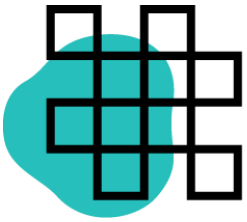
Pixel Primo User Manual v1.6

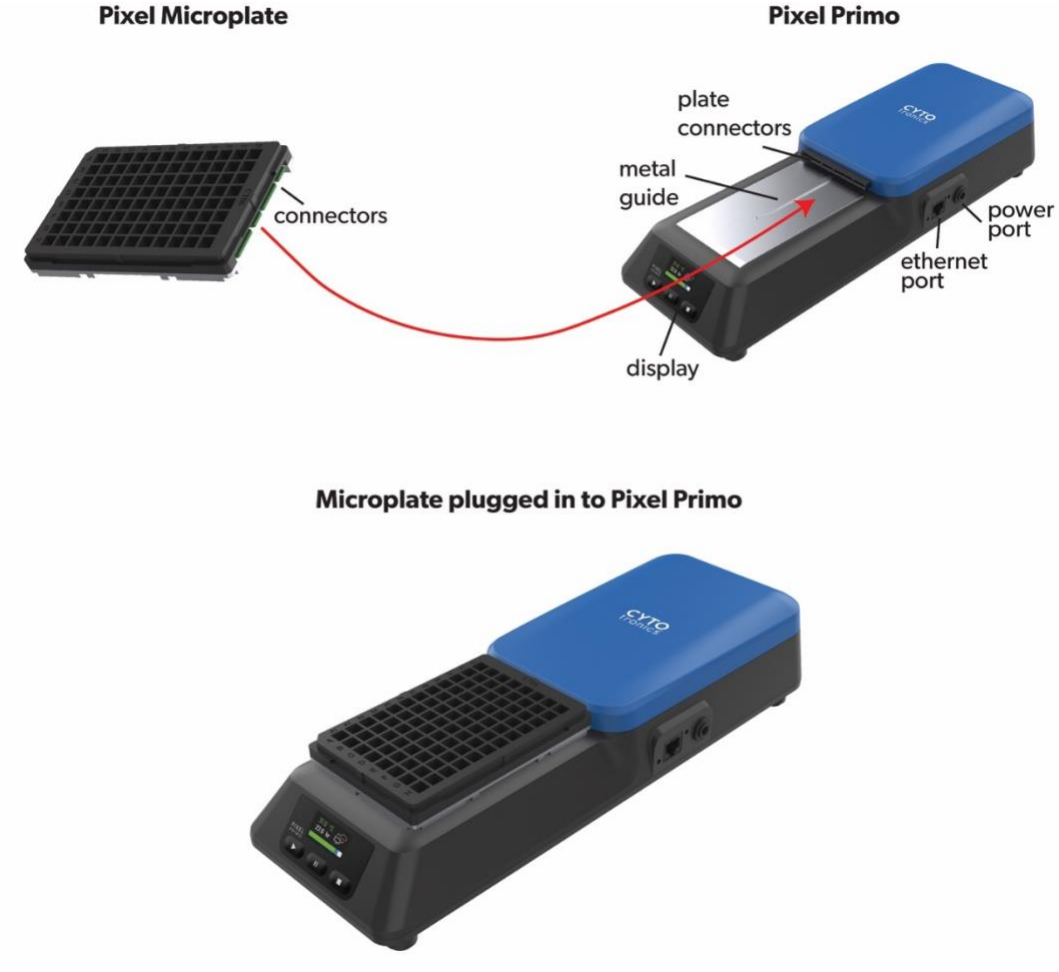


CytoTronics

Contents

1. Introduction to the Pixel Primo and Microplate

The Pixel Primo consists of two parts as shown below – the 96-well Pixel microplate and the Pixel Primo reader. Once cells have been plated on a microplate, it is inserted into the reader. Important elements of the Pixel Primo, such as the ethernet port and power port, are indicated. The entire unit is designed to run in an incubator at 35°C and 85% humidity.



A. Pixel Glossary

These terms will be used throughout this manual and will be important in the execution of both data acquisition and analysis.

