Multiple Iterative Labeling by Antibody Neodeposition (MILAN)

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Method Article

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Abstract

(What's new in protocol Version 5: an expanded troubleshooting section, more validated antibodies)

Multiplexing, labeling for multiple immunostains the very same cell or tissue section in situ, is of considerable interest. The major obstacles to the diffusion of this technique are high costs in custom antibodies and instruments, low throughput, scarcity of specialized skills or facilities. We have validated and detail here a method based on common primary and secondary antibodies, diffusely available fluorescent image scanners and routinely processed tissue sections \(FFPE). It entails rounds of four-color indirect immunofluorescence, image acquisition and removal \(stripping) of the antibodies, before another stain is applied. The images are digitally registered and the autofluorescence is subtracted. Removal of antibodies is accomplished by disulphide cleavage. In excess of 50 different antibody stains can be applied to one single section from routinely fixed and embedded tissue. This method requires a modest investment in hardware and materials and uses freeware image analysis software.

Introduction

Staining a section with multiple antibodies satisfy two different requirements: - to assess multiple analytes when the limitation is the number of sections \(e.g. a single one) - to classify single cells in tissue by high-dimensional methods, typically >15 markers. Both requirements are accomplished by this method, which relies on controlled antigen retrieval conditions \(once, at the beginning), antigen retention over the staining cycles, six-color immunofluorescent staining, bringing the total amount of stainings into the dozens. It employs unconjugated primary antibodies, commercial secondary antibodies and IF microscopes and scanners. It has thus the power to bring the two requirements mentioned above to a vast public of scientists, investigators, clinicians, etc. **2ME/SDS staining and stripping method** 1 perform ARx on dewaxed sections affixed to postively charged glass slides 2 allow to cool to about 50C or lower 3 acquire the AF image for all the channels deemed necessary \(optional) 4 perform the first IF stain in 100 mM Trehalose-containing dilution buffer 5 mount with 60% Glycerol in PBS, 0.2% N-propyl Gallate and 584 mM sucrose mounting medium containing DAPI 6 label the slide, acquire the images for all channels including DAPI and AF if not acquired before 7 unmount the slides in buffer/distilled water 8 transfer to Tris buffer 9 immerse for 30 min in pre-heated \(56C) 2ME/SDS buffer with agitation 10 transfer to Tris buffer and wash extensively with TBS-Ts buffer 11 repeat from #4 with additional positive and/or negative antibodies 12 store in 50% glycerol at -20C/-80C for extended storage, before returning to #4 or proceed as below 13 perform H&E or insoluble stainings if final A different stripping method, based on Guanidinium Hydrochloride and heath-mediated refolding may be used \(see Reference below). \(From Bolognesi MM, Manzoni M, Scalia CR, Zannella S, Bosisio FM, Faretta M, et al. "Multiplex Staining by Sequential Immunostaining and Antibody Removal on Routine Tissue Sections. Journal of Histochemistry & Cytochemistry.":http://journals.sagepub.com/doi/full/10.1369/0022155417719419 2017 Aug;65\(8):431–44; Figure 1)

Reagents

CHEMICALS

100x Tris-EDTA buffer, pH 8 (Sigma, T9285)

Trizma HCL (Sigma T3253) MW 157.60

Trizma base (Sigma T6066) MW 121.14

NaCl (Sigma 793566) MW 58.44

Sucrose (refined granulated sugar; *NB check for vegetable particles, e.g. in cane sugar)* MW 342.30

Tween-20 (Sigma P1379)

NaN_3 (Sigma 71290) MW 65.01

Bovine Serum Albumin (Sigma A2153) NB may substituted with gelatin.

Trehalose (Sigma 90210) MW 378.33

Glycerol (Sigma G9012)

N-propyl gallate (Sigma P3130) MW 212.20

N,N-dimethylformamide (Sigma D4551)

Phosphate buffered saline, tablets (Sigma P4417)

2-mercaptoethanol (Sigma M6250) MW 78.13 *(shelf life: 36 months)*

Sodium dodecyl sulphate, dust-free pellets (Sigma 74255)

DAPI dihydrochloride (D9542 Sigma) MW 350.25

Primary unconjugated antibodies (see attached table “Primary antibodies”)

Fluorochrome-conjugated secondary antibodies (see attached table “Secondary antibodies”)

Purified immunoglobulins (see attached table “Immunoglobulins”)

SOLUTIONS, PREPARATIONS AND BUFFERS

Washing Buffer (TBS-Ts) pH 7.5 stock solution 10X 1000ml

Tris-Buffered Saline - Tween20 sucrose

63,5 g Trizma HCL (403 mM)

11,8 g Trizma base (97.4 mM)

90 g NaCl (1.54 M)

342,3 g Sucrose (1 M)

2 ml Tween-20

NaN_3 0,1%

1000ml Distilled H2O

Dilute in distilled water 1:10 to 1X before use.

NB: do not use this buffer for Peroxidase-based IHC because the NaN_3 will inhibit the reaction. OK for AP-based IHC.

