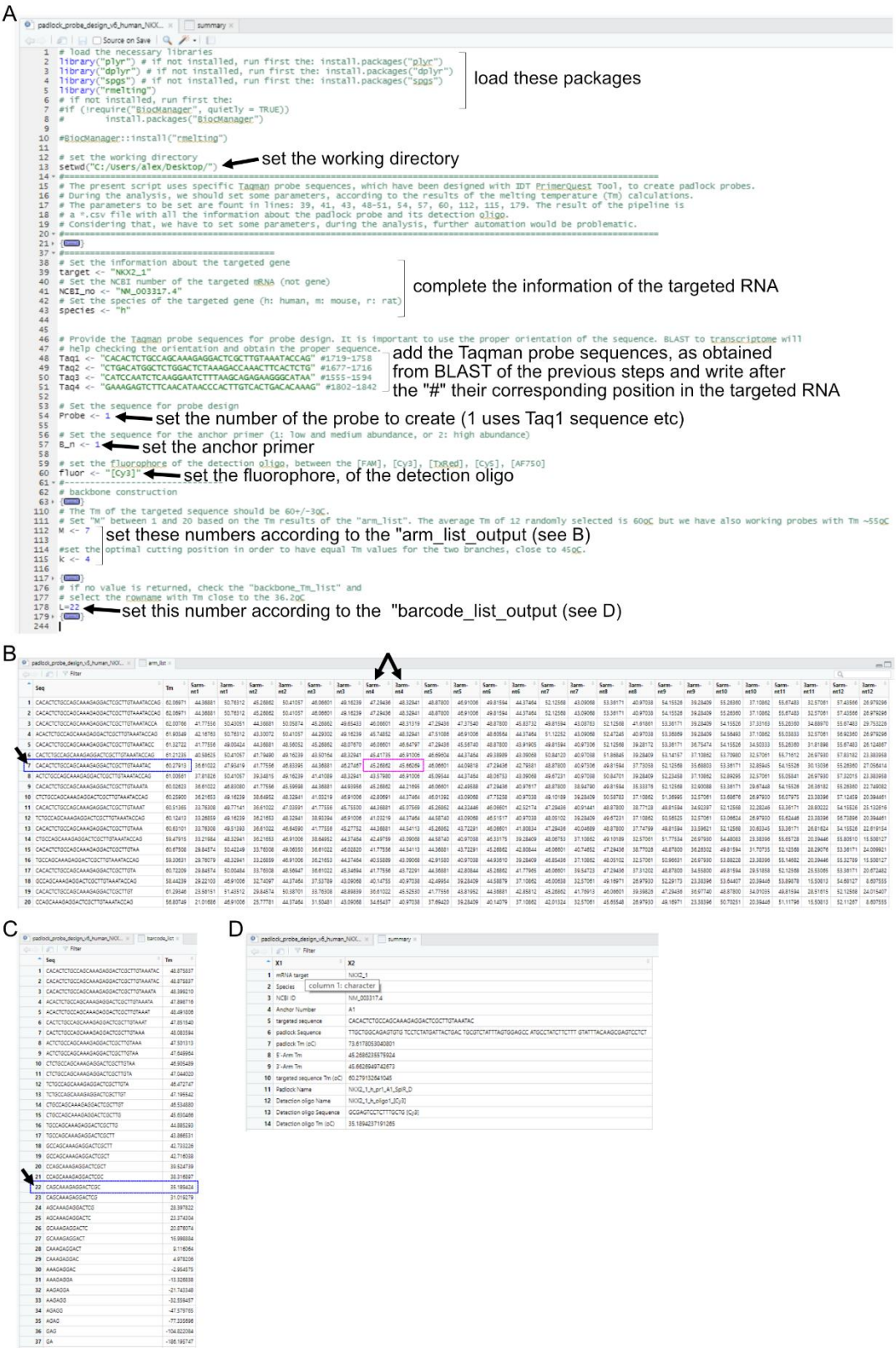


Figure 4. Steps for design of padlock probes and their corresponding detection oligos.

2. Tissue preparation

Tissues are (1) harvested and fixed, (2) frozen in OCT, and (3) sectioned and placed onto pre-coated glass slides. The following example describes the preparation and treatment of mouse lung. The same tissue treatment is used for both immunohistochemical assays and SCRINSHOT. All solutions, except the fixative, should be prepared using RNAse free reagents.

Reagents

PFA4% in PBS pH7.4

(i) Warm up 45ml mQ H_2O in a 50 ml FALCON tube, in a microwave oven for a few seconds in order to reach 60-70^oC (lid should be loose to avoid explosion).
(ii) Add 60µl NaOH 1M in hot H_2O and mix by inverting.
(iii) Add 2 g of PFA powder into the solution and mix well. PFA should completely dissolve. If the particles are still visible, warm the tube in the microwave oven for a few more seconds. **Avoid boiling**.
(iv) Add 5 ml 10X PBS (for example, Ambion, AM9625) into the solution and mix by gentle inversion.
(v) Place the solution on ice until it reaches room temperature (approximately 30 min).
(vi) Measure the pH using pH-strips and adjust, if needed, to 7.4-7.5.

PBS 1X pH7.4

Add 5 ml PBS 10X (Ambion, AM9625 or analogues) into 45 ml mQ H_2O (or other RNase free H_2O) and mix well.

Sucrose solution

Sucrose powder should be molecular biology grade. Use only disposable spoons to retain RNAse free condition. Prepare 30% sucrose in PBS 1X pH 7.4.

OCT

Cryomatrix Leica FSC22. Disposable syringes should be used for accurate volume measurements.

PFA-OCT mixture (2:1 v/v)

PFA 4% in PBS 1X pH 7.4 – 2 ml

OCT – 1 ml

Sucrose-OCT mixture (2:1 v/v)

30% sucrose in PBS 1X pH 7.4 – 20 ml

OCT (Cryomatrix Leica FSC22) – 10 ml

Isopentane

Sigma-Aldrich 277258-1L

Base Molds

Leica Surgipath Clear Base molds 3803025 or analogues. Different sizes can be used to accommodate tissue size.

2.1. Example of tissue collection and fixation: newborn and adult mouse lung

(i) Euthanize the animal, open the thoracic cavity and perfuse the lung through the heart to remove red blood cells from the organ (left atrium is cut and 1-5 ml of ice-cold PBS 1X pH7.4 is injected through the right ventricle).

(ii) Expose the trachea and pass a piece of surgical silk between the trachea and the esophagus in order to create a loose knot. Make a small hole in the trachea using either scissors or a needle and inject a PFA-OCT mixture into the lung using an insulin syringe with 20-24 G plastic catheter (e.g., B Braun 4251130-01) until the tip of the accessory lobe gets inflated. Tighten the knot around the trachea under the position of catheter insertion.

(iii) Carefully remove the lung with trachea from thoracic cavity. Do not cut or press the tissue to avoid collapse of the lung and compromised histology. Immerse the tissue in PFA4%, pH7.4 for 4-8 hours (e.g., newborn mouse: 2 hours, adult: 4 hours) at 4^{o}C with gentle rotation or shaking.

Extra steps to improve histology (applied to all the tissues)

Transfer the fixed organs into a new tube with a sucrose-OCT mixture and incubate at 4^{o}C for 12-16 hours with gentle rotation or shaking.

2.2. Tissue Freezing

(i) Place a small volume of isopentane into a beaker (500 ml) and add small pieces of dry ice into isopentane.

(ii) Place the beaker into a container with dry ice at the bottom. The liquid needs 5 min to equilibrate (temperature). Then it is ready to use.

(iii) Place the tissue into the plastic mold filled with OCT and place it into the beaker. Leave it until all the OCT becomes white.

(iv) Store the tissue blocks at -80^{o}C until sectioning.

2.3. Sectioning

Cut 10 µm-thick tissue sections using a cryostat (Leica CM3050S or analogue) and collect them onto poly-lysine coated slides (VWR Cat No. 631-0107), leaving 1.5-2 cm gap between samples (it is optimal to place a single sample per slide in order to provide enough space for sealing the chamber for *in situ* hybridization). Leave slides to dry in a container with silica gel and then store at -80^{o}C until usage.

3. SCRINSHOT hybridization protocol

The following steps describe the procedure of *in situ* hybridization of padlock probes for all selected genes on the tissue samples, followed by amplification of their sequences and hybridization of fluorophore-labeled detection oligos. The latest step is divided into cycles based on microscope filter setup. After the first set of genes is imaged, the probes are removed and the next set of detection probes is hybridized onto the tissue.

Solutions

(i) DEPC H_2O. 1 ml DEPC (Sigma-Aldrich, D5758-50ML) is added to 1 L milliQ H_2O and mixed for 12-16 hours using a magnetic stirrer at room temperature (RT). Solution is then autoclaved in order to deactivate DEPC.
(ii) 10X PBS (Ambion, AM9625 or analogue).
(iii) PBS-Tween 0.05% (500 ml): 450 ml DEPC H_2O + 50 ml PBS 10X + 250 µl Tween 20.
(iv) PFA 4%. See "Tissue Preparation" section.
(v) 0.1M HCl (30 ml). 30 ml DEPC H_2O and 250 µl 12N HCl.
(vi) 70% ETOH (40 ml): 28 ml ETOH and 12 ml DEPC H_2O.
(vii) 85% ETOH (40 ml): 34 ml ETOH and 6 ml DEPC H_2O.
(viii) RNAse Free H_2O (Sigma-Aldrich, W4502-1L).
(ix) 65% Formamide (1 ml): 650 µl deionized formamide (F9037-100ML) and 350 µl RNAse free H_2O. Avoid freeze-thawing formamide more than twice.
(x) 2X SSC (10 ml): 1 ml SSC Buffer 20× Concentrate (Sigma-Aldrich, S6639-1L) and 9 ml RNAse-free H_2O.
(xi) Washing buffer (1 ml): 900 µl 2X SSC and 100 µl deionized formamide.
(xii) Probes: ordered probes are usually lyophilized. Add RNAse-free H_2O and leave to dissolve for at least 4 hours at room temperature or overnight at 4°C. The concentration of padlock probe stock solutions is 10 µM and the concentration of detection oligos is 100 µM. All of them are stored at -20°C.

Notes: Ethanol solutions should be freshly prepared, at least for the "Permeabilization and dehydration" step.

Deionized formamide is aliquoted and stored at -80°C. After thawing, it can be kept at -20°C and refrozen one more time

All frozen reagents are thawed and mixed thoroughly before use. Master mix solutions are prepared in advance and kept at RT, but the enzymes are added last to the mixtures, just before application to the tissue.

