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Preparation, Deparaffinization, Antigen Retrieval and Staining of Human FFPE Kidney Tissue for Akoya Phenocycler Fusion Multiplex Analysis- Indiana University

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We use this protocol and it's working

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Abstract

This protocol describes the method for preparation and staining of FFPE kidney tissue on slides for Akoya Phenocycler-Fusion multiplex staining using barcoded antibodies. Included are the protocols for tissue preparation, sectioning, deparaffinization, antigen retrieval and tissue staining.

Purpose: Prepare FFPE tissue and perform multiplex antibody staining for Akoya Phenocycler-Fusion Imaging.

Guidelines

Procedure:

Tissue Cutting:
1) 5 µm sections are cut on Leica Histocore microtome and mounted on Fisher Superfrost Plus Gold slides (2×75mm) by floating on 42 °C water bath.
2) Slides are dried by propping at ~75° slant over-night on Kimwipes.

Deparaffinization and Antigen Retrieval:
3) When ready to deparaffinize, dry slide(s) at 60 °C on slide dryer for 1 hr.
4) Slides are deparaffinized and antigens retrieved according to Akoya Phenocycler Fusion User Guide v2.1.0 (https://www.akoyabio.com/support/) with xylene substituted for HistoChoice Clearing Agent:
a. Sequentially dip paraffin section slides in vertical slide holder in ~250 mL of the following fresh solvent solutions:
i. 100% Xylenes – 10 min
ii. 100% Xylenes – 10 min
iii. 100% Xylenes – 10 min
iv. 100% Ethanol/Reagent Alcohol – 10 min
v. 100% Ethanol/Reagent Alcohol – 10 min
vi. 100% Ethanol/Reagent Alcohol – 10 min
vii. 90% Ethanol/Reagent Alcohol – 5 min
viii. 70% Ethanol/Reagent Alcohol – 5 min
ix. 50% Ethanol/Reagent Alcohol – 5 min
x. 30% Ethanol/Reagent Alcohol – 5 min
xi. ddH2O – 5 min
xii. Citrate antigen retrieval buffer - while pressure cooker heats up (~10-15 min.)
5) Antigen retrieval is carried out by incubating slides in slide rack (covered with aluminum foil and lid) with citrate buffer in a TintoRetriever pressure cooker with 1 L ddH2O at a setting of 115 °C for 15 minutes.
6) After pressure comes down, remove slide rack with slide(s) and cool on bench top for 30 min.
7) Move slide(s) to coplin jar with 40 mL ddH2O and incubate for 5 min.
8) Move slide(s) to coplin jar filled with 1X PBS (pH 7.2) and incubate for 2 min.
9) Move slide(s) to a second coplin jar filled with 1X PBS (pH 7.2) and incubate for 2 min.
10) Move slide(s) to coplin jar with 40 mL 1X Akoya Fusion Hydration buffer and incubate for 2 min.
11) Move slide(s) to second coplin jar with 40 mL 1X Akoya Fusion Hydration buffer and incubate for 2 min.
12) Move slide(s) to coplin jar with 40 mL 1X Akoya Staining buffer and incubate for 20-30 min while preparing Antibody Staining Cocktail.

Preparation of Antibody Cocktail and Tissue Staining
13) Prepare a solution of Phenocycler Fusion Antibody Cocktail Solution (400 µL for each slide) according to Akoya FFPE protocol with previously developed conjugated antibody panel:

Phenocycler Fusion Blocking Buffer

- Staining Buffer [µL] 362
- N Blocker [µL] 9.5
- G2 (V3) Blocker [µL] 9.5
- J Blocker [µL] 9.5
- S Blocker [µL] 9.5
- Total [µL] 400

14) Add up the total volume of conjugated Phenocycler antibodies needed for staining each sample and remove that volume from the 400 µL of prepared Blocking buffer.
15) Add the appropriate volume of each antibody in the panel to the remaining blocking buffer solution.
16) Remove slide(s) from coplin jar, dry edges around tissue and slide back with kimwipe and place upright in humidity chamber.
17) Add 400 µL of Antibody Cocktail solution to tissue sections of each slide and incubate overnight at 4 °C.

Wash and Fix Stained Tissue
18) The following day, remove slide(s) from humidity chamber to a coplin jar with 40 mL of Phenocycler Staining buffer and incubate for 2 min.
19) Move slide(s) to second coplin jar with 40 mL Staining buffer and incubate for 2 min.
20) Move slide(s) to a coplin jar with 40 mL 1.6% PFA in Phenocycler Storage Buffer and incubate for 10 min.
21) Wash slide(s) in coplin jar with 40 mL 1X PBS by dipping 3 times.