What these chemical are there for? Tris is for buffering the solution; Salt is to weaken non-specific molecular interactions; Tween-20 is to reduce surface tension and evenly wet the slide, preventing section margin drying; sucrose is to prevent dehydration [6]

Antibody diluent 1x (100ml):

2 g BSA

90 ml Distilled H2O

50 mg or 1 ml NaN3 5% (stock)

3,8 g Trehalose (100mM)

10 ml TBS 10X

NB: Dissolve the BSA in distilled water first; then add the other chemicals.

Mounting fluid

100ml 60% glycerol / 40% distilled water x DAPI

60 ml Glycerol

10 ml PBS 10x pH 7.5

10 ml Sucrose saturated (200 g in 100 ml dist., 5.84M)

20 ml Distilled H_2O

Add 0,2% n-propyl gallate from a 20% stock solution, prepared by dissolving the compound in N,N-dimethylformamide (store at -20C)

N.B.: Other (hardening) mounting media cause antigen re-masking [3]

To dissolve DAPI, use DAPI dihydrochloride (D9542 Sigma, 1 mg size, 2.85 µM) [*carcinogen!*]. Resuspend in 285 µl of Dimethylformamide (DMF). [*You can skip DMF and proceed directly with Methanol by adjusting the molarity accordingly. Contributed by R.Perego's lab*]. The solution (DAPI 10 mM) will be turbid and yellow. Mix equal volumes of DAPI in DMF and "methanol":http://cshprotocols.cshlp.org/content/2007/10/pdb.rec11127.full?text_only=true : the solution (DAPI 5 mM) will become clear transparent.

Dilute to 2-10 µM in either TBS-Ts or mounting fluid.

NB: concentrations above 10 µM on FFPE material will be A) unmanageable for exposure (too short), B) DAPI signal will bleed into the Autofluorescence, FITC, and TRITC filters (in order).

Stripping buffer

200ml 1x (*prepare and refrigerate w/o the 2-ME*)

20 ml SDS 10%

12,5 ml Tris HCl pH6,8 (0,5M)

167,5 ml Distilled H2O

before use, add 0,8 ml 2-mercaptoethanol; once you added, the half-life is ~100 hrs.

NB: Work under a fume hood. Scale up or down the volumes and chemicals accordingly.

Storage Buffer (Glycerol 50%):

100ml 1x

50 ml glycerol (Sigma G9012)

10 ml Tris buffer 10x pH 7.5

10 ml 50% sucrose (half-saturated solution; 5%=300mM).

30 ml Distilled H2O

NaN_3 100x (5%) stock solution

12,5 mg NaN_3

250 ml distilled H2O

50% Saturated Sucrose

100 ml Distilled H2O

100 g sucrose

0.5% NaN_3

refrigerate

Equipment

- "Kartell slide boxes \(50-100 slides)":https://www.kartelllabware.com/en/products/plastilab/microscopy-and-microbiology/microscope-slide-boxes/ - Glassware - Shaking waterbath

Procedure

Step 1: GLASS SLIDES Standard 75.5±05 x 25.5±0.5 mm "microscope slides":https://en.wikipedia.org/wiki/Microscope_slide are required for all histopathology studies. _NB: Do not use slides with rounded smoothened corners because they tend to slip out of the scanner stage._ **Step 2: SECTIONS** \(Figure 1) See figure in Figures section. Sections for multiplexing of 3±1 µm thickness need to be placed on positively charged slides \(the ones used for immunohistochemistry) one section per slide, positioned toward the slide end opposite to the frosted end for label, at least 2-3 mm from the slide border. The scanning speed is maximal across the slide, thus prefer a transversal or oblique, rather than a longitudinal placement of the section. place sections on coated/charged glass slides Bake overnight in an upright position in oven 40°C or lower Dewax in Xylene \(2 changes 10 min each) -> graded alcohol \(99%-95%-70%-H20) _NB. An Hexane overnight step before the xylene has been recommended for complete paraffin extraction\[1]: hexane is volatile and may dry the sections before entering xylene. An advantage in immunoreactivity is antigen-dependent and modest._ **Step 3: ANTIGEN RETRIEVAL** See figure in Figures section. Perform antigen retrieval with 10 mM EDTA in Tris-buffer pH 8; use 800 ml distilled water in a MWO-proof glass container, to which add 8 ml of a 100x Tris-EDTA buffer, pH 8. - Insert the slides in a radiotransparent slide holder \(Figure 2) - Place in a household microwave oven \(MWO), set to “high” or 850W: should boil vigorously in 8 min. - Reduce power to “low” or 300W and allow 20-30 min. of intermittent radiation to maintain boiling. _This overcomes pH dependent retrieval\[2]_ - Cool to 50°C or below to allow antigen refolding before transferring to **washing buffer \(TBS-Ts)**, by checking with a kitchen thermometer. \(Figure 2) _Take precautions: hot fluid._ - Slides can be stored in 50% glycerol-sucrose-TBS at this step \(storage buffere) \[3] **Step 4: IF STAINING: Primary and Secondary Ab dilution and incubation** See figure in Figures section. Dilute all primary Ab’s to 1 µg/ml \(or equivalent by titration) in **Antibody diluent** \[3] Dilute all secondary Ab’s

