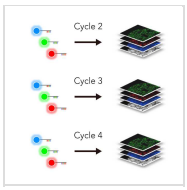

May 10, 2022

CODEX® Multiplexed Imaging | Microscope Setup and Tissue Imaging

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Vanderbilt Diabetes Research Center

Islet and Pancreas Analysis Core
Vanderbilt Diabetes Research Center

This protocol is adapted from the CODEX User Manual, revision C (Akoya Biosciences, Dec. 2020). See also: CODEX Run and Keyence 800 setup.

This protocol describes the imaging workflow currently in use by the Vanderbilt Diabetes Research Center Islet & Pancreas Analysis (IPA) Core and Powers/Brissova Research Group for CO-Detection by indEXing (CODEX®, now PhenoCycler™; Akoya Biosciences). See also **CODEX® Multiplexed Imaging | Modality overview**.

DOI

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Apr 08, 2022

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Apr 08, 2022 Conradvreihsmann

May 10, 2022 Islet and Pancreas Analysis Core Vanderbilt Diabetes Research Center

60514

In steps of
CODEX® Multiplexed Imaging | Modality Overview

Materials and Reagents:

- Prepared reporter plate
  - 10X CODEX Buffer **Akoya**
- **Biosciences Catalog #7000001**
  - Nuclear Stain **Akoya**
- **Biosciences Catalog #7000003**
  - Dimethyl sulfoxide ≥99.9% **Sigma**
- **Aldrich Catalog #472301-6X1L** Step 3
  - CODEX Gaskets v2 **Akoya**
- **Biosciences Catalog #7000010** Step 3
  - 22 x 22m Glass Cover Slips # 1 1/2 **Electron Microscopy**
- **Sciences Catalog #72204-01** Step 3
  - Bent-tip tweezers **Fine Science**
- **Tools Catalog #11251-33**
- MilliQ/ddH_2O
- 1.5-mL microcentrifuge tubes

Equipment:

CODEX® Instrument
Microfluidics system that integrates with microscope hardware
Akoya Biosciences **V1**

All-in-one Fluorescence Microscope
Benchtop imaging system
Keyence **BZ-X810**

NIKON OBJECTIVE LENS (20X) - CFI PLAN APO 20X LAMBDA **Nikon Instruments Catalog #MRD00205**

BZX DAPI Filter **Keyence Corporation Catalog #OP-87762**

BZX GFP Filter **Keyence Corporation Catalog #OP-87763**

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BZX TRITC Filter **Keyence Corporation Catalog #OP-87764**

BZX Cy5 Filter **Keyence Corporation Catalog #OP-87766**

*See **Figure 1** for microscope configuration.*

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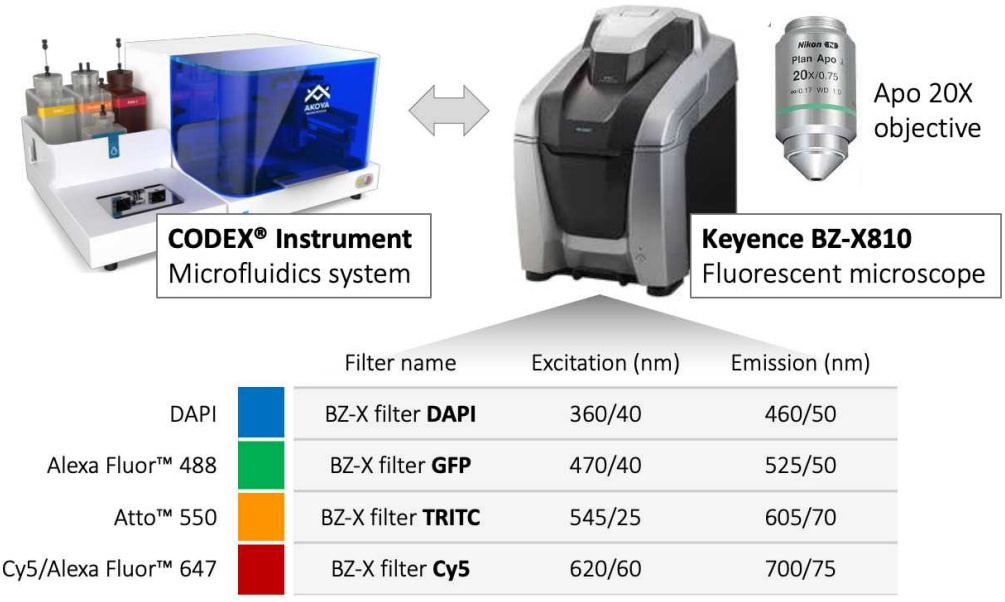
This protocol is adapted from the CODEX User Manual, revision C (Akoya Biosciences, Dec. 2020). See also: CODEX Run and Keyence 800 setup.

