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Complete CO-Detection by IndEXing (CODEX) Protocol for FF and FFPE Tissues

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1 *Works for me* This protocol is published without a DOI.

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SUBMIT TO PLOS ONE

ABSTRACT

This protocol contains the necessary information to run an entire CODEX protocol from tissue staining through imaging.

PROTOCOL CITATION

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46862

MATERIALS TEXT

CODEX staining kit (Akoya, 7000008)
Nuclear stain (Akoya)
Assay Reagent (Akoya)
10x CODEX Buffer (Akoya, 7000001)
CODEX Storage Buffer (Akoya, 232107)
96 well plates, black (Akoya, 7000006)
96 well plate foil seals (Akoya, 7000007)
PLL-coated 22mm x 22mm glass coverslips
01.% Poly-L-lysine solution (Sigma, P8920-500ML)
22mm x 22mm glass coverslips (Electron Microscopy Sciences, 72200-10)
CODEX Gaskets (Akoya, 7000010)
DMSO
Xylene
100% Ethanol
100% Methanol
16% Paraformaldehyde (Electron Microscopy Sciences, 15710)
Dumont bent tip forceps (Electron Microscopy Sciences, 0304-5/15-PO)
CODEX stage insert
Drierite beads
Slide oven
Tinto Retriever Pressure Cooker (BioSB, BSB 7008)
Coverslip staining rack (Electron Microscopy Sciences, 72240)
Microcentrifuge
Citrate Buffer (Vector Laboratories, H-3300)
6 Well Plates

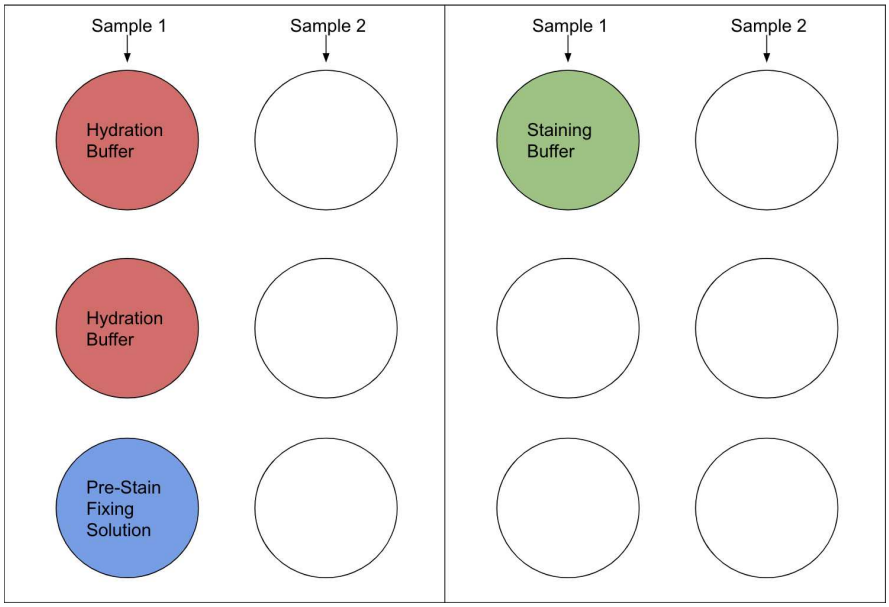
Pre-Staining (Fresh Frozen) 4h 15m

1 Prepare all reagents: 5m

Put all blockers (N, G, J, S) in an ice bucket to begin thawing.

Add 5 mL of Hydration and Staining Buffers to the 6-well plates to equilibrate to room temperature.

Prepare 5 mL **Pre-Stain** Fixing Solution per sample (0.5mL 16% PFA in 4.5 mL in **Hydration Buffer**) and add it to the 6-well plate.



This diagram is for 1 coverslip, repeat as necessary for total number of coverslips.

Retrieve coverslip from -80°C freezer and place tissue side up onto a bed of Drierite beads. 2m

2

Determine which side of the coverslip the tissue is located on by gently scraping the corner of the OCT layer. Mark your initials on the corner of the coverslip.

Let the coverslip sit on the beads for 2 minutes.

00:02:00

3 10m

Remove the coverslip from the Drierite beads.

Place in each coverslip in a beaker containing acetone with the tissue side facing up and incubate for 10 minutes.

00:10:00

Optional: place the coverslip in a coverslip staining rack and immerse in the acetone.

If the coverslip lays flat on the bottom of the beaker, the coverslip may suction to the beaker and become difficult to remove.

4 2m

Remove the coverslip from the acetone.

Place the coverslip on the humidity chamber, tissue side up, and let sit for 2 minutes.