to 5 µg/ml \(~1:200 – 1:300). _NB. Fluorochrome-conjugated antibodies used in double indirect IF \[3] have different concentration/signal curves. Alexa 488 conjugates tend not to increase signal above 5µg/ml, because of self-quenching; Rhodamine RedX and Alexa 647 do increase. BV480 conjugates tend to have an exponential increase of signal with increased concentration above 2-3 µg/ml; however, anti-isotype conjugates are much brighter than species-specific ones. If using BV480, beware of A) non-specific background increase, B) spillover of BV480 signal into Autofluorescence and FITC channels._ By using unconjugated primary antibodies in indirect immunofluorescence, the following combinations are permitted, based on species- or isotype-specific secondary antibodies and a filter combination as depicted in Fig. 5: - One each of rabbit, mouse, rat and goat antibodies - One rabbit Ab plus one each of the mouse IgG1, IgG2a, IgG2b or IgG3, up to one Rb + 3 mouse Abs. _NB: anti-isotype secondary antibodies are invariably raised in goat or rabbit; use secondary abs raised in donkeys or lamas for the first combination exemplified \(Rb, Mo, Rat, Gt)._ **Humid chamber Incubation** \(Figure 3) See figure in Figures section. check with a level/iPhone for perfectly horizontal placement of the chamber: this will prevent antibody solution slipping during prolonged incubations \(e.g. O/N). Do not let the slides touch each other. Use a closed container \(Kartell)\[4] with distilled water, Sodium Azide and a tissue to prevent floating. use a minimum of 100µl of antibody for a section of 1x1 cm or less and the volume multiplied accordingly for larger sections. _NB: you can recover and re-use the antibody from the slide, however the small volume of antibody and the relatively larger residual fluid on the slide after washing may alter the Ab concentration uncontrollably. This will not happen with the mailers \(see below)._ **Vertical 5-slide mailer Incubation** \(for high efficiency) \(Figure 3 and 4) See figure in Figures section. Fill a standard 5 slide vertical mailer \("Kaltek":https://www.kaltek.it/en/histology/slides/slide-mailers-2/, "MLS":https://www.mls.be/products/?lang=en&category=08&subcategory=8.1.1.9 or others) with 12 ml antibody solution. You are supposed to stain five slides simultaneously, so that the fluid can completely cover each section. This setup can be re-used for a total of approx. 10 rounds of staining \(= 50 slides total)\[3]; thus 12 µg of antibody is enough for one 50-slides experiment and 1 vertical mailer. **Procedure** - Incubate in primary Ab overnight at room T \(manual) or at +4C \(mailers). _NB: overnight incubation will increase the staining efficiency \[3]._ - Wash 2x in 15 min. with **TBS-Ts** in a coplin jar - Incubate in secondary Ab 30 min. - Wash 2x in 15 min. with TBS-Ts in a coplin jar - Incubate in negative primary Ab → double indirect staining _NB: double indirect staining will double the fluorescence yield \[3]. In order to save primary antibodies, use isotype- and species- matched irrelevant negative purified Ig._ - Wash 2x in 15 min. with TBS-Ts in a coplin jar - Incubate in secondary Ab 30 min. - Wash 2x in 15 min. with TBS-Ts in a coplin jar - Stain with DAPI \(2-10 nM) in TBS-Ts by immersing 1 min in a vertical mailer, then rinsing in TBS-Ts. If DAPI is in the mounting fluid, skip this step. - Mount slides with **mounting fluid** and 24x50 coverslips. Remove excess fluid from the edges, otherwise will interfere with the re-positioning of the slide on the stand. - Remove the disaccharide-containing fluid from the bottom of the slide with a distilled water-soaked pad, for smooth mechanical operations \(may be performed just before step 10). - Affix a label containing a 2D barcode for file name reading by the instrument and with other metadata \(date, experiment #, etc.)\[3] **Step 5: STRIPPING** \[3, 5] - Coverslip removal by soaking in coplin jar with washing buffer or distilled water \(either one, OK). This is one of the steps where scratching of the tissue sections may occur when re-positioning back the slide in the presence of a

coverslip. Act in a continuous vertical motion, expose the whole slide, transfer to a new coplin jar with buffer if the coverslip detached, reposition if unmoved, slip gently the coverslip if partially displaced with a continuous motion. - Transfer to Tris buffer pH 7.5, in order to remove disaccharides. - Preheat vertical containers with **stripping buffer** to exactly 56°C in closed, shaking water-bath. _Cave: tight temperature range for effective stripping\!_ _Stripping buffers contains chemicals with offensive odor; work under hood\!_ - Strip for 30 min. at 56°C with shaking - Transfer to TBS-Ts - Wash at least for one hour with washing buffer with several washes in the first quarter of an hour. **Step 6: STORAGE** Store at any step. If slides are not used for > 3 days store @ -20°C in **storage buffer** Prefer storage of unstripped slides after the last staining; stripping will get rid of autofluorescence or background formed during storage.