Experiment Preparation

1 Check and power up equipment: CODEX® Instrument and Keyence BZ-X810 microscope.



		Filter name	Excitation (nm)	Emission (nm)
DAPI		BZ-X filter **DAPI**	360/40	460/50
Alexa Fluor™ 488		BZ-X filter **GFP**	470/40	525/50
Atto™ 550		BZ-X filter **TRITC**	545/25	605/70
Cy5/Alexa Fluor™ 647		BZ-X filter **Cy5**	620/60	700/75

Figure 1. Integration and configuration of CODEX® Instrument integrated with the Keyence BZ-X810 microscope.

2 Equilibrate the following to **Room temperature** :

- Coverslip in storage buffer
- Prepared reporter plate

3 Gather additional supplies:

CODEX Gaskets v2 **Akoya Biosciences Catalog #7000010**

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22 x 22m Glass Cover Slips # 1 1/2 **Electron Microscopy**

- **Sciences Catalog #72204-01**

Dimethyl sulfoxide ≥99.9% **Sigma**

- **Aldrich Catalog #472301-6X1L**

4 Prepare **1 L** of 1X CODEX Buffer (**100 mL** 10X CODEX Buffer in **900 mL** MilliQ/ddH_2O).

5 Fill bottles for the CODEX Instrument accordingly:

- Bottle 1 - 1X CODEX Buffer
- Bottle 2 - DMSO

⚠ Make sure the **Waste Bottle** is empty.

6 Load the prepared reporter plate, noting the coordinates of the first well containing solution.

7 Launch the CODEX Instrument Manger (CIM) software. Load or create an experiment template and input appropriate labels under **Project**, **Experiment**, and **Operator** fields.

Instructions described here are based on CIM version 1.30.0.12; see CODEX® Support for more information.

8 Specify the number of cycles in **Total #Cycles** and enter the **Start Cycle Well** (the first well in the reporter plate for the experiment).

9 Double check template contents, including marker names, classes, and exposure times. Set **Z-Stack Planes** to 5.

10 Press *Start Experiment* button and follow the guided **Experiment Start Wizard** prompts as outlined below.

Experiment Start Wizard

11 **Prime the instrument:**

Briefly soak a gasket in 1X CODEX Buffer. Load a blank coverslip onto the stage plate and place gasket on top. Fit the stage insert, tighten, and place the assembly on a horizontal surface (in a plastic collection container or on the benchtop). Click *Prime* on the Experiment Start Wizard.

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12 Load sample:

Once the machine is finished priming, remove the stage insert and gasket and discard the blank coverslip. Carefully load the **sample coverslip** into the stage plate, place gasket on top, and tighten the stage insert. Remove any buffers or salts from bottom of the coverslip using a Kimwipe dipped in water. Next, situate the stage plate/stage insert into the holder of the Keyence BZ-X810 microscope, making sure fluidics lines can exit through the front of the microscope.

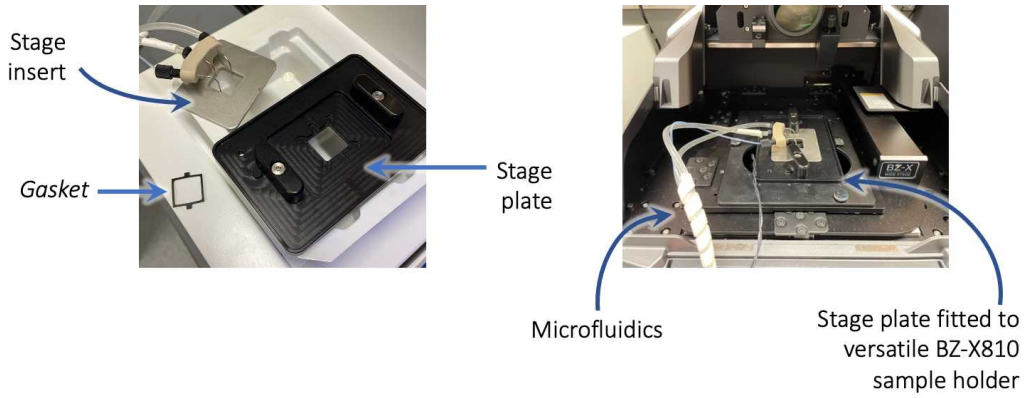

Figure 2. View of stage plate and stage insert before loading coverslip (left) and after being fitted into the Keyence BZ-X810 microscope (right).

13 Add DAPI:

Pipette **700 µL** of Nuclear Stain Solution (**0.067 % (v/v)** Nuclear Stain in 1X CODEX Buffer) onto the sample coverslip. Click *Immediately Wash Tissue* on the Experiment Start Wizard.

14 Configure microscope settings:

Minimize the CIM window and Experiment Start Wizard and launch the BZ-X Viewer software. Choose *Capture Still Images*, and select *Versatile* as the Sample Holder.