Before chamber mounting, all the incubations are done in 50 ml RNAse-free tubes. For all master mix solutions, prepare 10% more (to ensure the sufficient volume for the reaction).

To avoid drying of the tissue, remove the last PBS-Tween 0.05% just before the addition of the next step reaction mix.

3.1. Post-fixation of the slides

(i) Remove the slides from -80^{o}C and place them in a small slide box (Sigma-Aldrich Z708313-25EA) for transfer, then immediately place at 45^{o}C for 15 min to prevent moisture accumulation (the lid of the slide box should be open when slides are at 45^{o}C).
(ii) Incubate the slides in 4%PFA (freshly prepared) for 5 min. Use clean forceps to transfer the slides between solutions to avoid contamination.
(iii) Wash the slides for 2 x 5 min in PBS-Tween 0.05%.

3.2. Permeabilization of the tissue

(i) Incubate the slides in 0.1M HCl at RT for 3 min. Two slides can be placed back-to-back in a 50 ml FALCON tube. If tissue detaches, reduce the HCl incubation time or its concentration.
(ii) Wash the slides for 2 x 2 min with PBS-Tween 0.05%.

3.3. Mounting of hybridization chambers

In order to perform reaction in sterile conditions and facilitate solution application, the hybridization chambers (Gracebio, SA20-0.5-SecureSeal) should be mounted on top of the sample. To ensure the uniform chamber attachment the slide should be dehydrated with series of ethanol. Following the chamber mounting, the sample is rehydrated.

(i) Incubate the slides in 70% ETOH for 2 min
(ii) Incubate the slides in 85% ETOH for 2 min
(iii) Incubate the slides in 99.5% ETOH for 2 min
(iv) Place the slides horizontally and leave to dry. Put a protective cover over them to reduce contamination
(v) Peel off the thin adhesive liners of the hybridization chambers. If you want to reuse the chambers, label the tapes at the outer side with a marker, so it would be clear which way to stick them back, and keep them in a box for reuse. Mount the hybridization chambers onto the slides in such way that the holes are along the longer side of the slide. This prevents air trapping when the slides are later immersed in 50ml FALCON tubes with solution. Slightly press the chamber onto the slide to get rid of the air bubbles.
(vi) wash the slides for 3 x 2 min PBS-Tween 0.05% for rehydration

Handling notice

Hold the slide at a 45^{o} angle (approximately), and add and remove the solutions with a pipette through the lower hole of the chamber to ensure the coverage of tissue and to avoid bubbles.

3.4. Blocking

The blocking solution contains tRNA and an Oligo-dT sequence for blocking unspecific binding sites of probe binding.

Blocking master mix				
Reagents	**stock**	**final**	**1 x slide**	**x slides**
RNase-free H_2O			57.5 µl	
Ampligase Buffer	10x	1x	10 µl	
KCl	1 M	0.05 M	5 µl	
Formamide deionized	100%	20%	20 µl	
Oligo-dT	10 µM	0.1 µM	1 µl	
BSA	10 µg/ul	0.2 µg/µl	2 µl	
RiboLock (Thermo)	40 U/µl	1 U/µl	2.5 µl	
tRNA (Ambion AM7119)	10 µg/µl	0.2 µg/µl	2 µl	
Total			**100 µl**	

Oligo-dT:AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN

(i) Add the blocking solution into the chamber, mix by gentle pipetting (3-5 times) and incubate the slides at room temperature (RT) for 30 min.
(ii) Wash the slides for 2 x 1 min with PBS-Tween 0.05%.

3.5. Hybridization of Padlock probes

Prepare a list of all genes to be detected on the particular tissue sample. The following master mix contains 0.05 µM of each padlock probe for each of the genes. If a gene is expressed at high levels (like Scgb1a1 in the lung airways), a lower number and concentration of padlock probes should be used to avoid molecular and optical saturation of the signal (1 padlock and 0.01 µM). Transcript counts from single cell mRNA sequencing experiments provide a useful guideline for this step. It is important to keep in mind that signal reduction might give false negative cells, especially if they express low levels of the detected gene.

Reagents	**stock**	**final**	**1 x slide**	**x slides**
RNase Free H2O				
Ampligase Buffer	10x	1x	10 µl	
KCl	1M	0.05M	5 µl	
Formamide deionized	100%	20%	20 µl	
Probes 0.1 µl/padlock	40 µM	0.04 µM	0.1 µl x	
BSA	10 µg/µl	0.2 µg/µl	2 µl	
RiboLock (Thermo)	40U/µl	1U/µl	2.5 µl	
tRNA (Ambion AM7119)	10 µg/µl	0.2 µg/µl	2 µl	
Total			**100 µl**	

(i) Add the solution into the chamber, mix by gentle pipetting (3-5 times) and seal the two holes with PCR adhesive membrane strips (cut to the suitable size) to prevent evaporation.
(ii) Incubate the slides at **55ºC for 15 min** for denaturation and at 45ºC for 120 min for hybridization of the probes onto the target mRNA. For this step we use a PCR machine or an Eppendorf Thermomixer.
(iii) Wash the slides for 3 x 10 min with washing buffer (10% formamide in 2X SSC) to remove unhybridized probes.
(iv) Wash the slides for 3 x 1 min with PBS-Tween 0.05% to remove the remaining formamide, as it can deactivate the enzyme in the following step.

3.6. Ligation of Padlock probes

The ligation of the hybridized padlock probes is mediated by SplintR ligase (PBCV-1 DNA Ligase) which can function with DNA:RNA hybrid molecules.

Reagents	stock	final	1 x slide	x slides
RNase Free H_2O			82.5 µl	
T4 RNA Ligase Buffer	10x	1x	10 µl	
ATP	1 mM	10 µM	1 µl	
BSA	10 µg/µl	0.2 µg/µl	2 µl	
SplintR (NEB)	25 U/µl	0.5 U/µl	2 µl	
RiboLock (Thermo)	40 U/µl	1 U/µl	2.5 µl	
Total			**100 µl**	

(i) Add the solution into the chamber, mix by gentle pipetting (3-5 times) and seal the two holes with PCR adhesive membrane strips to prevent evaporation.
(ii) Incubate the slides at **25ºC** for 12-16 hours using a PCR machine or an Eppendorf Thermomixer (or any incubator).
(iii) Wash the slides for 2 x 1 min with PBS-Tween 0.05%.

3.7. Rolling circle amplification (RCA)

Φ29 polymerase is used to perform the rolling circle amplification (RCA). To avoid interference in the detection of low-abundance genes by the highly abundant ones, we used two distinct padlock probe backbones differing in their anchor sequence, one for low and one for high abundant genes. As a result, two RCA primers, which recognize the corresponding backbones, are used as initiators of RCA. If only one backbone is used, the other one should be omitted from the protocol. The thiophosphate modifications of RCA primers (indicated with "*") prevents the 3'-5' exonuclease activity of Φ29 polymerase, increasing the RCA efficiency [2].

Reagents	stock	final	1 x slide	x slides
RNase Free H_2O			68.5 µl	
Φ29 buffer (Lucigen)	10x	1x	10 µl	
Glycerol	50%	5%	10 µl	
dNTPs	10 mM	0.25 mM	2.5 µl	
BSA	10 µg/µl	0.2 µg/µl	2 µl	
RCA Primer1 (TAAATAGACGCAGTCAGT*A*A)	10 µM	0.1 µM	1 µl	
RCA Primer2 (CGCAAGATATACG*T*C)	10 µM	0.1 µm	1 µl	
Φ29 polymerase (Lucigen)	10 U/µl	0.5 U/µl	5 µl	
Total			**100 µl**	

(i) Add the solution into the chamber, mix by gentle pipetting (3-5 times) and seal the two holes with PCR adhesive membrane strips to prevent evaporation.
(ii) Incubate the slides at 30°C for 12-16 hours using a PCR machine or an Eppendorf Thermomixer.
(iii) Wash the slides for 2 x 1 min with PBS-Tween 0.05%.
(iv) Add 4% PFA, mix by gentle pipetting (3-5 times) and incubate for 15 min at RT to fix the RCA products on the tissue.
(v) Wash the slides for 3 x 1 min with PBS-Tween 0.05%.
(vi) Wash the slides for 3 x 10 min with 65% formamide at 30°C on a PCR machine block or an Eppendorf Thermomixer.
(vii) Wash the slides for 2 x 1 min with PBS-Tween 0.05%.

3.8. Hybridization of the first set of detection oligos

The following master mix contains the fluorophore-labeled oligos, which recognize the gene specific domain of the padlock probes. To prevent fluorophore bleaching avoid extensive light exposure after this step.

Reagents	stock	final	1 x slide	x slides
RNase Free H2O				
SSC	20X	2X	10 µl	
Formamide deionized	100%	30%	30 µl	
FITC-labeled probes	10 µM	0.02 µM	0.2 µl each	
Cy3-labeled probes	10 µM	0.01 µM	0.1 µl each	
Cy5-labeled probes	10 µM	0.01 µM	0.1 µl each	
TxRed-labeled probes	10 µM	0.01 µM	0.1 µl each	
Cy7-labeled probes	10 µM	0.01 µM	0.1 µl each	
DAPI	50 µg/ml	0.5 µg/ml	1 µl	
BSA	10 µg/µl	0.5 µg/µl	5 µl	
Total			**100 µl**	

(i) Add the solution into the chamber, mix by gentle pipetting (3-5 times) and incubate the slides in the dark for 60 min at 30°C.

(ii) Wash the slides for 3 x 5 min with washing buffer (see solutions). For more stringent washes formamide concentration in the washing buffer can be increased to 20%, and washes can be done at 30 degrees.
(iii) Wash the slides for 3 x 1 min with 2X SSC.

3.9. Dehydration and mounting

(i) Incubate the slides in 70% ETOH for 2 min.
(ii) Carefully remove the chambers and incubate the slides in 70% ETOH for 1 min.
(iii) Incubate the slides in 85% ETOH for 2 min.
(iv) Incubate the slides in 99.5% ETOH for 2 min.
(v) Place the slides horizontally and leave to dry in the **dark**.
(vi) Apply the SlowFade™ Gold Antifade mounting medium and put the cover-slip on the slides
(vii) Store the slides in the dark at 4^{o}C or RT, until imaging.
(viii) Image the slides following instructions in part 4.

Note: When the chambers dry, their adhesive liners can be placed back and the chamber can be stored for future reuse.

3.10. Removal of the detection oligos after imaging, before next hybridization

Immerse the slides in 50 ml FALCON tubes (one slide per tube) with ETOH 70% and place them horizontally in a 45^{o}C oven, with the coverslip facing the bottom. Incubate until the coverslip is detached.