Timing

Step 1-2: Tech Lab cutting time **Step 3:** approx. 100 min. **Step 4:** 1 overnight incubation plus approx. 4 hrs. **Step 5:** 2 hrs. **Step 6:** N/A

Troubleshooting

Inadequate or failure to remove antibodies (stripping) may occur because of these causes:

Failed temperature control during stripping

Dehydration (*may not be obvious on inspection, see the Ref Boi G. et al*)

E**xpired 2-Mercaptoethanol**. 2ME has a shelf-life of 36 months and a half-life of ~100 hours when diluted in a pH 6.5 buffer. Expired 2ME may still stinks, but the stripping efficiency of the 2ME/SDS mix may decrease considerably. Don’t trust your nose, read the label.

Low buffer/section area ratio. In some vertical mailers, the anterior or posterior wall may bend, leaving not enough room for sufficient buffer volume to overlay the section. This may results in insufficient stripping for the sections placed first or last, particularly when the section is very large.

Superdense antigens. As published in our leading paper, very dense antigens may strip with difficulty. Examples are cytoplasmic pentameric Ig or uromodulin (read the Wikipedia entry). One may encounter such antigens, for which we have few suggestions. Try to reduce antibody concentration below saturation (in the 0.1-0.01 µg/ml range) and/or counterstain only once.

Light-source induced chemical modification and protein precipitation during exposure, particularly with powerful lasers (see the "Science":http://science.sciencemag.org/content/361/6401/eaar7042.long paper)

A repetition of the stripping, preceded by 1 min boiling in the AR solution may help.

ADDITIONAL TROUBLESHOOTING

FFPE-proof antibody negative on MILAN. The epitope of about 1-3% of the antibodies tested, effective on FFPE are exquisitely inactivated by a single exposure to 2ME/SDS. Examples are the CD30 epitope of BerH2, the CD45RO epitope of UCHL1.

Section scratching. Being a manual technique, accidental scratching of the section is a dreadful occurrence. To avoid the most dangerous type of erasure, always insert and extract a slide in a container behind another slide, with the edge of the slide to be inserted facing the empty back of a previous slide.

Errors when investing a substantial amount of reagents in the vertical mailers method may be prevented by running a less crucial set of slides first. Best to test with the MILAN IF method primary antibodies used before only in immunohistochemistry: enzymatic IHC is a non-linear, signal thresholding method quite different from the linear quantitative IF method, on which MILAN is based. And in case of errors: **strip and repeat!** With this method you can do it.

Anticipated Results

You should be able to sequentially stain a single FFPE section with antibodies raised in the same species, directed against close epitopes, on the very same subcellular structure, provided you space each staining by a stripping cycle. Examples can be seen in the literature references provided.

References

1. Faolain, E.O., et al., _Raman spectroscopic evaluation of efficacy of current paraffin wax section dewaxing agents._ J Histochem Cytochem, 2005. 53\(1): p. 121-9 doi: 10.1177/002215540505300114. 2. Scalia, C.R., R. Gendusa, and G. Cattoretti, _A 2-Step Laemmli and Antigen Retrieval Method Improves Immunodetection._ Appl Immunohistochem Mol Morphol, 2015. 24: p. 436-446 doi: 10.1097/PAI.0000000000000203. 3. Bolognesi, M.M., et al., _Multiplex Staining by Sequential Immunostaining and Antibody Removal on Routine Tissue Sections._ Journal of Histochemistry & Cytochemistry, 2017. 65\(8): p. 431-444 doi: 10.1369/0022155417719419. 4. Dominguez-Sola, D. and G. Cattoretti, _Analysis of the Germinal Center Reaction in Tissue Sections._ Methods in molecular biology \(Clifton, N.J.), 2017. 1623: p. 1-20 doi: 10.1007/978-1-4939-7095-7_1. 5. Gendusa, R., et al., _Elution of High Affinity \(>10-9 KD) Antibodies from Tissue Sections: Clues to the Molecular Mechanism and Use in Sequential Immunostaining._ J Histochem Cytochem, 2014. 62\(7): p. 519-531 doi: 10.1369/0022155414536732. 6. Boi, G., et al., _Disaccharides Protect Antigens from Drying-Induced Damage in Routinely Processed Tissue Sections._ J Histochem Cytochem, 2015. 64\(1): p. 18-31 doi: 10.1369/0022155415616162.