14.1 Verify the following settings:

- Set to **Multi-Color**
- Enable all four fluorescent channels (DAPI, GFP, TRITC, Cy5)
- Set all channel pseudocolors to white
- Set Excitation Light to **100**% and enable **Low Photobleach**
- Set camera to **Mono**
- Set Resolution Sensitivity to **High Resolution**

In Options tab:

- Select **Z-Stack -> Multi-Color**
- Enable **Gain** and **Transmitted Light Power**
- Enable **Excitation Light Filter**

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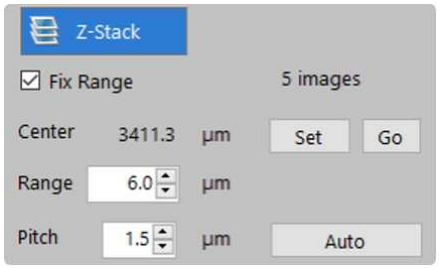
- Choose **14bit** under TIFF Image Setting
- Enable **Automatically Save Image** and set Format to **TIFF**

Under Capture Area Settings:

- Enable **Z-Stack**
- Enter **1.5 µm** for Pitch
- Enable **Stitching**
- Choose **Set Center and Number of Images**

14.2 Set Z-stack parameters:

Ensure that the tissue is situated over the objective, moving the stage using the BZ-X Viewer as needed. Switch to the DAPI channel and perform autofocus to locate the tissue. At multiple points across the tissue, find and note the optimal focus (Z position). If values are within ~5 µm of each other, scroll to the center Z position and under Capture Area Settings, select *Fix Range* and click *Set.* Range should correspond to 5 images (Z-stacks), approximately ~6 µm.



If values are **not** within 5 µm of each other, clean underside of coverslip and check that the stage plate/insert are securely in place. If the Z positions are still varied across different areas of the tissue, the Range (BZ-X Viewer) and Z-stack planes (CIM) may be increased as needed.

14.3 Set imaging region:

Bring up the **Navigation** window, adding regions as needed to locate the edges of the tissue. Use these macro stitched images to approximate the center of the tissue and click *Add.* Define region size by setting the number of X and Y images (tiles) under Capture Area Settings. Without changing positions, scroll back through stored regions and ensure that the green box (denoting the captured area) includes the tissue edges. Adjust center and/or X and Y numbers as needed.

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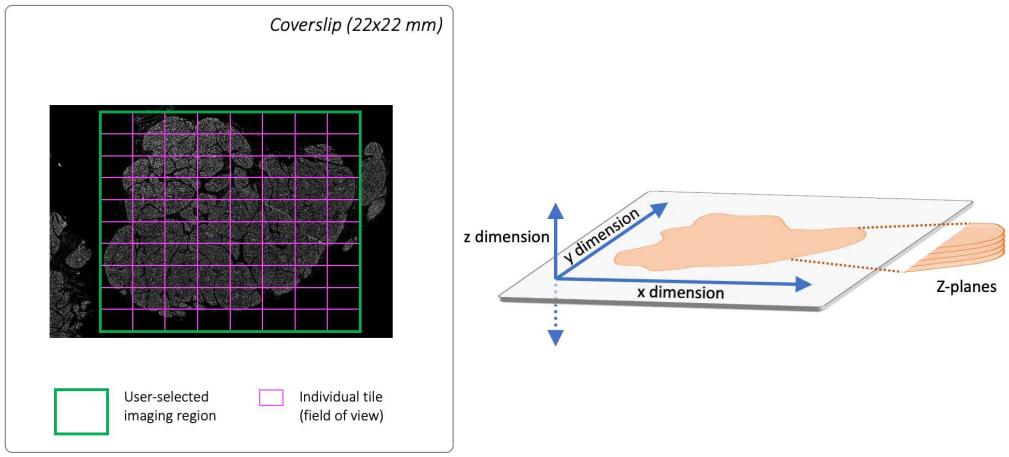

Figure 3. Schematic depicting the imaged region (green outline) across a series of tiles (magenta). These parameters represent the x and y dimensions, while the Z-Stack range corresponds to the z dimension.

Once you are satisfied with the imaging region, delete all other regions stored in the Navigation window so that only the central region remains. Select *Start Capture* and specify the file path for images to be saved (folder should match Experiment name). Press *Stop* after a few images.

15 **Microscope Pre-Check:**

Back in CIM Experiment Start Wizard, select *Microscope Pre-Check.* If pre-check fails, double-check microscope parameters outlined above and in the Quick Reference Guide.

16 Press *Start Run*. Stay several minutes to ensure sample well is not leaking before walking away.

Post-Run

17 Once run is complete, carefully remove coverslip and place back into **Storage Buffer** for preservation at 🌡 **4 °C** if future imaging or staining is to be performed. Otherwise, coverslip can be discarded.

18 Load a blank coverslip and perform a *Clean Instrument Wash* by navigating to the **Maintenance** tab. If another run will not be performed immediately, place Bottle 1 and Bottle 2 lines in a beaker of MilliQ water and perform a *Maintenance Wash*.

19 Empty the vacuum waste container and small solution containers under the hood. Rinse, and leave to dry on paper towels. Remove reporter plate.

20 Shut down BZ-X Viewer and CIM software.

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