00:02:00

5 2m

Place the coverslip in the first well (refer to plate configuration in step 1 if necessary) of Hydration Buffer, immerse 2-3 times to ensure complete removal of acetone, incubate the coverslip for 2 minutes.

00:02:00

6 2m

Place the coverslip into a second well containing 5 mL of Hydration Buffer, immerse 2-3 times, and incubate for another 2 minutes for a total of 2 washes.

00:02:00

7 10m

Place the coverslip in the well containing Pre-Staining Fixing Solution, immerse 2-3 times, and incubate for 10 minutes at RT.

00:10:00

8

Remove the coverslip from the Pre-Staining Fixing Solution.

Place the coverslip in the first well containing Hydration Buffer (used from the tissue hydration steps; Step 5). Lift and immerse the sample coverslip 2-3 times to ensure complete removal of the Pre-Staining Fixing Solution.

9

Place the coverslip to the second well containing Hydration Buffer and immerse 2-3 times.

10 Place the coverslip to well containing 5 mL Staining Buffer. 30m

Incubate coverslip for 20-30 minutes while preparing antibody cocktail.

00:30:00

11 Prepare Antibody Cocktail (Section 5.2) during Staining Buffer equilibration.

Remove selected antibodies from 4°C and keep them on ice until use. Spin down the tubes to collect any liquid from caps.

11.1 Prepare a stock solution of blocking buffer:

Blocking Buffer Stock Solution	1 sample
Staining Buffer	181 µL
N Blocker	4.75 µL
G Blocker	4.75 µL
J Blocker	4.75 µL
S Blocker	4.75 µL
Total Volume	200 µL

Prepare blocking buffer just before staining, **at maximum** 1 hour before. Multiply all values by n for n slips/samples.

Add blocking buffer to each of the antibody cocktail solution tubes, then add antibody. Mix by pipetting up and down only**, do not votex**.

The final volume of the Antibody Cocktail Staining Solution is a total of 200 µL per tissue.

Antibody dilutions we used are as recommended: 1 µl antibody per tissue, 1:200 ratio of each antibody. We remove blocking buffer volume equal to the total antibody volume.

For example: if we use 13 antibodies in our experiment, we add 187 µl of blocking buffer and 13 µl total of antibody (1 µl of each antibody) to make the Antibody Cocktail Staining Solution.

12 ⚠ 3h

Remove the coverslip from the staining buffer and place into the humidity chamber.

Add 190 µL of Antibody Cocktail to the center of the coverslip and ensure that the solution covers the entire tissue. Repeat for every slip.

Remove/minimize bubbles with a P2 pipette/forceps.

Place the lid on the humidity chamber once complete, and incubate for 3 hours.

03:00:00

The humidity chamber **must** be placed on a stable surface free of vibrations. If anything disturbs the surface tension of the staining droplet on the coverslip, the tissue could dry out.