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Figures

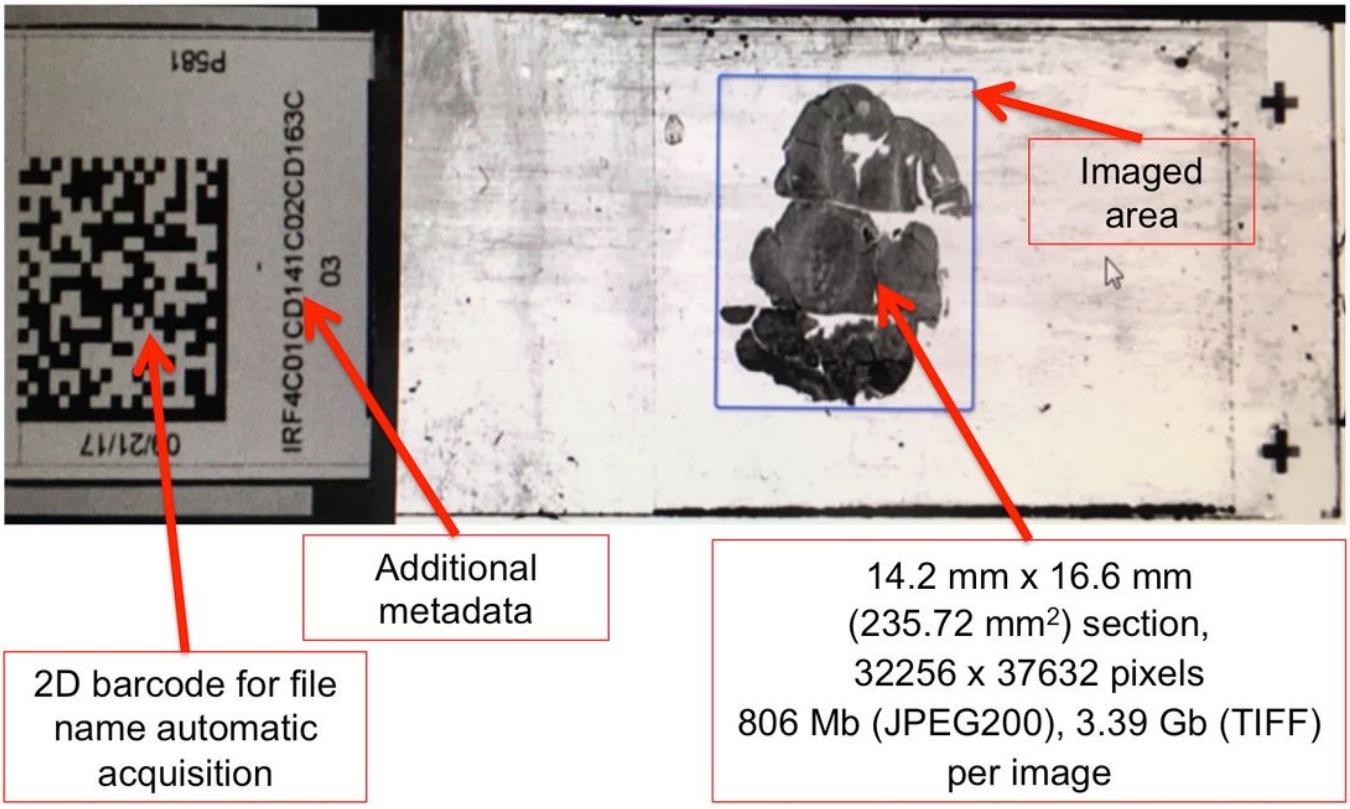

Fig. 1: slide setup for MILAN multiplexing

Figure 1

Slide setup for MILAN multiplexing.