(i) Incubate the slides in 85% ETOH for 2 min.
(ii) Incubate the slides in 100% ETOH for 2 min.
(iii) Place the slides horizontally on the bench and leave to dry.
(iv) Mount the chambers (the chambers can be reused, if their adhesive film is intact).
(v) Wash the slides for 3 x 2 min with PBS-Tween 0.05 %.

UNG treatment

The detection probes contain 2-3 U-nucleotides, which can be recognized and removed by Uracil DNA Glycosylase (UNG) enzyme. As a result, the detection probes are destabilized and washed away with the formamide.

Reagents	stock	final	1 x slide	x slides
RNase Free H2O			86 µl	
UNG buffer	10X	1X	10 µl	
BSA	10 µg/µl	0.2 µg/µl	2 µl	
UNG (Fermentas)	1U/µl	0.02U/µl	2 µl	
Total			**100 µl**	

(i) Add the solution into the chamber, mix by gentle pipetting (3-5 times) and seal the two holes with PCR adhesive membrane strips to prevent evaporation.
(ii) Incubate the slides at **37ºC** for 60 min using a PCR machine or an Eppendorf Thermomixer. *For removal of the detection probes of highly abundant genes, it is beneficial to do the UNG treatment step twice.*
(iii) Wash the slides for 3 x 10 min with 65% formamide at 30ºC using a PCR machine or an Eppendorf Thermomixer.
(iv) Wash the slides for 2 x 1 min with PBS-Tween 0.05%.
(v) Add the master mix of the second set of probes and repeat the steps of the hybridization of detection oligos (go to step 3.8)

4. Imaging

In order to detect the probe signal in the tissue, the imaging can be done with a widefield fluorescent microscope at 40x magnification (20x magnification can also be used) acquiring a full Z stack of a sample (depending on sample thickness) with ≤0.8 µm step. An exemplar setup is described below.

Zeiss AxioImager Z2 microscope, equipped with a Zeiss AxioCam 506 Mono digital camera and an automated stage. Zeiss LED Colibri2 and external HXP120 light sources are used with the following Chroma filters: DAPI (49000), FITC (49002), Cy3 (49304), Cy5 (49307), Texas Red (49310) and Atto740 (49007). To have an overview of the whole tissue section, a 10x lens, (Zeiss Plan Apochromat" 10x / 0.45 M27, 420640-9900-000) is used for initial imaging. Higher or lower magnification can be used for this step, depending on tissue size. Then, specific areas of interest are selected and imaged with 40x lens (Zeiss EC "Plan-Neofluar" 40x/1.30 Oil Ph3, 440451-9903-000), using tiling function and Z-stack acquisition. The datasets are saved as *.czi files, using ZEN 2.5 Blue edition software. The coordinates and the acquisition settings (e.g. LED intensity and camera exposure time) of the acquired areas can be recalled from saved *.czi files, allowing the acquisition of the same positions in all detection cycles (there may be a slight shift of sample/stage between cycles, which depends on the stage accuracy and should be less than 500 pixels in both X and Y axes). For the consistency in Z-axis it is useful to use the auto focus function of Zen software and re-adjust for every acquisition.

5. Image preparation for visualization and analysis

Acquisition of the same areas of sample using Zen produces either a set of images in a form of a Z-stack (in case of small sample), or a large number of images (tiles) in a Z-stack (in case of large sample) that should be combined and saved in a suitable format in order to detect the

signal co-localization in the same cells. After acquisition the steps of preparation for image analysis are the following:

1. Creation of maximal-orthogonal projections of the Z-stacks of *.czi files
2. Stitching of the tiled images in case of large samples
3. Alignment of images from all detection cycles using DAPI channels
4. Combination of all aligned images into one multichannel image to visualize the signal colocalization (optional)
5. Export of single-channel aligned images for further signal detection

5.1. Orthogonal projection and stitching

Both steps (projection and stitching) for all detection cycles of the same area are described on the example of a large tissue sample using Zen blue 2.5 free version. It is necessary to activate “Panorama module” in Modules Manager to perform the stitching step. Select the file that you plan to work with in the ‘Input’ panel before applying the settings described in Figure 5. All the stitched projected datasets are saved as *.czi files, which will be used for the following steps of the analysis (the original files can be stored as a backup) (Figure 5).

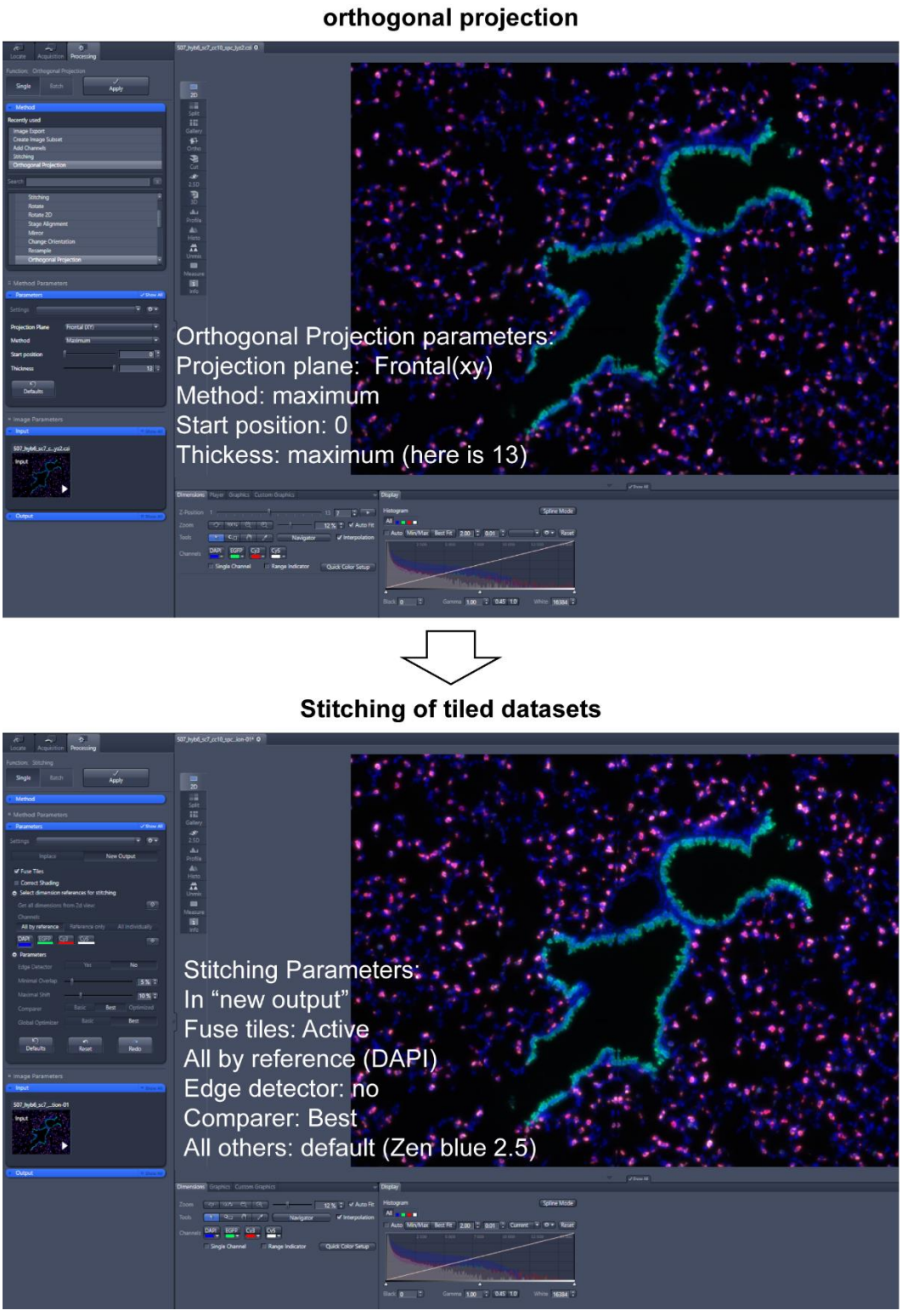


Figure 5. Maximal projection and stitching of the acquired images, using Zen blue 2.5 software.

5.2. Alignment of the image datasets of the same tissue area after multiple detection cycles

To be able to measure the number of the detected RNA transcripts with cellular resolution, the images of all detection cycles must be properly aligned. The usage of automated microscope stages, which can recall the coordinates of the acquired areas to reuse them, facilitates this. However, slight shifts, which depend mainly on the stage accuracy, remain in most cases. Nuclear staining (DAPI) serves as a reference. It is used to measure the shift between different acquisitions and to correct it. We have successfully used 2 different approaches for image alignments, the first is manual and in works through Zen blue 2.5 and the second is automatic and uses the ImageJ and the HyperStackReg (https://github.com/ved-sharma/HyperStackReg).

5.2.1. Manual Alignment

(i) Use the dataset of one hybridization as a reference (in the given example, it is the hybridization-3) and open it in Zen blue 2.5.
(ii) Use the “Create Image Subset” function to create a new image with DAPI channel only, being smaller than the initial image by approximately 500 pixels in every dimension (top, bottom, left and right). It should not start from position 0 (x=0, y=0), because alignment will be impossible if the stage shift, in the other detection cycles, has occurred to the left of the reference image (in the given example x=400, y=400, width=6700, height=5000 pixels).
(iii) Do the same for the interrogated detection cycle dataset.
(iv) Merge the two DAPI-channel images, with “Add Channels” and measure the shift between the reference and the second image, using an easily-recognizable landmark (e.g., a nucleolus), preferentially in the middle of the image. In theory, there should be no rotation in the imaging process. However, in case of accidental camera rotation, align the samples in the center and measure the angle of rotation (Graphics => Angle). Then use ‘Rotate’ function to align images.
(v) Make another crop of the thirddetection cycle dataset, using the adjusted x,y coordinates and create a new merge to evaluate the alignment.
(vi) If the result is fine, select all channels of the dataset, create and save the result for downstream analysis.

The manual alignment example is shown in Figure 6 and “Manual_alignment_procedure_Zen.mp4” in https://github.com/AlexSount/SCRINSHOT_scripts_for_EMBO_course. This laborious method has an advantage of the opportunity to handle any image size limited only by RAM.