13 Proceed to **Post-Stain (FF and FFPE)** section (**step 29**).

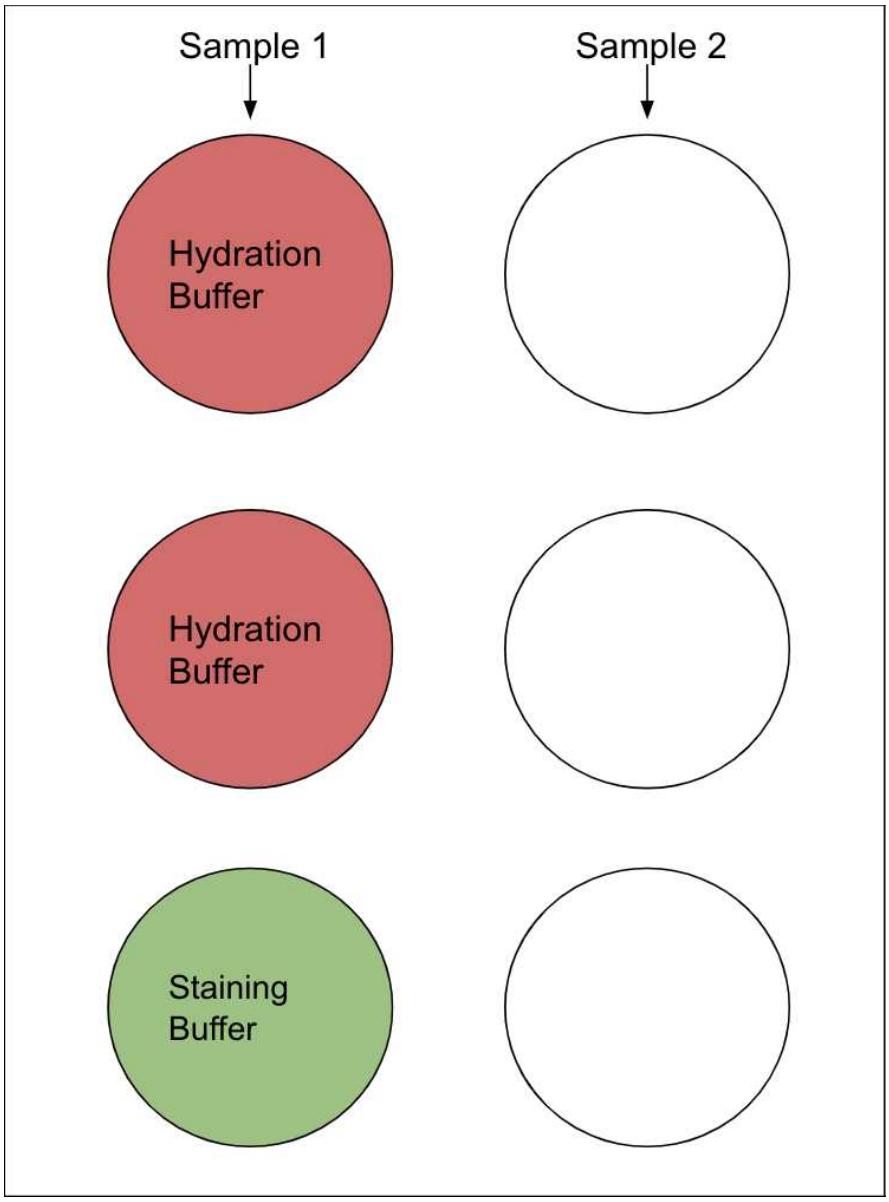
Pre-Staining (FFPE) 6h

14 Preheat slide oven and tray to 55°C 5m

Prepare all reagents:

Put all blockers (N, G, J, S) in an ice bucket to begin thawing.

Add 5 mL of Hydration and Staining Buffers to the 6-well plates to equilibrate to room temperature.



This configuration is for 1 coverslip only. Repeat as necessary depending on number of slips.

15 Heat coverslip in a slide oven at 55°C with tissue facing up for 25 minutes. 25m

00:25:00

We rest the coverslip on an angled slide baking rack. This helps the wax can drip off the edge while it is melting. This can easily be made by folding aluminum foil into shape.

16 After the wax has been sufficiently melted, place the coverslip on a coverslip staining rack and wait 1 minute to briefly cool down. 1m

00:01:00

17 Immerse the staining rack in the container containing the following reagents for 5 minutes each: 50m

1. Xylenes
2. Xylenes
3. 100% Ethanol
4. 100% Ethanol
5. 90% Ethanol
6. 70% Ethanol
7. 50% Ethanol
8. 30% Ethanol
9. ddH_2O
10. ddH_2O

00:50:00

Agitate the solution halfway through each step (at 2.5 minutes in each 5 minute cycle).

Preheat the pressure cooker at step 7
Fill the pressure cooker with water to the 6 cup line with the metal rack inside
Seal with the lid and open the pressure release valve
Set to the **high-pressure** protocol and press "start"

18 In an 80 mL glass beaker, prepare 1x citrate buffer (0.01 M).

We found that 40 mL is enough for an 80 mL glass beaker (39.6 mL of ddH_2O, 400 µl 100x citrate buffer).

19 Immerse the staining rack in 1x citrate buffer and wrap it completely with aluminum foil to ensure the best sealing possible (in order to prevent both the Citrate buffer from evaporating and the water from evaporating and diluting the citrate buffer).

20 Remove the lid of the pressure cooker, quickly place the covered beaker inside, and seal with the lid to retain heat. 20m

Set the pressure cooker to the **high-pressure protocol** for 20 minutes and press "Start". Ensure the pressure release valve is now **closed**.

00:20:00

21 After antigen retrieval, open the pressure release valve and wait until all pressure is relieved. 10m

The lid **cannot** be removed until all pressure is relieved.

Use a hot glove to carefully remove the beaker from the pressure cooker and let it sit to equilibrate at RT for 10 minutes.

00:10:00

22 Remove the staining rack from the beaker and quickly immerse it in a beaker/container filled with ddH_2O and incubate for 2 minutes at RT. 2m

00:02:00

23 Place the staining rack in a second beaker/container filled with ddH_2O and incubate for 2 another minutes. 2m

00:02:00

24 Remove the coverslip from the staining rack. 2m

Place the coverslip in the well containing 5 mL Hydration Buffer, immerse 2-3 times, and incubate for 2 minutes.

00:02:00

25 Place the coverslip to the second well containing 5 mL Hydration Buffer, immerse 2-3 times, and incubate for an additional 2 minutes for a total of 2 washes. 2m

00:02:00

26 Place the coverslip to well containing 5 mL Staining Buffer. 30m

Incubate coverslip for 20-30 minutes.