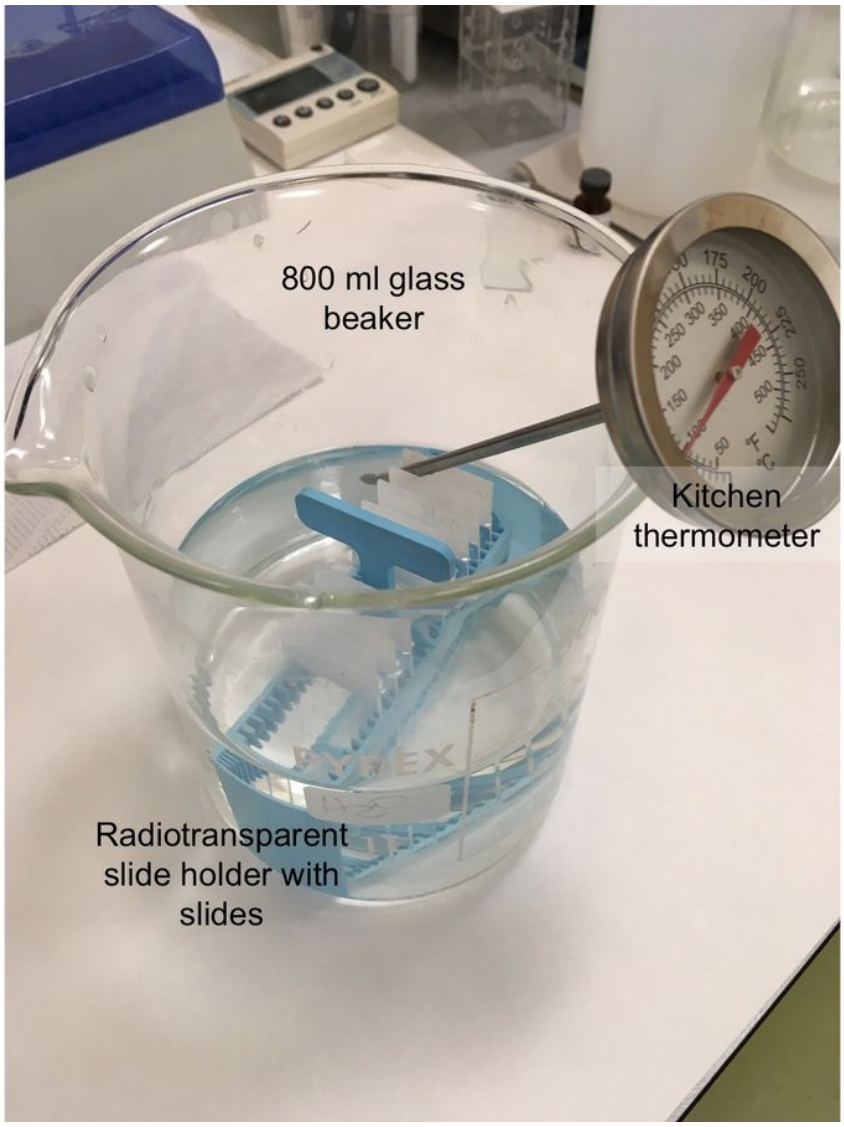

Fig. 2: AR setup for MILAN multiplexing.