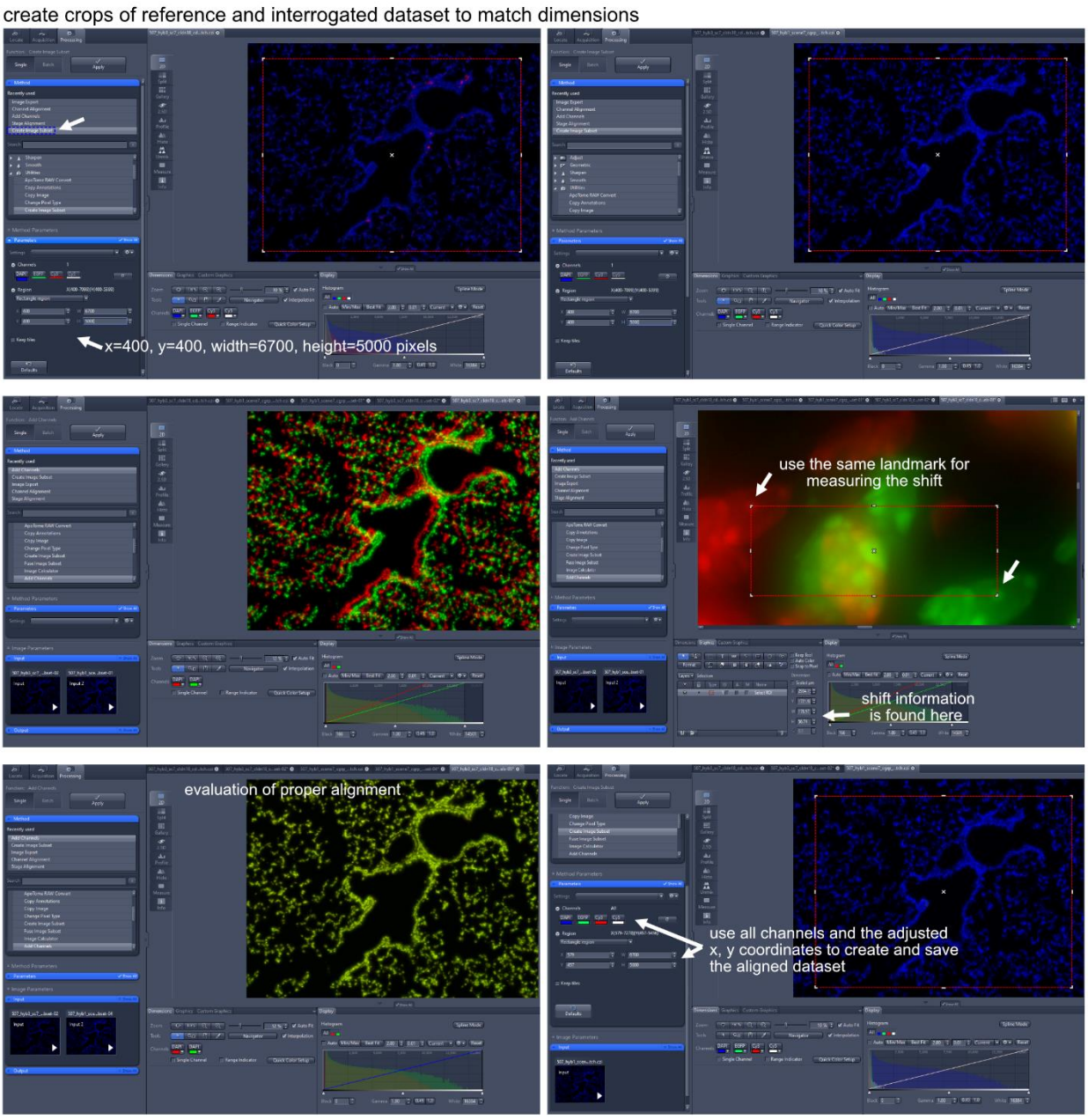


Figure 6. Manual alignment using Zen Blue 2.5.

5.2.2. Automated alignment with ImageJ and HyperStackReg

The automatic segmentation works with original ImageJ [3] (its Fiji version causes java-related problems with HyperStackReg plugin). It works well but it needs large amount of RAM, for big datasets (Automated_alignment_ImageJ.mp4 in https://github.com/AlexSount/SCRINSHOT_scripts_for_EMBO_course).

(i) Export the images from the *.czi files of each detection cycle as original, 16-bit *.tiff format images, using Zen blue 2.5. Name the images according to the corresponding hybridization number (e.g., hyb1, that will result to hyb1_c01_ORG.tif etc)
(ii) Open the images of each detection cycle in ImageJ and create a “Stack” (Image => Stacks => Images to Stack)
(iii) Transform “Stack” to “HyperStack” (Image => Hyperstacks => Stack to Hyperstack), setting the number of channels accordingly (if a channel is missing, using a black image with the appropriate dimension and color depth). The Slices equal to 1 and the frames to the number of detection cycles (in the given example there are 4 channels and 8 frames).
(iv) Save the Hyperstack and open it as a “virtual stack” to limit RAM usage (File => Import => TIFF virtual stack)
(v) Run HyperStackReg plugin, setting “Transformation”: Rigid Body and “Choose channels for transformation matrix computation”: Channel1 (or the number that corresponds to the DAPI-channel). The duration of the procedure mainly depends on the specification of the computer (for the given example, an intel-i7 (6^{th} gen) with 32GB RAM and Windows10 needs 14 min for an 7614x5718 pixels image with 4 channels and 8 detection cycles.
(vi) Make a crop with the appropriate size to ensure that there are no empty areas at the image borders, because of the adjustments during the alignment.
(vii) Finally, visually inspect the overlap of DAPI-channels to ensure that alignment was successful, transform Hyperstack to Stack (Image => Hyperstacks => Hyperstack to Stack) and save the result

5.3. Image export and renaming for further analysis

The measurement of signal-dots is based on the analysis of the images from all channels. From the *.czi files (manual alignment) or Stack file (automated alignment) export each channel as original, 16-bit *.tiff format image, using Zen blue 2.5 or ImageJ, respectively. For convenience in next steps of analysis, use the same prefix of the exported files (in the example, it is “hyb1”). Also, remove the “zeros” from the names of the first 9 images (e.g., hyb1_c01_ORG.tif => hyb1_c1_ORG.tif).

Prepare an excel file with the information about the analyzed genes, their corresponding channel and gene number (Figure 7). The column names should be “Hyb”, “Channel_Order”, “Channel”, “Gene_Order” and “Gene”. It will be used later for data merging in RStudio.

Preparation of Channel order list

Hyb	Channel Order	Channel	Gene Order	Gene
3	1	DAPI	nuclei	dapi
3	2	FITC	gene1	Cldn18
3	3	Cy3	gene2	Cd74
3	4	Cy5	gene3	Empty-Cy5
1	5	Cy3	gene4	Empty-Cy3
1	6	Cy5	gene5	Ascl1
2	7	FITC	gene6	Empty-FITC
2	8	Cy3	gene7	Etv5
2	9	Cy5	gene8	Lgi3
4	10	Cy5	gene9	Fgfr2
5	11	FITC	gene10	Rfp
5	12	Cy3	gene11	Ager
5	13	Cy5	gene12	Spry2
6	14	FITC	gene13	Scgb1a1
6	15	Cy3	gene14	Sftpc
6	16	Cy5	gene15	Lyz2
7	17	FITC	gene16	Cyp2f2
7	18	Cy3	gene17	Axin2
8	19	FITC	gene18	Calca
8	20	Cy3	gene19	Napsa

Figure 7. Example of prepare a list of the analyzed genes.

6. Image analysis

The single channel images are further analyzed for the presence of signal-dots. In order to do that, signal thresholds need to be defined, followed by quantification of the signal of each gene. The following steps are performed:

(i) Definition of signal-dots (threshold setting based on shape and intensity of the signal)
(ii) Signal quantification
(iii) Definition of cell-ROIs (nuclear segmentation and expansion)
(iv) Assignment of signal-dots to cell-ROIs

The analysis of the images requires the Zen blue 2.5, CellProfiler 4.1.3, ImageJ and Fiji and R with RStudio.

6.1. Threshold setting for all analyzed RNA species

The analysis of SCRINSHOT signal-dots is based on the “4_fixed_probe_analysis_pipeline_V6_1-19genes_CP413.cpproj” custom CellProfiler [4] script, which identifies and measures fluorescence signals with specific intensities and sizes. To determine a suitable threshold for each gene, a CellProfiler custom pipeline was created (“1_threshold_V2.cpproj”). It tests how many signal-dots are recognized using different thresholds. The analysis is first done in a small representative area, which includes positive cells for all the analyzed markers. If not possible, multiple areas can be used to provide a more

accurate result. The most convenient way to prepare the images for the threshold analysis is to do a crop of the *.tiff file that was exported in step 5.3.

To run the “1_threshold_setting_v2.cpproj” script, drag and drop the image of each gene for analysis at the “Images” section of the pipeline in CellProfiler. Then, set an output directory where the results are saved (“View Output Settings” tab). Name the output folders according to the corresponding analyzed gene.