00:30:00

Start by incubating for 20 minutes, then add 10 minutes once that time has elapsed if the antibody cocktail preparation is not complete.

26.1 Prepare antibody cocktail. 20m

Remove antibodies from 4°C and keep them on ice. Spin down the tubes to collect any liquid from caps.

26.2 Prepare a stock solution of blocking buffer:

Blocking Buffer Stock Solution	1 sample
Staining Buffer	181 µL
N Blocker	4.75 µL
G Blocker	4.75 µL
J Blocker	4.75 µL
S Blocker	4.75 µL
Total Volume	200 µL

Prepare blocking buffer just before staining, **at maximum** 1 hour before. Multiply all values by n for n slips/samples.

Add blocking buffer to each of the antibody cocktail solution tubes, then add antibody. **Only** pipette mix.

The final volume of the Antibody Cocktail Staining Solution is a total of 200 µL per tissue.

Antibody dilutions we used are as recommended: 1 µl antibody per tissue, 1:200 ratio of each antibody. We remove blocking buffer volume equal to the total antibody volume.

For example: if we use 13 antibodies in our experiment, we add 187 µl of blocking buffer and 13 µl total of antibody (1 µl of each antibody) to make the Antibody Cocktail Staining Solution.

27 ⚠ 3h

Remove the coverslip from the staining buffer and place onto the humidity chamber.

Add 190 µL of Antibody Cocktail to the center of the coverslip and ensure that the solution covers the entire tissue. Repeat for every slip.

Remove/minimize bubbles with a P2 pipette or forceps.

Place the lid on the humidity chamber once complete, and incubate for 3 hours.

03:00:00

The humidity chamber **must** be placed on a stable surface free of vibrations. If anything disturbs the surface tension of the staining droplet on the coverslip, the tissue could dry out.

28 Proceed to **Post-Stain (FF and FFPE)** section (**step 29**).

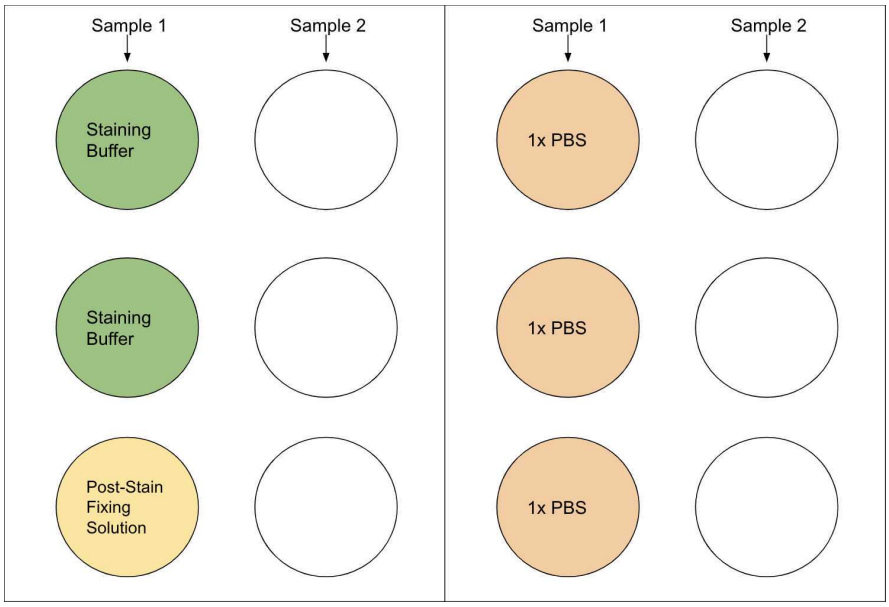
Post-Stain (FF and FFPE) 50m

29 Gather reagents. 5m

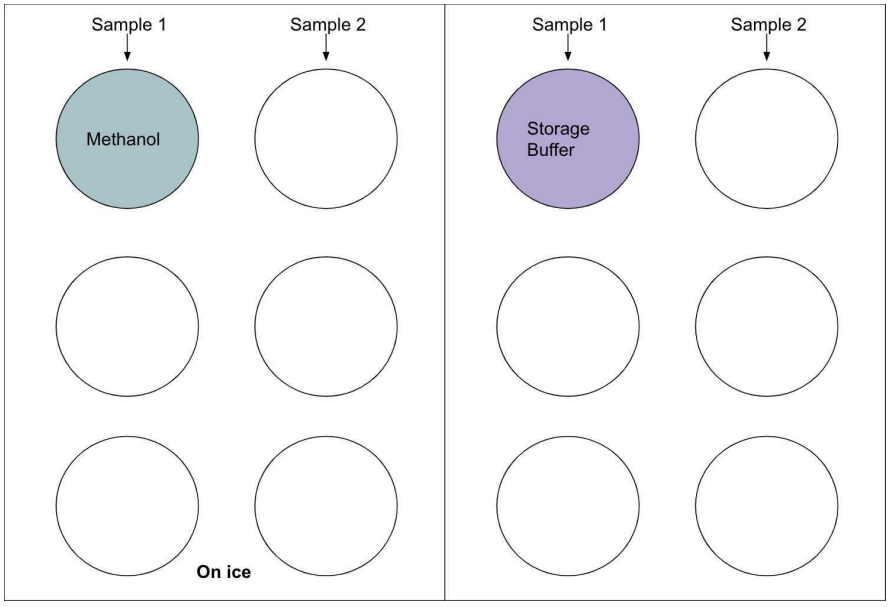
Add 5 mL of Staining Buffer, 1x PBS, and Storage Buffer to the 6-well plates to equilibrate to room temperature.

