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Akoya Phenocycler-Fusion Tissue Staining and Imaging Protocol for Formalin-Fixed Paraffin-Embedded Samples adapted from Indiana University

Forked from Indiana University adapted Akoya Phenocycler-Fusion Tissue Staining and Imaging Protocol for Fresh Frozen Kidney Samples

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We use this protocol and it's working

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Abstract

This protocol presents the Yale University TMC Akoya Phenocycler-Fusion Tissue Staining & Imaging Protocol for Formalin-Fixed Paraffin-Embedded Human Lymph Node Samples. This protocol was adapted from Indiana University adapted Akoya Phenocycler-Fusion Tissue Staining and Imaging Protocol for Fresh Frozen Kidney Samples.
This protocol has been used for FFPE human lymph node samples for the Cellular Senescence Network (SenNet) Program and the Human BioMolecular Atlas Program consortia. The size of the marker panels has ranged between 35 to 47 targets and includes cell type markers for B cells, T cells, macrophages, and other immune cell types. that will label various cell types Multiple sections have been placed on a single slide and imaged simultaneously.

Materials

Akoya Biosciences Phenocycler-Fusion and Staining Kit
Antibodies of choice
Coplin jars
Methanol

Tissue Baking - Day 1

12h

1 Bake tissue slides in a 60°C drying oven overnight for optimal tissue adherence to the glass slide. For some tissue samples, baking at 60°C for 1 hour will be sufficient to stick to the glass slide.

- For optimal use of reagents, 2-3 tissue slides are prepared in one batch.

Tissue deparaffinization and rehydration

1h

2 Locate and/or label 10 plastic Coplin jars and fill them with the following reagents: 5m
Two Coplin jars filled with Xylene (HistoChoice can be used as an alternative)
Two Coplin jars filled with 100% Ethanol
One Coplin jar filled with 90% Ethanol
One Coplin jar filled with 70% Ethanol
One Coplin jar filled with 50% Ethanol
One Coplin jar filled with 30% Ethanol
Two Coplin jars filled with double-distilled water

3 Remove tissue slides from the 60°C drying oven and proceed with the tissue deparaffinization steps. 1m

4 Place the tissue slide into the first Coplin jar filled with Xylene for 5 minutes. 5m

5 Remove the tissue slide from the first Coplin jar filled with Xylene and place it into the second Coplin jar filled with Xylene for 5 minutes. 5m

6 Remove the tissue slide from the second Coplin jar filled with Xylene and place it into the first Coplin jar filled with 100% ethanol for 5 minutes. 5m

7 Remove the tissue slide from the first Coplin jar filled with 100% ethanol and place it into the second Coplin jar filled with 100% ethanol for 5 minutes. 5m

8 Remove the tissue slide from the second Coplin jar filled with 100% ethanol and place it into the Coplin jar filled with 90% ethanol for 5 minutes. 5m

8.1 Remove the tissue slide from the Coplin jar filled with 90% ethanol and place it into the Coplin jar filled with 70% ethanol for 5 minutes. 5m

9 Remove the tissue slide from the Coplin jar filled with 70% ethanol and place it into the Coplin jar filled with 50% ethanol for 5 minutes. 5m

10 Remove the tissue slide from the Coplin jar filled with 50% ethanol and place it into the Coplin jar filled with 30% ethanol for 5 minutes. 5m

11 Remove the tissue slide from the Coplin jar filled with 30% ethanol and place it into the first Coplin jar filled with water for 5 minutes. 5m

12 Remove the tissue slide from the first Coplin jar filled with water and place it into the second Coplin jar filled with water for 5 minutes. 5m

Antigen retrieval and pre-staining steps 2h

13 Akoya Biosciences provides two antigen retrieval buffers: AR6 (Citrate buffer, pH 6) and AR9 (Tris EDTA buffer, pH 9). The choice of antigen retrieval buffer depends on the target antigen. In our experience, AR9 buffer works optimally for most antigens.

A pressure cooker is used for antigen retrieval. Alternatives could be a microwave or a hot plate.

14 Prepare 50 ml 1X AR9 buffer from the stock 10X AR9 buffer. (Dilute with water)

15 Place the slide into the 1X AR9 buffer in and seal the Coplin jar.
Fill the pressure cooker with water to approximately a quarter or a third full.

16 Place the coplin jar into the pressure cooker and set the timer for 20 mins at high pressure. 20m

17 After 20 minutes, turn the pressure valve on the pressure cooker to release pressure.
Apply caution since high temperatures could potentially lead to burns

18 Remove the Coplin jar with the slide from the pressure cooker and allow it to cool to room temperature for 30 minutes to 1 hour. 1h

19 While the coplin jar cools to room temperature, locate and/or label 5 plastic coplin jars and fill them with the following reagents:
Two Coplin jars filled with double-distilled water
Two Coplin jars filled with hydration buffer
One Coplin jar filled with staining buffer

20 Move the slide into the first coplin jar filled with water and dip the slide 2-3 times to remove the AR9 buffer solution. 1m
- this is not an incubation step, just a quick rinse

21 Move the slide into the next coplin jar filled with water and incubate the slide for 2 minutes to thoroughly remove any remaining AR9 buffer solution. 2m

22 Move the slide into the first Hydration Buffer coplin jar and incubate the slide for 2 minutes. 2m

23 Move the slide into the second Hydration Buffer coplin jar and incubate the slide for another 2 mins. 2m

24 Place slide in Staining Buffer and incubate for 20-30 minutes 30m
- timing depends on how quickly the antibody cocktail can be made

25 During the Staining Buffer incubation, prepare the Antibody Cocktail

Preparing Antibody Cocktail

26 Remove the selected antibodies, spin down if necessary, and place on ice

27 Prepare the appropriate volume of Blocking Buffer for the number of slides being stained
*See attached file for Blocking Buffer components