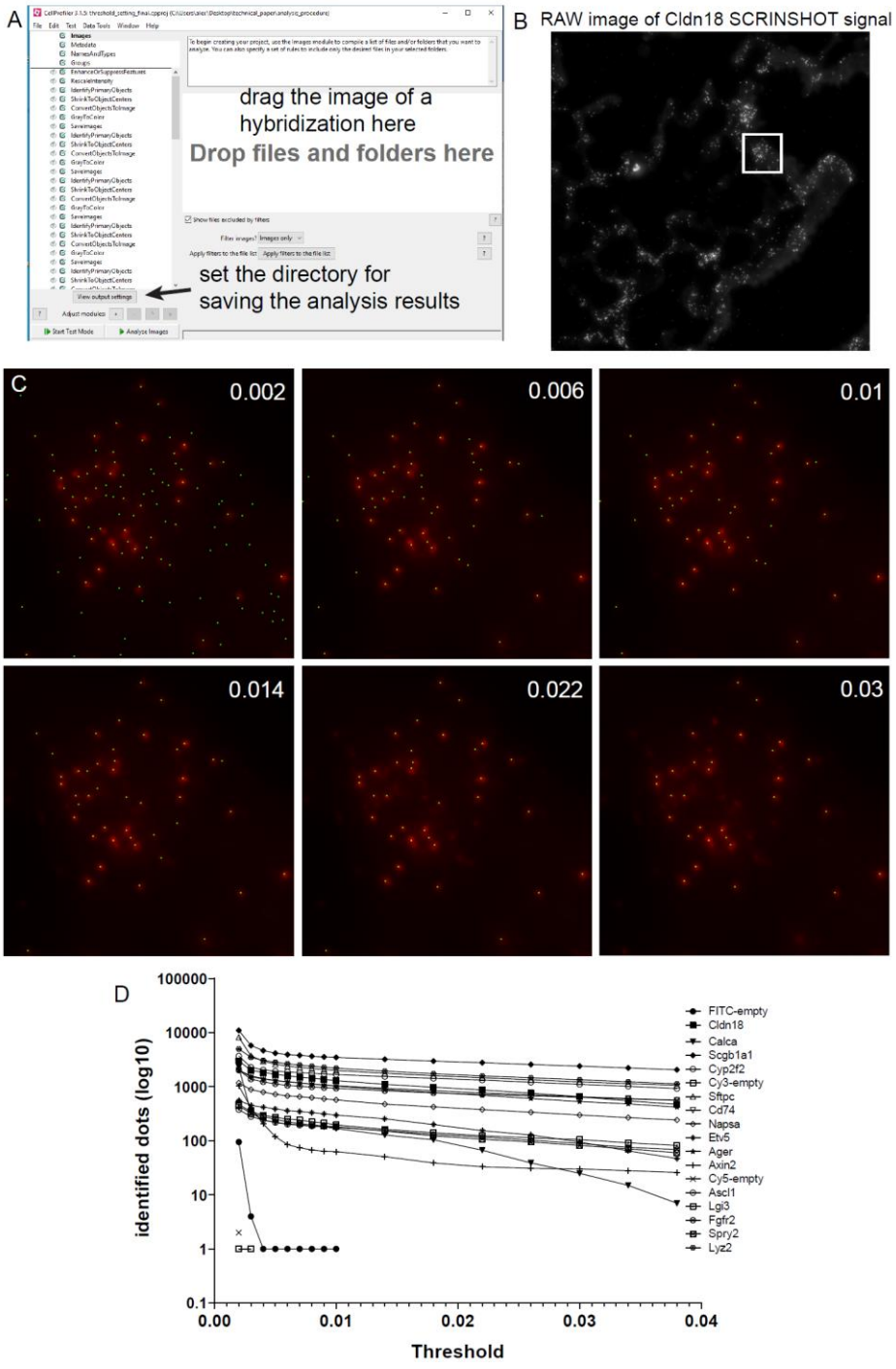


Figure 8. Example of the analysis for threshold estimation. (A) Interface of 1_threshold_setting_v2.cpproj CellProfiler script, showing which parameters should be set for the analysis. (B) Cldn18 RAW image of SCRINSHOT signal, which is used as an example for the threshold analysis. (C) Six representative images of the analysis, showing the fluorescence signal (red) and the identified signal-dots (green). The corresponding thresholds are written on the images. (D) Histogram of the signal-dot identification plotted againstthreshold values. The y-axis is log10 transformed for visualization.

The script produces a number of *.csv files in the “output” directory, containing the coordinates of the identified signal-dots, but the summary is included in the “MyExpt_Image.csv” file. The “Count_IdentifiedBlobs_thrs” values correspond to the number of identified dots for the tested thresholds. The “threshold_test” folder in the “output” directory contains merged images of the raw signal (red) and the identified signal-dots (green) for every threshold. In the example in Figure 8, different thresholds (0.002-0.038) were tested. As expected, low threshold values detect background fluorescence levels and high threshold values are correlated with loss of positive signal.

In conclusion, this step provides a method to estimate suitable threshold values for signal-dot identification and measurement of gene expression values. Images and quantitative results in *.csv files provide complementary information, and it is recommended to **interpret both** for threshold choice.

Optionally, the same area can be acquired without any detection oligos in the used channels (FITC, Cy3 and Cy5), allowing more accurate distinction of signal from tissue autofluorescence (Figure 8 D).

6.2. Signal quantification

6.2.1. Image tiling to reduce file size

Large images might require sufficient RAM in the computer to be processed by CellProfiler in further steps. In that case, it is necessary to reduce the file/image size, dividing it into smaller regions, which facilitates processing.

This process is done with the “2_tiling_with_fiji_v2.ijm” ImageJ script that allows automatic tiling of all the images in a selected folder (Figure 9 A).

For the quantification of the dots with CellProfiler, we need a file with the tile positions in the original image. For that reason, we use the “3_csv_creation_script_v4_for_cellprofiler.R” that produces a *.csv file with the order and location (in the disk) of the produced tile-images (for the given example, its name is “s507_s7_tiling.csv”).

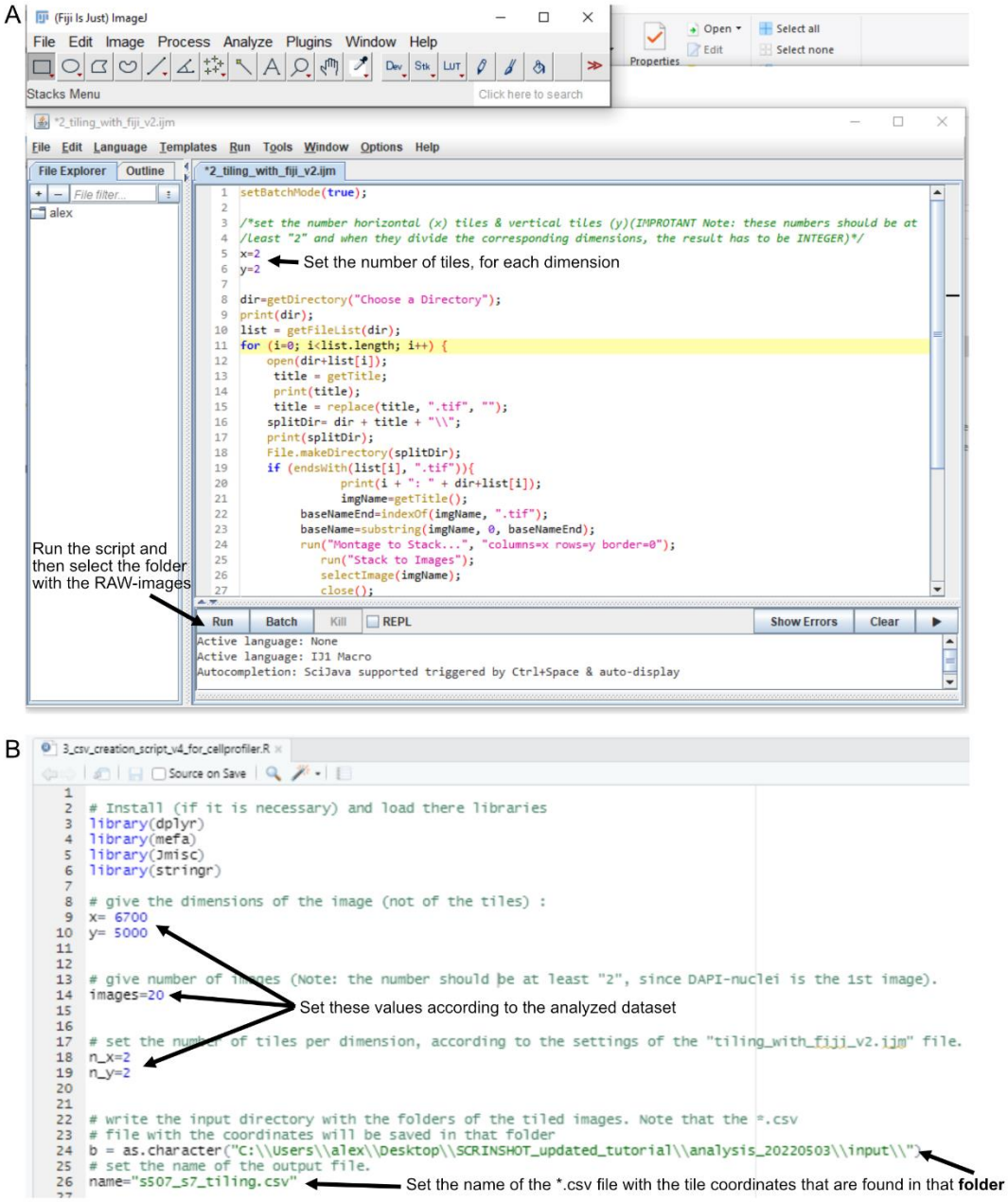


Figure 9. Tilling with ImageJ of all channel images and creation of a *.csv file with tile coordinates, for next step.

6.2.2. SCRINSHOT signal quantification with CellProfiler

The custom CellProfiler script, “4_fixed_probe_analysis_pipeline_V6_1-19genes_CP413.cpproj” is used to automatically count the dots of all genes in the selected dataset. The *.cpproj file provided here has been written and tested with CellProfiler 4.1.3 and performs the analysis of

19 genes (Figure 10). For more genes, more modules can be added by copy/paste in the script, and for fewer genes, modules can be deleted. To run the analysis:

(i) Set the input (folder with the tiled images) and the output (folder for saving the results) directories from “View Output Settings” tab.

(ii) In “Name of the file” field of “LoadData” module use the *.csv file, which has been produced by the “3_csv_creation_script_v4_for_cellprofiler.R” script of the previous step (in the given example, it is “s507_s7_tiling.csv”).

(iii) Set the “Manual threshold” value in the “IdentifyPrimaryObjects” module for each gene, as it has been determined in the corresponding section. The threshold for each given gene should be the same for all analyzed datasets of the same experiment, if the values are later compared or combined.

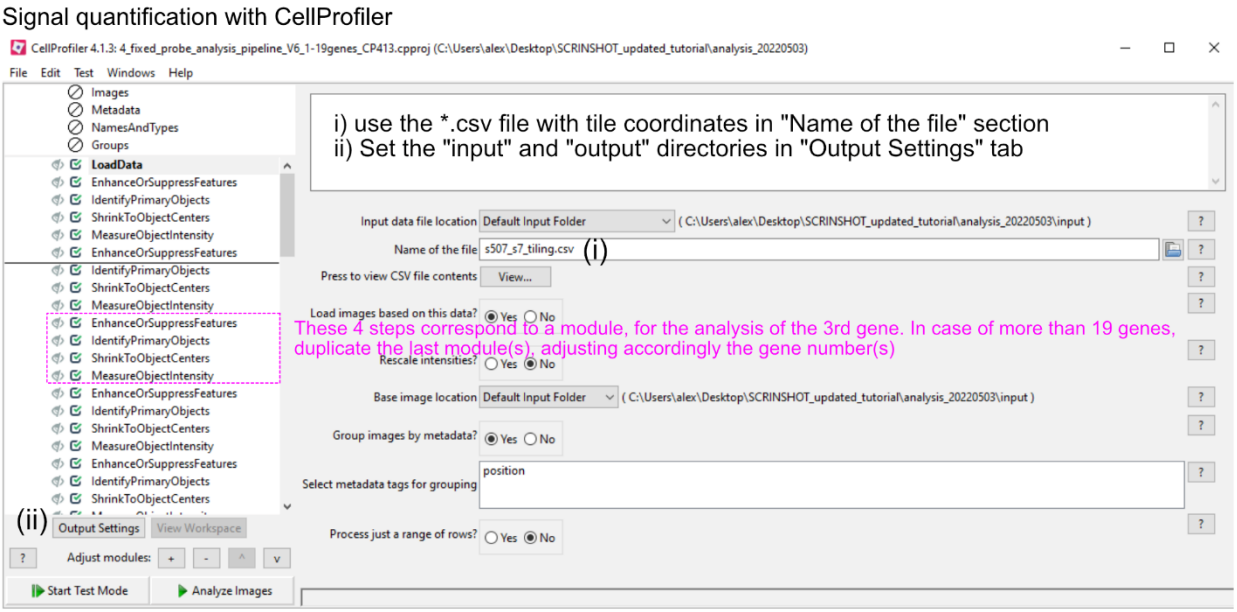


Figure 10. Example of the analysis for signal-dot identification and quantitation. (A) Interface of the “4_fixed_probe_analysis_pipeline_V6_1-19genes_CP413.cpproj” CellProfiler script, showing which parameters should be set for the analysis.