Add 5 mL 100% methanol to a 15 mL Falcon tube and place it at -20°C

Prepare 5 mL **Post-Stain** Fixing Solution (0.5mL 16% PFA in 4.5 mL **Storage Buffer**, 5 mL per sample) and add it to the 6-well plate.



This configuration is for 1 coverslip only. Repeat as necessary depending on number of slips.



This configuration is for 1 coverslip only. Repeat as necessary depending on number of slips. Ensure to get an ice bucket for the methanol plate.

30 Following the 3 hour antibody incubation, place the coverslip in the first well containing 5 mL Staining Buffer. 2m

Immerse the coverslip 2 to 3 times to ensure the complete removal of the Antibody Cocktail and incubate for 2 minutes.

00:02:00

31 Place coverslip in the second well containing 5 mL Staining Buffer, immerse 2-3 times, and incubate for another 2 minutes for a total of 2 washes. 2m

00:02:00

32 Remove coverslip from Staining Buffer 10m

Place the coverslip in the well containing Post-Stain Fixing Solution and incubate for 10 minutes.

00:10:00

32.1 During this time, prepare methanol on ice according to plate configuration in step 12.

Retrieve methanol from -20°C and to add to the 6-well plate on ice

33 Remove coverslip from the well containing Post-Staining Fixing Solution.

Place coverslip in the first well containing 5 mL 1x PBS and immerse the coverslip 2-3 times to ensure the the complete removal of the Post-Staining Fixing Solution.

34 Place the coverslip to the second well containing 5 mL 1x PBS and immerse the coverslip 2-3 times.

35 Place the coverslip to the third well containing 5 mL 1x PBS and immerse the coverslip 2-3 times for a total of 3 washes.

36 Remove the coverslip from the well containing 1x PBS 5m

Place the coverslip in the well containing ice-cold methanol and incubate on ice for 5 minutes.

00:05:00

36.1 During this time, rinse and dry the humidity chamber's tray to prepare for the next step.

37 Place the 6-well plate containing the previously used 1x PBS next to the ice bucket and quickly transfer the coverslip from methanol to the first corresponding 1x PBS well.

Ensure the coverslip is fully immersed in PBS (as methanol drying will cause the coverslip to float on the surface).

Immerse the sample coverslip 2-3 times to ensure the complete removal of methanol.

38 Place the coverslip in the second well containing 1x PBS and immerse the coverslip 2-3 times.

39 Place the coverslip in the third well containing 1x PBS and immerse the coverslip 2-3 times for a total of 3 washes.

40 Prepare Fixative Reagent. 2m

Add 1 mL of 1x PBS to an Eppendorf tube.

Retrieve one aliquot of Fixative Reagent tube from storage in -20°C freezer. Each aliquot contains ~20 µL of reagent.

One aliquot is good for 5 coverslips.

Spin down the Fixative Reagent to collect any liquid from the cap and add the entire 20 µL to the 1x PBS.

Pipette up and down/invert the tube to mix thoroughly, as the Fixative Reagent is quite viscous.

Fixative Reagent cannot be reused/re-frozen, so the entire aliquot must be used, and then thrown out. **Do not** thaw the entire strip.

Fixative Solution	1-5 samples	6-10 samples
1x PBS	1 mL	2 mL
Fixative Reagent	20 µL	40 µL

41 Remove the coverslip from the 1x PBS and place the coverslip on the humidity chamber. 20m

Add 200 µL of Fixative Solution to the center of the coverslip and ensure that the solution covers the entire tissue. Repeat for every slip.

Remove/minimize bubbles with a P2 pipette tip or forceps.

Place the lid on the humidity chamber once complete, and incubate for 20 minutes.

00:20:00

42 Remove the coverslip from the humidity chamber.

Place the coverslip in the first well containing 1x PBS and immerse 2-3 times to ensure complete removal of the Fixative Solution.