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Figure 2

Antigen Retrieval setup for MILAN multiplexing

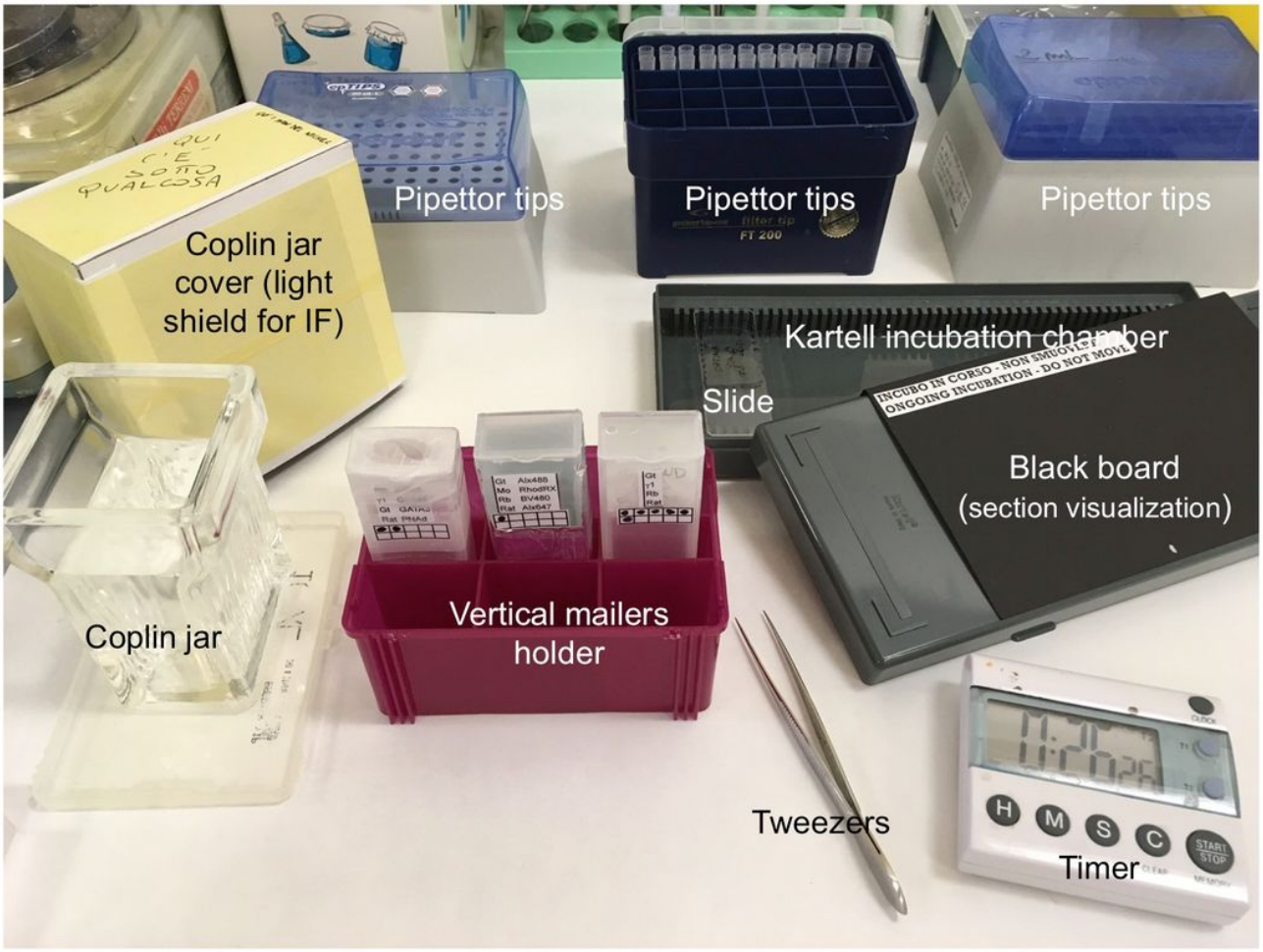

Fig. 3: bench setup for MILAN multiplexing

Figure 3

Bench setup for MILAN multiplexing

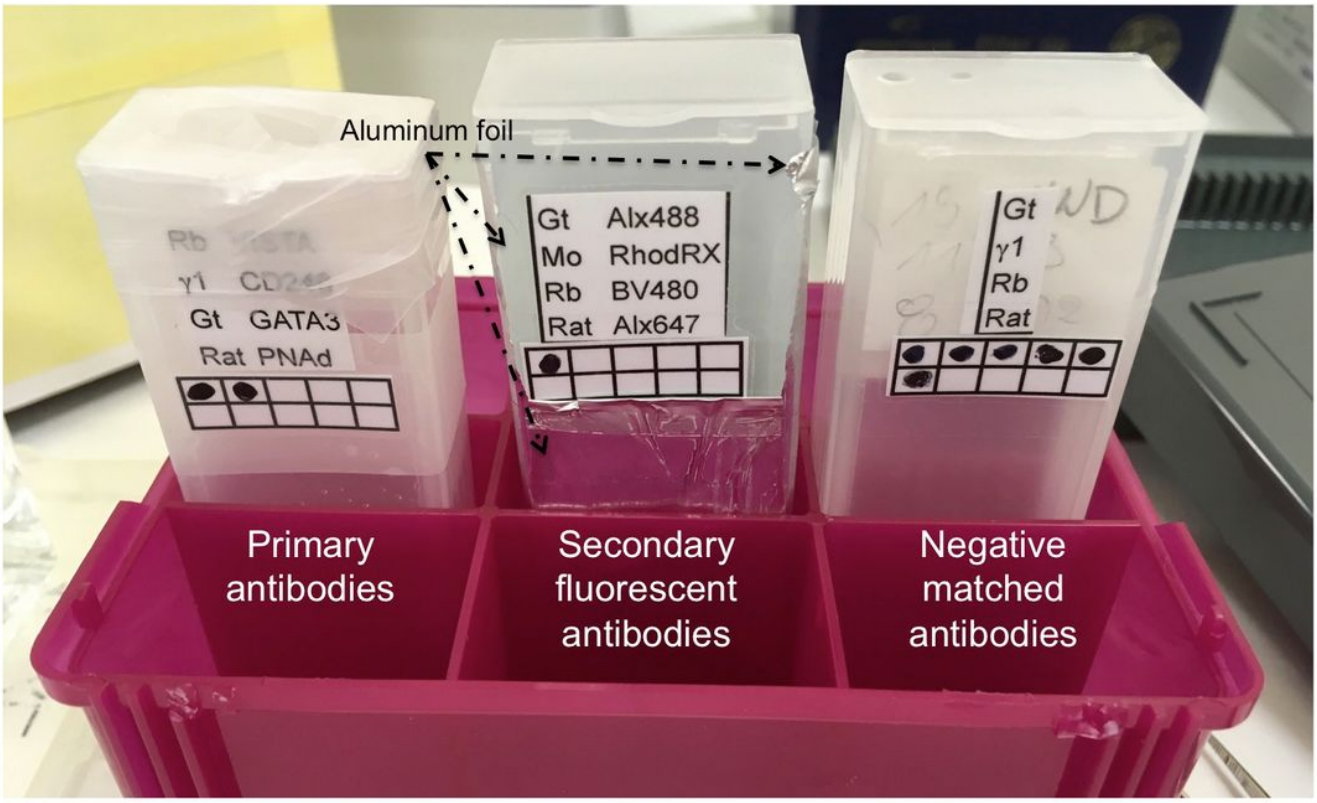

Fig. 4: vertical mailers setup for MILAN multiplexing.
Each mailer contains four 1st Abs, 2nd Abs and isotype- and species-matched negative Abs. Note the grid to record the number of 5-slide runs each mailer has undergone. Max runs for 2nd Abs is five (2 passages each).This mailer is wrapped in aluminum foil.

Figure 4

Vertical mailers setup for MILAN multiplexing Each mailer contains four 1st Abs, 2nd Abs and isotype- and species-matched negative Abs. Note the grid to record the number of 5-slide runs each mailer has undergone. Max runs for 2nd Abs is five (2 passages each).This mailer is wrapped in aluminum foil.