After completion of the analysis, in the output directory, there will be *.csv files with the coordinates of all identified signals (their prefix is “MyExpt_IdentifiedBlobs”). To further proceed with the visualization of the results and downstream analyses, signal-dots of all analyzed genes are incorporated to one *.csv file using the “5_dot_list_creation_v3.R” script, that will create a new *.csv file with all dot coordinates for all analyzed genes and annotate them according to the gene number and name based on the “channel_order.xlsx” file (Figure 7). It will also create new *.csv files for each of the analyzed genes.

6.3. Visualization of the results with TissUUmaps

Use of TissUUmaps allows convenient and interactive projection of the results. The program can be downloaded and installed from the https://tissuumaps.github.io/, instructions available in the same link. Since it is also available for Windows OS, installation can be done using the of "TissUUmaps_winInstaller.exe" file. Any image can be used as a background (in the given example, we used the DAPI-channel image to visualize the nuclei, Figure 11).

(i) Open the TissUUmaps => File => Open Image and select the corresponding file. The image will be loaded quickly and an additional file (e.g., "*_TMAP.tif") will be created that corresponds to a pyramid type of image, that allows fast load, zoom in and out, for future projections of the results.

(ii) Load the *.csv file with the dot coordinates (in the given example, it is "all_gene_marker_coordinates.csv"), set the variables at the right panel of the program and press "Load Markers".

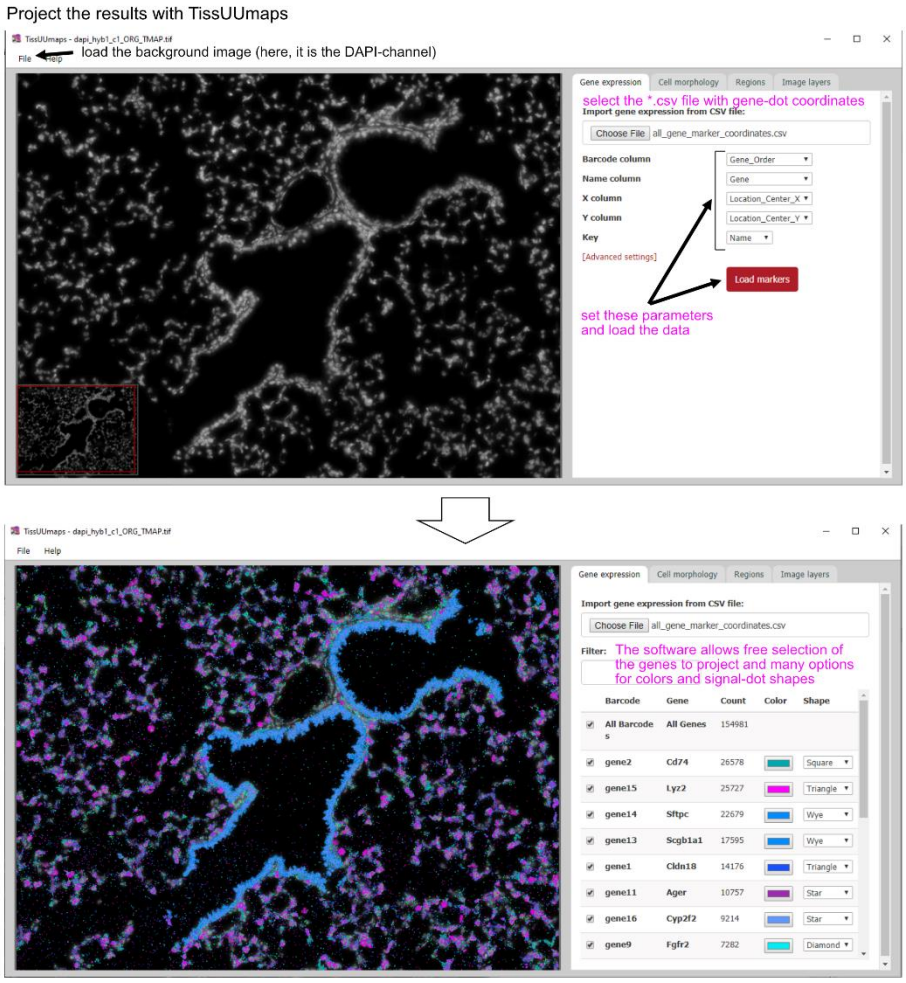


Figure 11. Projection of the identified signal-dots with TissUUmaps.

6.4. Definition of regions of interest (ROI) for single cell resolution

To add single-cell resolution in SCRINSHOT analysis, it is necessary to set specific regions of interest (ROIs), which are accounted as cells and register the identified signal-dots of the detected RNA transcripts to them. Cell segmentation on tissue sections is one of the most challenging tasks for spatial gene-expression methods, because of the difficulty to identify the real cell borders (for example the alveolar region of adult mouse lung contains overlapping and elongated cells, making the real cell border definition almost impossible even with 3D confocal imaging strategies). To circumvent this problem, ROIs are based on nuclear outlines, which are (i) segmented manually, and (ii) expanded by 2 µm in all dimensions (avoiding the overlap of cell regions). ROIs represent a region around the nucleus (including the nucleus itself) that contains most SCRINSHOT signals, further referred to as cell-ROIs. To create quantitative data, we registered the detected signal-dots of all genes to these cell-ROIs, producing count matrices. We also are working in an automated, versatile solution for nuclear segmentation and signal-dot recognition and quantification, with BIAS deep-learning software of Single-Cell Technologies (https://single-cell-technologies.com/).

6.4.1. Manual nuclear segmentation

Segmentation of all nuclei in the analyzed dataset was done with ROI Manager in Fiji, using the reference DAPI channel (hyb1_c1_ORG.tif). The procedure is time consuming but digital pen devices can help. The result is saved as a *.zip file, which contains *.roi files, corresponding to all drawn nuclear ROIs. The *.roi files can be extracted and used partially, but to open them with ROI Manager in Fiji, they have to be in a *.zip file format (Figure 12). It is important to avoid overlapping nuclear ROIs because their intersection will be recognized as a separate object.

After completion of nuclear segmentation, Fiji is used to create a binary image with nuclear outlines, which will be used in the following steps.

(i) Create a white RGB image with the same dimensions as the analyzed image.
(ii) Change the color options in Fiji (edit=>options=>colors: Foreground: black, Background: white, Selection: white).
(iii) Import in Fiji the saved .zip with the *.roi files.
(iv) In ROI manager=> properties: stoke color: white, width: 0, fill color: black, show outlines.
(v) Flatten the image and in ROI manager=> properties: stoke color: white, width:2, fill color:none. This step introduces some distance between the neighboring ROIs.
(vi) Flatten the image.
(vii) Process=> Binary => Make Binary (in options of this tab, ensure that “Black background: is activated, and the colors are not inverted)

(viii) Save the image as “hyb1_c1_ORG.tif” and use it to replace the DAPI channel in the “input” directory. The original DAPI channel is saved for future reference.

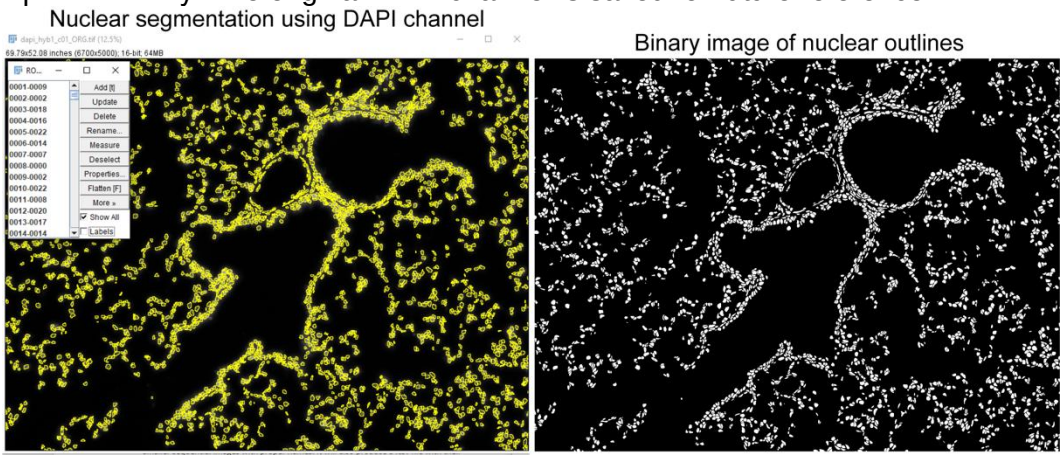


Figure 12. Manual segmentation of all nuclei in acquired tissue areas and creation of a binary image with the nuclear outlines.

6.4.2. Creating cell-ROIs by expansion of nuclear-ROIs

In order to partially capture cytoplasmic region around the nucleus, which contains SCRINSHOT signals, the “6_expand_nuclei_V2.cpproj” CellProfiler script uses the binary image with nuclear outlines from the previous step to expand the ROIs by 2 μm without necessary tiling (Figure 13).

(i) Open the “expand_nuclei_V2.cpproj” script in CellProfiler.
(ii) Drag and drop the binary nuclear-ROI image (not tiled) into the “Images” field.
(iii) Set the output folder at the “View output settings” tab.

