MAR 24, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.8epv5jdbnl1b/v1

Protocol Citation: Angela R. Sabo, Connor J Gulbronson, Daria Barwinska, Michael J. Ferkowicz, William Bowen, Seth Winfree, Tarek Ashkar 2023. Indiana University adapted Akoya Phenocycler-Fusion Tissue Staining and Imaging Protocol for Fresh Frozen Kidney Samples. **protocols.io** https://dx.doi.org/10.17504/protocols.io.8epv5jdbnl1b/v1

Protocol status: Working
We use this protocol and it's working

Created: Feb 13, 2023

Last Modified: Mar 24, 2023

PROTOCOL integer ID:
76905

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

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ABSTRACT

This protocol presents the Indiana University adapted Akoya Phenocycler-Fusion Tissue Staining & Imaging Protocol for Fresh Frozen Kidney Samples that is used both for the Kidney Precision Medicine Project and the Human BioMolecular Atlas Program consortia. The marker panels spanned 41 targets that will label various cell types (epithelial, immune, stromal) and cell states (injury, cycling, repair). This protocol has been used on tissues up to 5-7mm x 12-15 mm in dimension. Multiple sections have been placed on a single slide and imaged simultaneously. For post-imaging H & E Staining please see dx.doi.org/10.17504/protocols.io.kqdg397yeg25/v1 .

MATERIALS

Akoya Biosciences Phenocycler-Fusion and Staining Kit
Antibodies of choice

Setup - Day 1

1 Prepare humidity chamber - we use an empty 1000uL tip box and place water and a wet paper towel under the tray.

2 Prepare Drierite absorbent beads - locate a second empty box with a lid and add an even layer of beads

3 Get an ice bucket - for use later when preparing Antibody Cocktail

4 Locate and/or label 5 plastic coplin jars for the following reagents:
1 x Acetone
2 x Hydration Buffer
1 x Pre-stain fixative
1 x Staining Buffer

5 Prefill the acetone, hydration buffer, and staining buffer jars

Preparing Tissue for Staining

1h 2m

6 Remove sample slides from the -80 freezer and immediately place on Drierite beads for 5 minutes - make sure slide is tissue-side up

5m

7 Remove slide from Drierite beads and place in Acetone coplin jar for 10 minutes

10m

8 Place slide in the humidity chamber to dry for 2 minutes

2m

9 Place slide in the first Hydration Buffer coplin jar and incubate for 2 minutes 2m
- Dip slides 2-3 times to make sure acetone is rinsed off adequately

10 Place slide in the second Hydration Buffer coplin jar and incubate for 2 minutes 2m

10.1 During this incubation, prepare the Pre-staining Fixative solution
- For one coplin jar, use 36 mL of hydration buffer and 4 mL of fresh 16% PFA

11 Move slide into the Pre-staining fixative solution coplin jar and incubate for 10 minutes 10m

12 Move slide back into the first Hydration Buffer coplin jar and dip slide 2-3 times to remove the fixative solution 30s
- this is not an incubation step, just a quick rinse

13 Move into the second Hydration Buffer coplin jar and dip slide 2-3 times to remove the fixative solution 30s
- this is not an incubation step, just a quick rinse

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

15 During the Staining Buffer incubation, prepare the Antibody Cocktail

Preparing Antibody Cocktail

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

17 Prepare appropriate volume of Blocking Buffer for the number of slides being stained
*See attached file for Blocking Buffer components
MasterMixTable.xlsx

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

18.1 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

19 Add the desired amount of antibodies to the appropriate tubes
- Our concentrations are listed in the attached table. Antibody clones and vendor available upon request
MasterMixTable.xlsx

20 Pipette gently to mix the solution

Tissue Staining

1d

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

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

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

24 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

25 Optional step: Gently place parafilm over tissue sample

26 Incubate Sample Slide overnight at 4C
- We place our samples in a cold room to ensure there is no disturbance

1d

Set Up - Day 2

27 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)

28 Fill Methanol coplin jar and place in freezer until needed

Post Staining

51m

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

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

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

31.1 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

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

33 Remove slide from the Post-Stain fix and wash in the first PBS coplin jar 30s
- this is not an incubation step, just a few quick dips to rinse the tissue

34 Repeat step 33 in the second and third PBS coplin jars 30s

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

36 Place slide in the ice cold Methanol for 5 minutes 5m

37 After Methanol incubation, immediately place slide in the first PBS coplin jar to rinse
- As in step 33, this is not an incubation, just a quick rinse

38 Repeat step 37 with the second and third PBS coplin jars

39 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

40 Preload a pipette with 200 uL of final fixative solution

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

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

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

44 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

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

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

47 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

48 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

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

50 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

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

52 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)

52.1 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

MasterMixTable.xlsx

53 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

54 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 (exposures and cycle set up) is shown in the attached spreadsheet.

Imaging Parameters.xlsx

Analysis Options Available

55 Analysis conducted by IU follows the pipeline(s) described in the following papers:
- Winfree et al 2017
- Ferkowicz et al 2020
- Winfree et al 2023
(links are listed in references tab)

Segmentation and cytometry analysis for each sample is conducted using Volumetric Tissue Exploration and Analysis (VTEA, https://vtea.wiki/).

A pipeline for combining individual sample datasets into one analytical space using R Studio will be available soon.