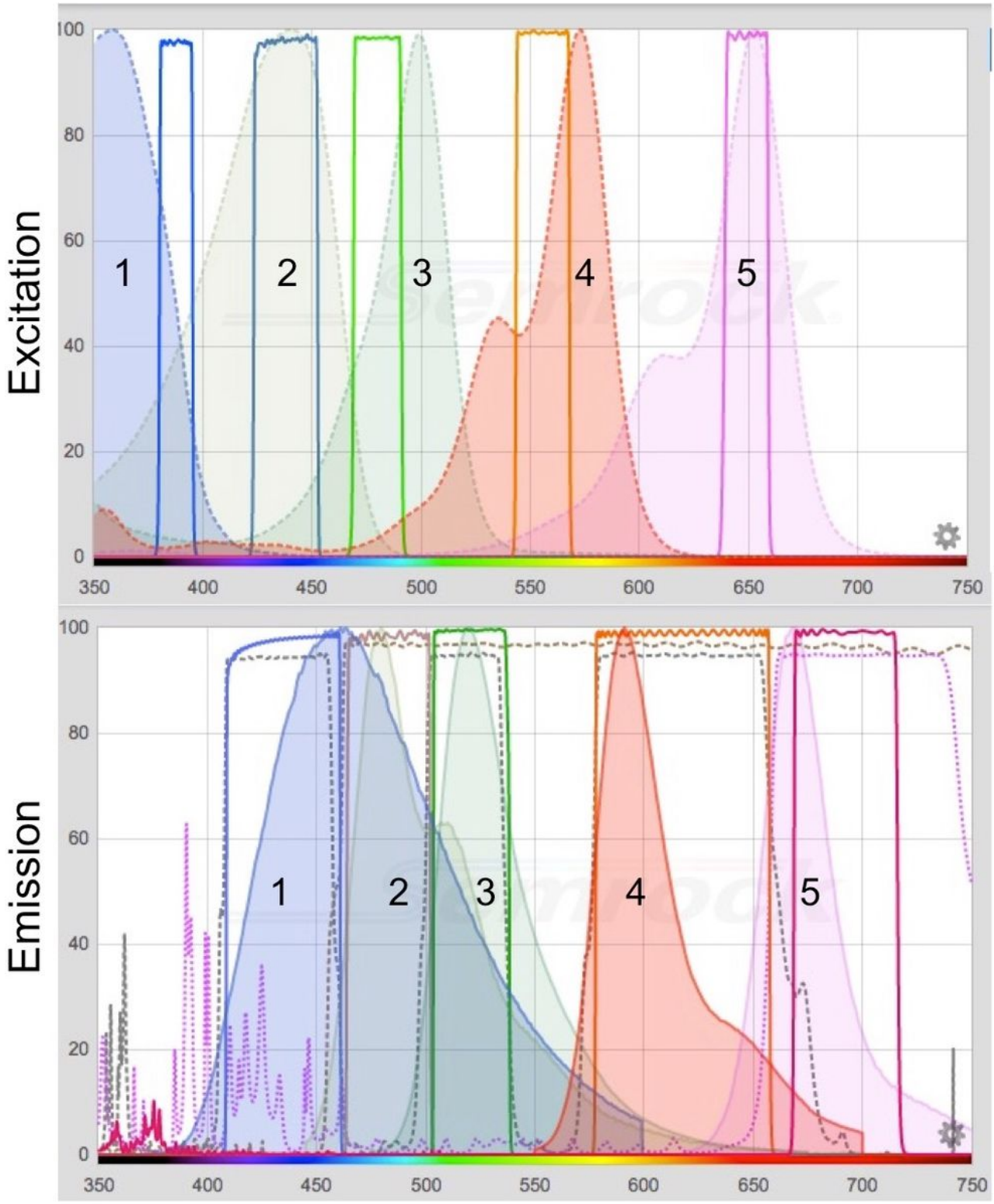

Figure 5

Six-color filter combination for multiplexing The figure represents filter combinations, housed in two 6-filter wheels and three turrets, to allow six independent fluorochrome acquisitions. The reference instrument is a S60 scanner from Hamamatsu. The composite panel represents the excitation filters and fluorochrome spectra (top) and the emission filters, dichroic mirrors and fluorochrome spectra (bottom). Excitation spectra are represented by a dashed profile, emission spectra by a solid profile. The filter

profiles are represented by solid lines, the dichroic ones by a dashed line. *1*: DAPI (359/461) [exc/em]; *2*: BV480 (437/478); *3*: Alexa 488 (499/519); *4*: Rhodamine RedX (570/590); *5*: Alexa 647 (652/668). The filter combination depicted are *DAPI*: 387/11- 435/40 [exc/em], *BV480*: 438/24 - 483/32; *FITC*: 480/17 - 520/28; *TRITC*: 556/20 - 617/73; *Cy5*: 650/13 - 694/44; *AutoFluorescence (AF)*: 438/24 - 617/73. The dichroic mirrors are: FF403/497/574-Di01 (triband), 458-Di02 and FF655-Di01. These filters can be obtained by companies like Semrock or Chroma. Alexa ® dyes are a Life Technologies patent. BV480 dye is a BD Biosciences patent. The spectra images are obtained with the Searchlight Semrock web application.

Figure 6

The MILAN logo. Please tag your posters, presentations and publications with this logo, documenting the source of the technique. The logo was created by Veerle Haemels, Phd Student, Laboratory for Precision Cancer Medicine - www.lpcm.be, Translational Cell and Tissue Unit, Department of Imaging and Pathology, KU Leuven, Herestraat 49, B - 3000 Leuven

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Immunoglobulins.xlsx
- PrimaryAntibodiesv5.xlsx
- SecondaryAbs.xlsx
- PinocchioBoxv4.xlsx