The produced “Cell_outlines1.tiff” image is used in Fiji to create a new cell-ROI mask with the “Analyze Particles” function. The results are introduced to ROI Manager and are saved as *.zip, similarly to the nuclear ROIs in previous step (Figure 13).

(iv) Analyze => Analyze Particles: Size (pixel^2):100-infinity, Circularity:0-1, Show: nothing, Display Results:yes, Add to Manager: yes
(v) ROI Manager: more=> save as “507_s7_all_cell_rois.zip”
(vi) ROI Manager: more=> list => save “Overlay Elements” as “all_cell_roi_list.csv”

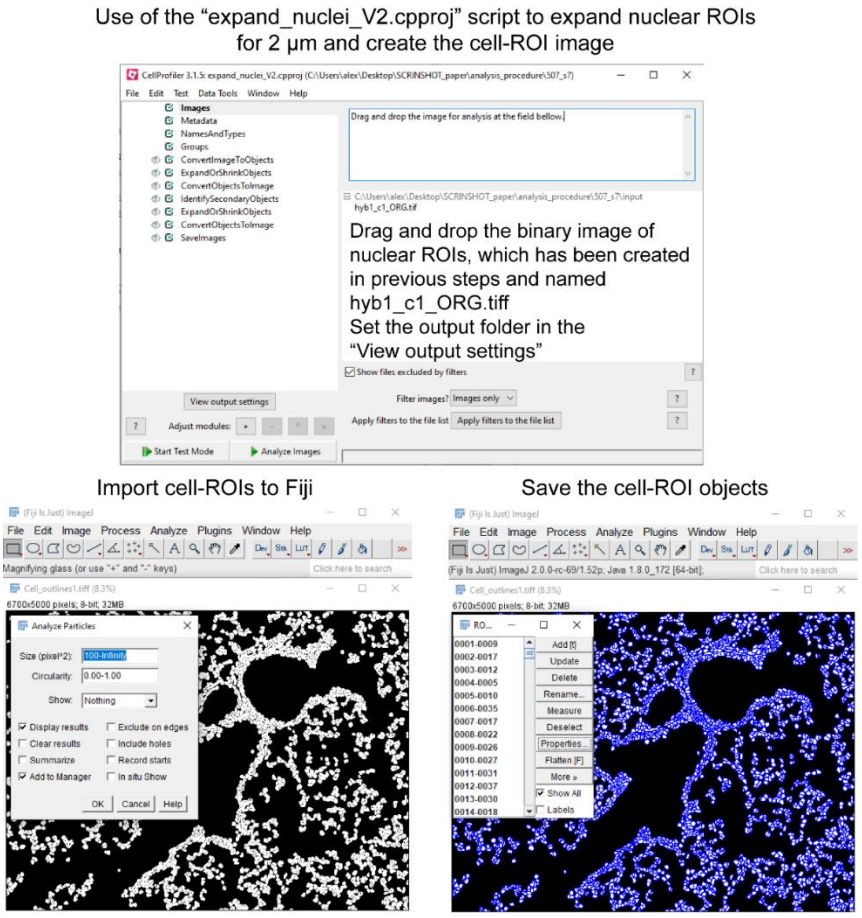


Figure 13. Characteristic example of nuclear-ROI expansion with “6_expand_nuclei_V2.cpproj” script and preparation of the file with the cell-ROI outlines, using ROI-Manager in Fiji.

6.5. Assigning of signal-dots to ROIs

To obtain quantitative data regarding the detected RNA molecules in cell-ROIs, we use the “7_signal_registration_to cell_rois.ijm” ImageJ script, that uses the results from the section 6.2.2 for each gene to register its dots to the cell-ROIs (Figure 14).

(i) Open the script in Fiji and set the input and output directories accordingly (lines 5-6). If needed create the output directory manually.
(ii) In the line-9, set the x,y dimensions of the analyzed image (in the given example, it is x=6700 pixels and y=5000 pixels).
(iii) In the line-25, set the path of the ROI Manager *.zip file with the cell-ROIs, that has been created in step 6.4.2-(v).

The script creates a number of *.csv files with the analyzed genes in the output directory. These files contain five columns: “no-title”: ascending measurement number, “Area”: the cell-ROI surface area in pixels2, “Mean”: mean fluorescence intensity in each cell-ROI, “Min”: minimum fluorescence intensity in each cell-ROI (8-bit), “Max”: maximum fluorescence intensity in each

cell-ROI (8-bit). From these values, the “Area” and the “Mean” will be used for the following steps.

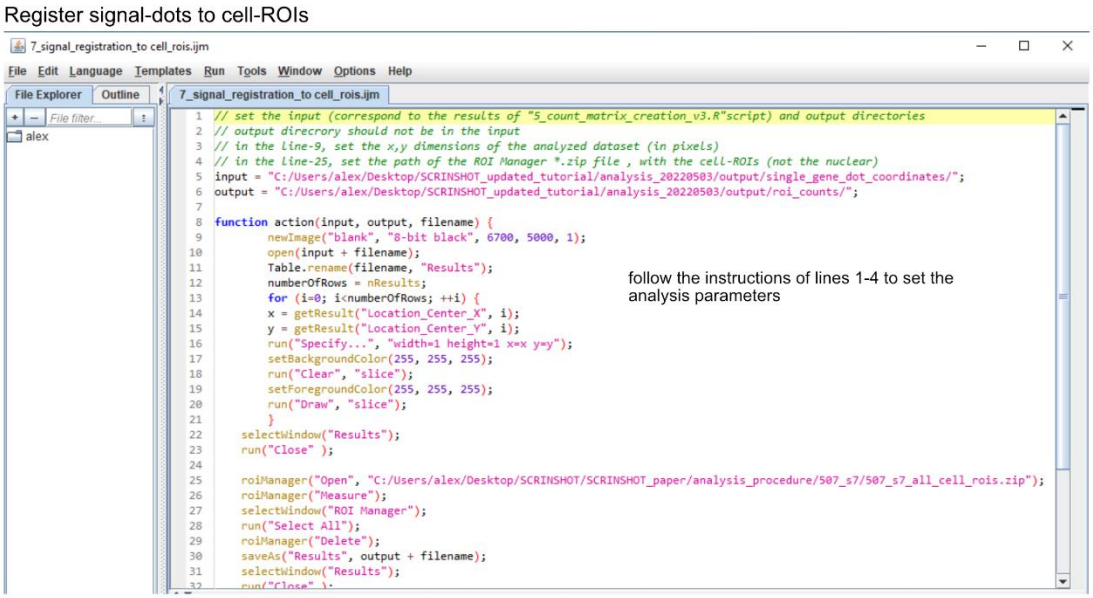


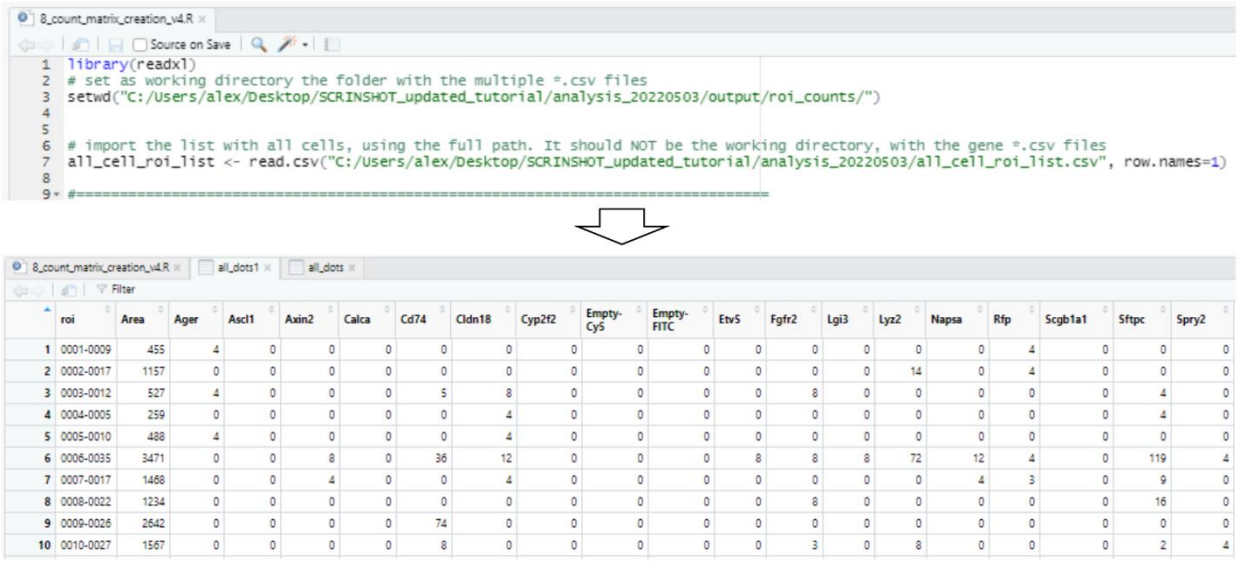
Figure 14. Registration of signal-dots to cell-ROIs, using the “7_signal_registration_to cell_rois.ijm” ImageJ script.

To transform the Mean Fluorescence Intensity (MFI) values to number of dots, we use the “8_count_matrix_creation_v4.R” script. The transformation is based on the fact that the 1pixel images have 8-bit color-depth and that each dot has intensity 255. The mean fluorescence intensity of the cell-ROIs stems from the division of the number of the 1-pixel dots (with intensity 255) by the surface of the corresponding cell-ROI in pixels^2. As a result, the reverse transformation gives the number of the measured dots.

(i) Open the *.zip file with the cell-ROIs (NOT the nuclear-ROIs) in Fiji and export a list with all the cell-ROIs through ROI manager as a *.csv file in ROI-Manager => More => List. Name it “all_cell_roi_list.csv”.
(ii) Set the folder of the output directory as a working directory in the “7_signal_registration_to cell_rois.ijm” ImageJ script (line-3).
(iii) Provide the full path of the “all_cell_roi_list.csv” file in the line-7.