43 Place the coverslip in the second well containing 1x PBS and immerse the coverslip 2-3 times.

44 Place the coverslip in the third well containing 1x PBS and immerse the coverslip 2-3 times for a total of 3 washes.

45 Place the coverslip in corresponding well containing 5 mL Storage Buffer with the tissue facing up.

Seal the plate with parafilm and store at 4°C until ready to image.

If planning to image immediately, keep the sample in the final PBS well at RT.

Reporter Plate (FF and FFPE) 40m

46 Remove the Assay Reagent and Nuclear Stain from -20°C and place in an ice bucket to thaw completely.

Prepare the Reporter Stock Solution based on the total number of cycles for the experiment.

Prepare solution in 1.5 mL amber tube or a 15 mL conical tube covered with foil, depending on the volume/amount of cycles. Mix by gently pipetting up and down so as not to introduce bubbles.

Reporter Stock Solution	1 cycle
ddH2O	244 µL
10x CODEX Buffer	30 µL
Assay Reagent	25 µL
Nuclear Stain (Hoescht)	1 µL
Total Volume	300 µL

Multiply all values by n for n cycles. 1 cycle = 1 well.

46.1 Remove Reporters from -20°C and place in an ice bucket to thaw while preparing the Reporter Stock Solution.

47 For each cycle, label a new tube with the associated cycle number or the well number (for example, "Cycle 1" or “A1”).

Add the Reporter Stock Solution to each tube. The volume of Stock Solution will vary depending on on the total number of Reporters in that cycle.

	3 Reporters per cycle	2 Reporters per cycle	1 Reporter per cycle	Blank cycle
Amount of Reporter Stock Solution per cycle for 1 reporter plate	235 µL	240 µL	245 µL	250 µL

Multiply all values by n for n cycles. 1 cycle = 1 well.

We have a blank cycle at the beginning and the end of every CODEX run (the first and last wells of the reporter plate) for background reference. You can also include additional blank cycles in between certain antibodies that have very high signal to limit bleed over.

48 Add 5 µL of each Reporter to its corresponding amber tube.

Mix the contents of the tube by gently pipetting up and down or gently inverting the tube.

Repeat **step 46** for the total number of cycles.

49 Obtain a new black 96-well plate.

Gently pipette 245 µL of each tube into its corresponding well in the 96-well plate, taking care not to introduce any bubbles.

The CODEX machine doesn't take up the entirety of the reporter solution from each well, so you can sacrifice a small volume to avoid introducing bubbles.

50 Seal the reporter plate with a foil seal, using a credit card/squeegee across the surface to seal. Then, press firmly on each well to ensure the strongest seal possible.

51 Place reporter plate in 4ºC until ready to image.

CODEX Machine Setup 1h

52 Remove the stained coverslip and reporter plate from 4ºC to equilibrate to RT while setting up the machine.

53 Make 1x CODEX buffer (10:1 ddH2O to 10x CODEX buffer):

1x CODEX Buffer	1 cycle
Water	56.2 mL
10x CODEX Buffer	6.2 mL
Total Volume:	62.4 mL

Multiply all values by n for n cycles. Make a few cycles worth of additional 1x CODEX buffer.

Put 2 rubber gaskets in a 50 mL Falcon tube with filled with 1x CODEX buffer for at least 5 minutes.

We store our gaskets in 1x CODEX buffer, 1 pair at time, to ensure they're properly hydrated. We reuse our gaskets a maximum of 3 times before replacing with a new pair.

Put 5-10 mL of 1x CODEX buffer in a new 15 mL Falcon tube to use when preparing the nuclear stain (see step 71.1).

Add the remaining CODEX buffer to Bottle 1.

We fill our Bottle 1 completely with 2 L of 1x CODEX Buffer to ensure it doesn't run out during the experiment.

Fill Bottle 2 with DMSO.

We fill our Bottle 2 completely with 1 L of DMSO to ensure it doesn't run out during the experiment.

54 Open up the CODEX Instrument Manager (CIM) software.

Put blank coverslip in stage insert (outside of the microscope), verify all bottles are closed tightly, and no lines are tangled.

Run an initial "Clean Instrument Wash" protocol to make sure the fluidics are clear and running well.

55 Once "Clean Instrument Wash" is complete, click "experiment" and "new template". Enter the **project name**, **experiment name**, and **operator name**.

Adjust start well and number of cycles depending on the experiment being run/reporter plate used.

In the table, enter the the marker names and exposure times for each cycle

If the experiment warrants/microscope requires a Z stack, choose the number of Z-planes.

Click either "Save" or "Save As" depending on whether you want to re-write over an earlier project's settings.

Click "Start Experiment", read through the CODEX Start Wizard popup, and press "next".