FILE CODEX_Master_Mix.xlsx 18KB

28 Label one tube for each **unique** Antibody Cocktail being prepared

28.1 The FINAL volume for each tube should be 200 uL - counting antibodies. Remove 1 uL of Blocking Buffer per 1 uL of antibody being added.
- Amounts will vary depending on experiment and desired antibody concentration

29 Add the desired amount of antibodies to the appropriate tubes

- Our concentrations are listed in the attached table. Antibody clones and vendors available upon request CODEX_Master_Mix.xlsx 18KB

30 Pipette gently to mix the solution

Tissue Staining

3h

31 Optional step: Cut a rectangular piece of parafilm that is approximately the same size as the sample slide

32 Pre-load a pipette with 190 uL of the prepared Antibody Cocktail

33 Remove slide from Staining Buffer and use a kimwipe to gently dry the slide, without touching the tissue sample(s)

34 Place slide on the humidity chamber, tissue side up, and dispense the Antibody Cocktail
- make sure the tissue sample is completely covered, and there are no air bubbles present

35 Optional step: Gently place parafilm over tissue sample

36 Incubate Sample Slide at room temperature for 3 hours.

3h

FFPE Tissue post-staining

37 Locate and/or label the following plastic coplin jars:
2 x Staining Buffer
1 x Post-Stain Fixative
1 x 100% Methanol
4 x PBS (1x concentration)
1 x CODEX Buffer + Buffer Additive (1x concentration)

38 Fill Methanol coplin jar and place in freezer until needed

Post Staining

51m

39 If parafilm steps were included, gently remove parafilm from sample slide

40 Place sample slide in Staining Buffer coplin jar and incubate for 2 minutes
- Dip slide 2-3 times to ensure Antibody Cocktail is fully washed away

2m

41 Place sample slide in the second Staining Buffer coplin jar and incubate for 2 minutes

2m

41.1 During this incubation, prepare the Post-Staining fixative solution
- For one coplin jar, use 36 mL of storage buffer and 4 mL of fresh 16% PFA

42 Move slide into the Post-Stain coplin jar and incubate for 10 minutes

10m

43 Remove slide from the Post-Stain fix and wash in the first PBS coplin jar
- this is not an incubation step, just a few quick dips to rinse the tissue

30s

44 Repeat step 43 in the second and third PBS coplin jars

30s

45 With slide still in the third PBS coplin jar, remove Methanol coplin jar from the freezer

46 Place slide in the ice cold Methanol for 5 minutes

5m

47 After Methanol incubation, immediately place slide in the first PBS coplin jar to rinse
- As in step 43, this is not an incubation, just a quick rinse

48 Repeat step 43 with the second and third PBS coplin jars

49 With the slide still in the third PBS coplin jar, prepare the final fixative solution
- for up to 5 slides, use 1000 uL of 1xPBS and 20 uL of Fixative Reagent

50 Preload a pipette with 200 uL of final fixative solution

51 Remove sample slide from the third PBS coplin jar and place in the humidity chamber, tissue side up

52 Dispense 200 uL of Final Fixative solution and incubate for 20 minutes 20m

53 After the 20 minute incubation, rinse the slides in each of the three PBS coplin jars, as was done previously. 1m

54 There are two options for how to proceed next:
a) if imaging is not taking place immediately, sample slide(s) can be stored in Storage Buffer at 4C for up to 5 days.
b) if imaging is taking place the same day staining is finished, proceed to step 45

Cover-slipping the Slide for Imaging 10m 30s

55 Place slide to be cover-slipped in a fresh jar of 1x PBS and incubate for 10 minutes 10m

56 After the 10 minute incubation, carefully dry the slide with a kimwipe, so the coverslip can adhere properly

57 Place coverslip - sticky side up - on the stage. Make sure the coverslip is straight, as overhang will create an inadequate seal or cause the slide to not fit in the flow cell

58 Gently place the slide - tissue side facing the sticky side of the coverslip - on the stage, making sure the slide is lined up with the coverslip

59 Push the stage under the pressure arm, lower the arm, and let rest with for 30 seconds 30s

60 Place the now cover-slipped slide into 1x CODEX Buffer for 10 minutes to allow for equilibration and proper adherence of coverslip to take place. 10m

Preparing the Reporter Plate

61 Prepare the reporter plate stock solution as described in the attached spreadsheet.

62 There will be one unique well per cycle, including the blanks. Each well gets a different volume of stock solution based on the amount of reporter to be added. Final volume of each well should total 250 uL.
In general, the volumes are as follows, per number of reporters being added
3 reporters -> 235 uL of stock solution
2reporters -> 240 uL of stock solution
1 reporter -> 245 uL of stock solution
0 reporters -> 250 uL of stock solution (typically the two blank cycles)

62.1 The stock solution for the first and last cycles go into wells H1 and H2, respectively.
Stock solution + reporters will start in A1 and continue in order until all cycles are accounted for.

*An example of a reporter plate set up is shown in the attached spreadsheet

CODEX_Master_Mix.xlsx 18KB

63 Once all the necessary wells are filled, cover wells with foil plate seal to protect from debris and prevent the wells from drying out

*we have prepared reporter plates up to a week in advance, but generally try to prepare them no sooner than the day before an imaging session

Imaging the Slides

64 When reporter plates and slides are ready to use, follow the instructions on the Phenocycler-Fusion to begin the imaging experiment. An example of our experimental settings is shown in the attached spreadsheet.

Imaging Parameters.xlsx

Analysis Options Available

65 Segmentation and analysis for each sample are conducted using Qupath software (**https://qupath.github.io/**).

Downstream analysis following cell segmentation and phenotyping is done using Seurat in a custom R Studio script that can be made available.