The results include two *.csv files (“all_dots.csv” and “all_dots_integer.csv”) that can be found in the working directory (Figure 15). The count matrix can be further used for cell-ROI clustering, in a similar way to scRNA-Seq data clustering. The distinct cell-ROIs can be plotted back on the tissue.

creation of count matrix



8_count_matrix_creation_v4.R

```
1  library(readxl)
2  # set as working directory the folder with the multiple *.csv files
3  setwd("C:/Users/alex/Desktop/SCRINSHOT_updated_tutorial/analysis_20220503/output/roi_counts/")
4
5
6  # import the list with all cells, using the full path. It should NOT be the working directory, with the gene *.csv files
7  all_cell_roi_list <- read.csv("C:/Users/alex/Desktop/SCRINSHOT_updated_tutorial/analysis_20220503/all_cell_roi_list.csv", row.names=1)
8
9  #============================================================================
```

8_count_matrix_creation_v4.R | all_dots1 | all_dots

	roi	Area	Ager	Ascl1	Axin2	Calca	Cd74	Cldn18	Cyp2f2	Empty-Cy5	Empty-FITC	Etv5	Fgfr2	Lgi3	Lyz2	Napsa	Rfp	Scgb1a1	Sftpc	Spry2
1	0001-0009	455	4	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0
2	0002-0017	1157	0	0	0	0	0	0	0	0	0	0	0	0	14	0	4	0	0	0
3	0003-0012	527	4	0	0	0	5	8	0	0	0	0	8	0	0	0	0	0	4	0
4	0004-0005	259	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	4	0
5	0005-0010	488	4	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
6	0006-0035	3471	0	0	8	0	36	12	0	0	0	8	8	8	72	12	4	0	119	4
7	0007-0017	1468	0	0	4	0	0	4	0	0	0	0	0	0	0	4	3	0	9	0
8	0008-0022	1234	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	16	0
9	0009-0026	2642	0	0	0	0	74	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0010-0027	1567	0	0	0	0	8	0	0	0	0	0	3	0	8	0	0	0	2	4

Figure 15. Creation of the count matrix of all detected signal-dots in the cell-ROIs, using the "7_signal_registration_to cell_rois.ijm" and "8_count_matrix_creation_v4.R" scripts.

7. Literature

1. Ke, R., et al., *In situ sequencing for RNA analysis in preserved tissue and cells.* Nat Methods, 2013. **10**(9): p. 857-60.
2. Dean, F.B., et al., *Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification.* Genome Res, 2001. **11**(6): p. 1095-9.
3. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ: 25 years of image analysis.* Nat Methods, 2012. **9**(7): p. 671-5.
4. McQuin, C., et al., *CellProfiler 3.0: Next-generation image processing for biology.* PLoS Biol, 2018. **16**(7): p. e2005970.

SCRINSHOT protocol (short version) date:

Post-fixation of the slides

1) Slides are removed from -80°C and they are placed in a small slide-box (Sigma-Aldrich Z708313-25EA). We transfer them to the lab and place them in 45°C for 15 minutes to prevent moisture accumulation on the tissue.
2) Slides are fixed in room temperature PFA 4% in PBS 1x pH 7.4 (freshly prepared) for 5 min at room temperature (RT).
3) 2 x 5 min washes with PBS-Tween 0.05% (PBS-T: 450ml DEPC H_2O + 50 ml PBS 10x + 250µl Tween20).

Permeabilization/dehydration of the tissue

1) Incubate in 0.1M HCl in DEPC H_2O at RT for 3 min (in 30ml H_2O add 250µl 12N HCl)
2) Wash 2 x 2 min with PBS-T
3) 70% ETOH for 2 min, 85% ETOH for 2 min and 100% ETOH for 2 min

70% ETOH: 28ml ETOH + 12 ml DEPC H_2O, 85% ETOH: 34ml ETOH + 6 ml DEPC H_2O

Note: Ethanol solutions should be freshly prepared, at least for that step. If tissue detaches, reduce the HCl incubation time or its concentration.

Mounting of the chambers

Seal the chambers of suitable sizes over the tissue sections. Calculate the total solution volume (different size chambers have different volume). Calculation tables are added below for every reaction solution.

Rehydration

3 x 2 min PBS-T, leave in the last PBS-T until next reaction solution is added.

Blocking

Prepare

Reagents	**stock**	**final**	**1 x slide (µl)**	**x slides**
RNase Free H_2O			57.5	
Ampligase Buffer	10x	1x	10	
KCl	1M	0.05M	5	
Formamide deionized	100%	20%	20	
Oligo-dT	10µM	0.1µM	1	
BSA	10µg/µl	0.2µg/µl	2	
RiboLock (Thermo)	40U/µl	1U/µl	2.5	
tRNA (Ambion AM7119)	10µg/µl	0.2µg/µl	2	
Total			**100 µl**	

Note: deionized Formamide is aliquoted and stored at -80°C. After thawing, it can be refrozen at -20°C and should not be re-thawn more than 2 times.

Oligo-dT: AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN

1) Remove the PBS-T
2) Incubate at RT for 30 min
3) 2 x 1 min washes with PBS-T

PADLOCK PROBE HYBRIDIZATION & LIGATION

Prepare

Reagents	**stock**	**final**	**1 x slide (µl)**	**x slides**
RNase Free H_2O				
Ampligase Buffer	10x	1x	10	
KCl	1M	0.05M	5	
Formamide deionized	100%	20%	20	
Probes 0.1 µl/padlock	40µM	0.04 µM	0.1 x	
BSA	10µg/µl	0.2µg/µl	2	
RiboLock (Thermo)	40U/µl	1U/µl	2.5	
tRNA (Ambion AM7119)	10µg/µl	0.2µg/µl	2	
Total			**100 µl**	

1) Remove the PBS-T
2) Add the Probe solution into the chamber, mix by gentle pippeting (5-10 times) and seal the two holes with PCR adhesive membrane
3) Incubate at **55°C for 15 min** and 45°C for 120 min for hybridization
4) Wash 3 x 10 min with 10% Formamide in 2X SSC
5) Wash 3 x 1 min with PBS-T and leave in the last solution until the next step

Ligation with SplintR

Prepare

Reagents	**stock**	**final**	**1 x slide (µl)**	**x slides**
RNase Free H_2O			82.5	
T4 RNA Ligase Buffer	10x	1x	10	
ATP	1mM	10µM	1	
BSA	10µg/µl	0.2µg/µl	2	
SplintR	25U/µl	0.5U/µl	2	
RiboLock (Thermo)	40U/µl	1U/µl	2.5	
Total			**100 µl**	

1) Remove the PBS-T
2) Add the solution into the chamber, mix by gentle pippeting (5-10 times) and seal the two holes with PCR adhesive membrane
3) Incubate at 25°C O/N
4) Wash 2 x 1 min with PBS-T and leave in the last solution until the next step

RCA

Prepare the RCA master mix

Reagents	**stock**	**final**	**1 x slide (µl)**	**x slides**
RNase Free H_2O			68.5	
Φ29 buffer	10x	1x	10	
Glycerol	50 %	5 %	10	
dNTPs	10 mM	0.25 mM	2.5	
BSA	10µg/µl	0.2 µg/µl	2	
RCA Primer1 (TAAATAGACGCAGTCAGT*A*A)	**10 µM**	0.1µM	1	
RCA Primer2 (CGCAAGATATACG*T*C)	**10 µM**	0.1µM	1	
Φ29 polymerase (Lucigen)	10 U/µl	0.5 U/µl	5	
Total			**100 µl**	

Note: RCA Primers are TS modified. The modification is indicated with ""*

1) Remove the PBS-T
2) Add the solution into the chamber, mix by gentle pippeting (5-10 times) and seal the two holes with PCR adhesive membrane
3) Incubate at 30°C O/N
4) Remove the reaction mix
5) Wash 2 x 1 min with PBS-T and leave in the last solution
6) Fix for 15 min with PFA 4% in PBS 1X pH7.4 at RT
7) Wash 2 x 1 min with PBS-T and leave in the last solution
8) Wash 3 x 10 min with 65% formamide in H_2O at 30°C (for 1500µl: 975µl Formamide + 525µl H_2O)
9) Wash 2 x 1 min with PBS-T and leave in the last solution until the next step

Detection probe Reactions (light sensitive reagents - protect from light)

First Hybridization

Hybridization master mix preparation

Reagents	**stock**	**final**	**1 x slide**	**x slides**
RNase Free H_2O				
SSC	20X	2X	10 µl	
Formamide deionized	100%	30%	30 µl	
FITC-labeled probes	10µM	0.02µM	0.2 µl each	
Cy3-labeled probes	10µM	0.01µM	0.1µl each	
Cy5-labeled probes	10µM	0.01µM	0.1µl each	
Texas Red-labeled probes	10µM	0.01µM	0.1µl each	
AlexaFluor750-labeled probes	10µM	0.01µM	0.1µl each	
DAPI	50µg/ml	0.5µg/ml	1 µl	
BSA	10µg/µl	0.5µg/µl	5 µl	
Total			**100µl**	

Probe hybridization

1) Remove the PBS-T
2) Add the Detection oligo solution into the chamber, mix by gentle pippeting (5-10 times) and seal the two holes with PCR adhesive membrane
3) Incubate at 30°C for 60 min
4) Wash 3 x 5 min with 20% Formamide in 2X SSC (1200µl 2x SSC + 300µl Formamide)
5) Wash 3 x 1 min with 2 x SSC and leave in the last solution until the next step

Dehydration, Mounting & storage

1) Dehydrate the tissue with a series of ETOH: 70% for 2 min, remove chamber, 70% for 1 min, 85% for 2 min and 100% for 2 min. Air-dry in the dark.
2) Apply Slowfade mounting medium and coverslip
3) Store in the dark at 4°C or RT until imaging

Second and the followinghybridizations

Remove the coverslip and the mounting medium

1) Place the slide in ETOH 70%, horizontally at 45°C, with coverslip facing down, until the coverslip is detached. Place the slide in 85% ETOH for 2 min and 100% ETOH for 2 min. Air-dry and mount chambers
2) Wash 3 x 2 min in PBS-T and leave in the last solution until UNG reaction solution is added.

STRIP OFF 1st PROBES

Reagents	**stock**	**final**	**1 x slide (µl)**	**x slides**
RNase Free H_2O			86	
UNG buffer	10x	1x	10	
BSA	10µg/µl	0.2µg/µl	2	
UNG (Fermentas)	1U/µl	0.02U/µl	2	
Total			**100µl**	

1) Remove the PBS-T
2) Add the UNG solution in the chamber, mix by gentle pippeting (5-10 times) and seal the two holes with PCR adhesive membrane
3) Incubate at 37°C for 30-45min
4) Wash 3 x 5 min with 65% formamide in H_2O at 30°C (for 500µl: 325µl Formamide + 175µl H_2O)
5) Wash 2 x 1 min with PBS-T and leave in the last solution until the next step
6) Then continue as with the 1st hybridization

Important note: *For removal of the detection probes of abundant genes use the same buffer as above but incubate for 45 min at 37°C, gently pipette several times and* ***add fresh reaction mix*** *for additional 45 min at 37°C. We have noticed that the detection oligos of very abundant genes are not completely removed after UNG incubation for 90 min. So, the addition of fresh enzyme is beneficial.*