56 Click "Check Fluidics". If there is a pop up window prompting you to adjust any of the bottle volumes, do so and press "Check Fluidics" again. When you have the correct volumes for your planned experiment, the window with read "Fluidics Test Passed" will pop up.

Click "next" and then "Prime Instrument".

56.1 While instrument is priming, prepare the Nuclear Stain at 1:1000 (Hoescht : 1x CODEX buffer) in an 1.5 mL amber tube.

For one sample, 1 µL in 1ml 1x CODEX buffer

57 Once instrument prime is complete, click "next" which will prompt the nuclear stain.

Remove the blank coverslip from the stage insert.

Place the first gasket into the stage insert, pressing the edges down and into the well with the bent-tip forceps to ensure it's level and will seal properly.

Carefully place the stained coverslip into the stage insert. The coverslip may not fit perfectly when you first place it. Gently try to coax, **but do not force** it into the well until it falls flat. Gently press down on the edges of the coverslip with the bent-tip forceps to ensure it's level and will seal properly.

We have noticed that some coverslips have rough edges, likely from manufacturing, that will not allow them to fit into the stage insert. When we encounter this issue, we use a diamond pen to VERY carefully cut a sliver of glass away from the rough edge (enough to remove the rough edge but not enough to make the coverslip too small to fit into the gaskets).

Place the first gasket into the stage insert, pressing the edges down and into the well with the bent-tip forceps to ensure it's level and will seal properly.

Place the top plate on the stage insert and tighten both locks a the same time, applying equal pressure.

Once the stage insert is sealed, add 700 µL of nuclear stain solution to the coverslip and cover.

Click "Wait and Wash".

This step is where the coverslip is most likely to break. Inspect the coverslip before and after sealing the stage insert to look for cracks or defects.

Moving quickly while placing the coverslip into the stage insert is important to ensure that the tissue doesn't dry out.

58 Once the nuclear stain step is complete, click "next" to proceed to the microscope setup.

Microscope Setup (Andor Dragonfly 200 Spinning Disk Confocal Microscope) 1h

59 Turn on the microscope scope and install your objective.

We use a Nikon CFI Plan Apo Lamda 20x/0.75 Air objective

Place the CODEX stage insert on the microscope stage.

Open the Fusion software.

Click "Preferences" and "File Manager" to set the save path for your files.

60 Utilize this script with the Fusion software for fully automated fluidics and imaging using this microscope (similar to the commercial functionality with Zeiss and Keyence microscopes).

This takes advantage of the Andor Dragonfly REST-API. More info can be found here.

```
import time
import argparse
import sys, os
import ctypes
import sys
from Python_Examples import fusionrest as fusionrest

parser = argparse.ArgumentParser()
parser.add_argument("cycle", help="cycle parameter")
parser.add_argument("c", type=int, help="cycle number")
args = parser.parse_args()

print(str.format("cycle #{0}", args.c))
time.sleep(5)

if args.c == 4:
    fusionrest.run_protocol_completely("CODEX_single camera_protocol2") #4 will run
protocol2 on cycle 5
elif args.c == 7:
    fusionrest.run_protocol_completely("CODEX_single camera_protocol3") #7 will run
protocol3 on cycle 8
elif args.c == 8:
    fusionrest.run_protocol_completely("CODEX_single camera_protocol2") #make more elif
blocks if more protocols are needed
else:
fusionrest.run_protocol_completely("CODEX_single camera") #default protocol, will run on
all the cycles other than the ones included above

print("success")
sys.exit(0) # Exit code = 0: success

# Wait for N seconds
# time.sleep(10)
```

Ignore the italicized portion if using the same exposure time/laser intensity for all antibodies in a given channel. If not, create separate protocols in the Fusion software for the desired different settings and adjust in the script as necessary.

61 Under the **Protocol Manager** tab, create a CODEX imaging protocol with the desired lasers and camera choices. Adjustments can be made to individual channels under the **Channel Manager** tab.

As Akoya Biosciences only sells reporters in 488, 555, 657, and 750, we created channels with the following parameters:

Hoescht: 405 nm laser line, 445-46 nm filter
488: 488 nm laser line, 521- 38 nm filter
555: 561 nm laser line, 594-43 nm filter
647: 637 nm laser line, 698-77nm filter

Dichroic Mirror:
405-13 nm
488-13 nm
561-6 nm
640-16 nm

62 Under the **Protocol Manager** tab, select "Time-Series" or "Z-scan", depending on whether you want to acquire a Z-stack.