22) Repeat washes with 3 dips in coplin jar with 1X PBS two more times.
23) Transfer slide(s) to a coplin jar with 40 mL ice-cold methanol and incubate for 5 min. on ice.
24) Wash slide(s) 3X in coplin jars with 40 mL 1X PBS by dipping 3 times as above.
25) Leave slide(s) in last 1X PBS wash and prepare Phenocycler Final Fixative solution by adding 20 µL Phenocycler Fixative Reagent to 1 mL 1X PBS.
26) Remove slide(s) from 1X PBS, dry back and edges with Kimwipe, and place in humidity chamber.
27) Add ~400 µL Fixative solution to the tissue on each slide and incubate for 20 min at room temperature.
28) Remove the slide(s) from humidity chamber and repeat 3X washes in coplin jars with 1X PBS as above, dipping 3 times in each jar.
29) Transfer slides to coplin jar with 40 mL Phenocycler Storage buffer or begin process of imaging according to Akoya Phenocycler Fusion Manual.

Materials

Reagents and consumables listed on these pages:
- Tissue Path Superfrost Plus Gold slides (1518848)
- 20X Citrate Buffer (BioSB: BSB0020)
- 100% Xylenes (Fisher Chemical: X3P-1GAL)
- 100% Ethanol (Avantik: RS4029)
- ddH2O
- 1X PBS (pH 7.2)
- 1X Akoya Fusion Hydration Buffer
- 1X Akoya Staining Buffer
- Phenocycler Fusion Antibody Cocktail Solution (prepare 400 µL per slide)
- Phenocycler Fusion Blocking Buffer components (table below)
- 1.6% PFA in Phenocycler Storage Buffer
- Methanol (ice-cold) for post-staining dehydration step
- Phenocycler Fixative Reagent (for preparation of Phenocycler Final Fixative solution)

Phenocycler Final Fixative solution: prepare by adding 20 µL Phenocycler Fixative Reagent to 1 mL 1X PBS (prepare ~400 µL Fixative solution per slide and add ~400 µL to each slide during fixation step).

Equipment and supplies mentioned:
- Leica Histocore microtome
- Fisher Superfrost Plus Gold slides (2×75mm)
- 42 °C water bath (for floating sections)
- Slide dryer (capable of 60 °C)
- Kimwipes
- Vertical slide holder and slide rack
- Coplin jars (multiple)
- TintoRetriever pressure cooker (or equivalent)
- Aluminum foil and lid for covering slide rack
- Humidity chamber

Phenocycler Fusion Blocking Buffer (as shown in table):
- Staining Buffer [µL]: 362
- N Blocker [µL]: 9.5
- G2 (V3) Blocker [µL]: 9.5
- J Blocker [µL]: 9.5
- S Blocker [µL]: 9.5
- Total [µL]: 400

Troubleshooting

Before start

Preparatory notes appearing on these pages:
- Purpose: Prepare FFPE tissue and perform multiplex antibody staining for Akoya Phenocycler Fusion Imaging.
- Prepare citrate antigen retrieval buffer (20X stock indicated) and preheat pressure cooker (allow ~10-15 min to heat up) prior to antigen retrieval.
- Ensure slides (Fisher Superfrost Plus Gold) are ready and microtome set to cut 5 µm sections; float sections on 42 °C water bath and dry slides propped at ~75° slant overnight on Kimwipes.
- Dry slides at 60 °C on slide dryer for 1 hr immediately prior to deparaffinization.
- Prepare Phenocycler Fusion Blocking Buffer and plan Antibody Cocktail volumes (400 µL per slide) before starting staining steps.

Tissue Cutting

1 5 µm sections are cut on Leica Histocore microtome and mounted on Fisher Superfrost Plus Gold slides (2×75mm) by floating on 42 °C water bath.

Tissue Cutting

2 Slides are dried by propping at ~75° slant over-night on Kimwipes.

Deparaffinization and Antigen Retrieval

4h 8m

3 When ready to deparaffinize, dry slide(s) at 60 °C on slide dryer for 1 hr. 1h

4 Slides are deparaffinized and antigens retrieved according to Akoya Phenocycler Fusion User Guide v2.1.0 (https://www.akoyabio.com/support/) with xylene substituted for HistoChoice Clearing Agent:

4.1 Sequentially dip paraffin section slides in vertical slide holder in ~250 mL of the following fresh solvent solutions:

4.2 100% Xylenes – 10 min 10m

4.3 100% Xylenes – 10 min 10m

4.4 100% Xylenes – 10 min 10m

4.5 100% Ethanol/Reagent Alcohol – 10 min 10m

4.6 100% Ethanol/Reagent Alcohol – 10 min 10m

4.7 100% Ethanol/Reagent Alcohol – 10 min 10m

4.8 90% Ethanol/Reagent Alcohol – 5 min 5m

4.9 70% Ethanol/Reagent Alcohol – 5 min 5m

4.10 50% Ethanol/Reagent Alcohol – 5 min 5m

4.11 30% Ethanol/Reagent Alcohol – 5 min 5m

4.12 ddH2O – 5 min 5m

4.13 Citrate antigen retrieval buffer - while pressure cooker heats up (~10-15 min.) 15m

5 Antigen retrieval is carried out by incubating slides in slide rack (covered with aluminum foil and lid) with citrate buffer in a TintoRetriever pressure cooker with 1 L ddH2O at a setting of 115 °C for 15 minutes. 15m

6 After pressure comes down, remove slide rack with slide(s) and cool on bench top for 30 min. 30m

7 Move slide(s) to coplin jar with 40 mL ddH2O and incubate for 5 min. 5m

8 Move slide(s) to coplin jar filled with 1X PBS (pH 7.2) and incubate for 2 min. 2m

9 Move slide(s) to a second coplin jar filled with 1X PBS (pH 7.2) and incubate for 2 min. 2m

10 Move slide(s) to coplin jar with 40 mL 1X Akoya Fusion Hydration buffer and incubate for 2 min. 2m

11 Move slide(s) to second coplin jar with 40 mL 1X Akoya Fusion Hydration buffer and incubate for 2 min. 2m

12 Move slide(s) to coplin jar with 40 mL 1X Akoya Staining buffer and incubate for 20-30 min while preparing Antibody Staining Cocktail. 30m

Preparation of Antibody Cocktail and Tissue Staining

13 Prepare a solution of Phenocycler Fusion Antibody Cocktail Solution (400 µL for each slide) according to Akoya FFPE protocol with previously developed conjugated antibody panel:

Phenocycler Fusion Blocking Buffer	
Staining Buffer [µL]	362
N Blocker [µL]	9.5
G2 (V3) Blocker [µL]	9.5
J Blocker [µL]	9.5
S Blocker [µL]	9.5
Total [µL]	400

14 Add up the total volume of conjugated Phenocycler antibodies needed for staining each sample and remove that volume from the 400 µL of prepared Blocking buffer.

15 Add the appropriate volume of each antibody in the panel to the remaining blocking buffer solution.

16 Remove slide(s) from coplin jar, dry edges around tissue and slide back with kimwipe and place upright in humidity chamber.

17 Add 400 µL of Antibody Cocktail solution to tissue sections of each slide and incubate overnight at 4 °C.

Wash and Fix Stained Tissue 45m

18 The following day, remove slide(s) from humidity chamber to a coplin jar with 40 mL of Phenocycler Staining buffer and incubate for 2 min. 2m

19 Move slide(s) to second coplin jar with 40 mL Staining buffer and incubate for 2 min. 2m

20 Move slide(s) to a coplin jar with 40 mL 1.6% PFA in Phenocycler Storage Buffer and incubate for 10 min. 10m

21 Wash slide(s) in coplin jar with 40 mL 1X PBS by dipping 3 times. 3m

22 Repeat washes with 3 dips in coplin jar with 1X PBS two more times. 1m

23 Transfer slide(s) to a coplin jar with 40 mL ice-cold methanol and incubate for 5 min on ice. 5m

24 Wash slide(s) 3X in coplin jars with 40 mL 1X PBS by dipping 3 times as above. 1m

25 Leave slide(s) in last 1X PBS wash and prepare Phenocycler Final Fixative solution by adding 20 µL Phenocycler Fixative Reagent to 1 mL 1X PBS.

26 Remove slide(s) from 1X PBS, dry back and edges with Kimwipe, and place in humidity chamber.

27 Add ~400 µL Fixative solution to the tissue on each slide and incubate for 20 min at room temperature. 20m

28 Remove the slide(s) from humidity chamber and repeat 3X washes in coplin jars with 1X PBS as above, dipping 3 times in each jar. 1m

29 Transfer slides to coplin jar with 40 mL Phenocycler Storage buffer or begin process of imaging according to Akoya Phenocycler Fusion Manual.

Protocol references

Akoya Phenocycler Fusion User Guide v2.1.0 (https://www.akoyabio.com/support/) — xylene substituted for HistoChoice Clearing Agent as noted in protocol.