With the Dragonfly, acquiring a Z-stack is **not necessary to deconvolve your images** but may be desirable if you are interested in specific biology/morphology

63 Click "Live" and bring the tissue into focus on the DAPI channel.

When the objective is close enough the the coverslip, the "PFS" light will blink on the Nikon stage. This indicates that the microscope has detected the coverslip and the Perfect Focus (PFS) can be engaged. At this point, the tissue may go out of focus if the offset displayed on Fusion is far from where your current focal plane is.

For the Nikon CFI Plan Apo Lamda 20x/0.75, the general offset is ~9700. If you set your drift stabilization offset to 9700, you should be close to/in focus

Adjust the focus until the tissue comes back into focus. After this, your sample should stay in focus as you move across the tissue.

64 Under the **Montage** section, ensure that the "Montage Enabled" box is checked and click "Remove All".

Navigate to the upper left corner of the tissue and click the "+" button to add this point. Navigate to the bottom right portion of the tissue and click the "+" button to add this point.

Similarly, add these point to the "Specimen Bounds" under the **Acquisition Control** tab.

Set image name to "Preview" and click "Preview Montage" to acquire a DAPI-only whole tissue image

Ensure that the stitched preview scan encompasses a sufficient amount of tissue. If not, readjust the area by changing corner points and re-acquire until you're satisfied.

65 Once satisfied with the preview scan, make copies of the protocol and adjust the laser intensity/exposure times as desired in the experiment.

Skip this step if only using one protocol.

66 Perform final checks before beginning the experiment

Change image name to "Cycle".

Check that all settings in all protocols are as desired.

Verify that all lasers work.

Go to the final step in the CODEX machine setup (**step 77**).

Microscope Setup (Zeiss Axio Observer Inverted Microscope) 30m

67 Turn on the microscope and install your objective

We use a Zeiss Plan-Apochromat 20x/0.8 M27 Air objective

68 Open the Zeiss Zen Blue software.

When prompted, click "calibrate" to calibrate the stage.

69 Under the **Locate** tab, turn on the DAPI channel and bring the tissue into focus via the eye pieces.

Once the tissue is in focus, turn off the DAPI channel and switch to the **Acquisition** tab.

70 Select the "Z-stack" and "Tiles" checkboxes, and select "Full Z -Stack per Channel"

71 In the **Imaging Setup** and **Channels** sections, set up the LEDs, filter wheel, beam splitter, and light path as desired.

Create a CODEX imaging protocol with the desired settings.

As Akoya Biosciences only sells reporters in 488, 555, 657, and 750, we created channels with the following parameters:

Hoescht: 385 nm LED, 450/40 nm filter
488: 475 nm LED, 514/31 nm filter
555: 555 nm LED, 632/100 nm filter
647: 630 nm LED, 690/50nm filter

Beam Splitter:
405 nm
493 nm
575 nm
653 nm

72 In the **Focus Strategy** section, select "Use Focus Surface/Z Values by Tile Setup"

Under "Reference Channel and Offsets", set "DAPI" as the Reference Channel

Under "Stabilization Event Repetitions and Frequency", select "Expert". Then, select the "Tile Regions" and "Repeat Every __ Tile Region" checkboxes and set the value to 1. Finally, select "Center of Regions".

73 In the **Definite Focus** section, click "Find Surface" and, when the focus has been found, click "Store Focus"

74 In the **Tiles** section, click "Advanced Setup"

Under the **Tile Region Setup** tab at the bottom of the screen, select "Predefined", "Tiles", set the X and Y vales to 5 and click the "+" button to set a 5 x 5 tile region

Under the **Preview Scan** tab at the bottom of the screen, deselect all channels but "DAPI" and click "Start Preview Scan"

74.1 Under the **Support Points** tab at the bottom of the screen, set "Generic" with 3 x 3 (columns x rows) and click "distribute"

74.2 When satisfied with the position/tile region, click "Verify Tile Regions...", select "Definite Focus" as your Verification Helper Method, click "Move to Current Point" and click "Run DF and Set Z".

After the first point has been verified, click "Use DF to Verify the Remaining" to verify the rest of the support points.

When all points have been verified, a green check will appear at the bottom of the window.

75 In the **Z-Stack** section, set how many planes you want to acquire for your Z-stack and set the interval between each pane.

We typically acquire a 17 plane Z-Stack with an interval of 0.49 µm

76 In the **Auto Save** section, set the file name and save folder.

77 On the CODEX Instrument Manager (CIM) software, click "Microscope Pre-check" and take your hands off the mouse. An automated program will click through Zen Blue to ensure that the protocol is properly set.

When the program is complete, click "next".

Go to the final step in the CODEX machine setup (**step 77**).

CODEX Machine Setup (continued)

78 Once the microscope is set up, press "Start" and verify everything in the "Confirm CODEX Setup" pop-up window.

Click "Close" to begin the run.

Monitor the first cycle (through fluidics and imaging) to ensure there are no early issues with the run.