Microplate: CytoTronics' CMOS-based 96-well plate

Scan: a user-defined set of measurements which will be acquired at user-defined intervals; includes Barrier-Vertical Field (0.25, 1 kHz), Vertical Field (4, 12, 28, 60 kHz), Lateral Field (4, 12, 28, 60 kHz), Lateral Field D2 (4, 12, 28, 60 kHz), Radial Field (4, 12, 28, 60 kHz)

Scan Manager: the cloud-based interface through which scans are defined and managed (available at cytotronics.io)

Project: a user-defined group of experiments

Experiment: a user-defined group of scans; a single experiment can contain data from multiple microplates

Schedule: the user-defined scans and their intervals to be run on a microplate

Calibration Scan: a scan to be taken prior to cell seeding in order to assess the overall quality of the plate and electrodes

Scan Template: a preset scan schedule with user-defined measurements and their intervals; can serve as a shortcut in setting up scans

Dashboards: a set of cloud-based tools (available at cytotronics.io) for viewing scans and analyzing experiments; includes Plate Viewer, Video Creator, and Data Plotter

B. Primo Maintenance

If the Primo reader will not be used for 48 hours or more, it should be removed from the incubator and stored in a dry location at room temperature.

The Primo microplate connectors can degrade over time if exposed to the environment inside of an incubator. If necessary, the connector can be cleaned with isopropyl alcohol by using a cotton swab.

The surface of the Primo can be sterilized by spraying it with 70% ethanol and wiping it dry. Doing so may cause slight discoloration of the plastic but will not damage the instrument.

Due to the temperature difference between the incubator and the Primo cooling system, the surface of the Primo may accumulate water and start dripping. To keep the Primo dry, wipe off the water with a paper towel and put a drip plate under the Primo.

2. Installation

This section details how to install the Pixel Primo system in your incubator. These steps should be executed prior to beginning an experiment in order to ensure that the system is functioning.

A. Connecting to the Internet

Since scans are managed in a web-based application and all data is stored in the cloud, it is necessary for the Pixel Primo to be connected to the internet.

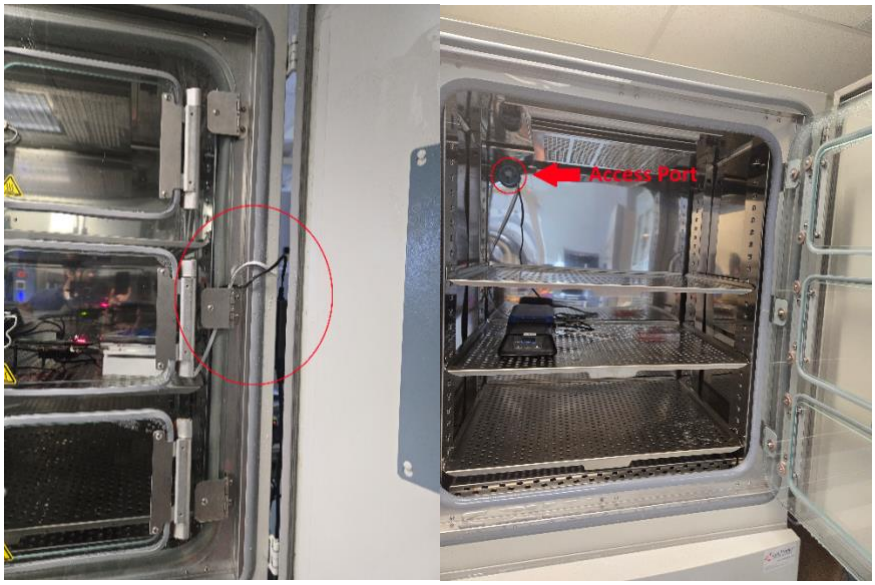
Prior to receipt of the Pixel Primo and microplates, the CytoTronics IT team (IT@cytotronics.com) will be in contact with your IT team to ensure that the Pixel Primo can be properly recognized by your network. For convenience, network setup can be completed with the device outside of the incubator.

CytoTronics will also establish accounts for all users. Each user will need a unique login name and password to access scans and data.

Once network setup is completed, ensure that you can successfully log into http://cytotronics.io and the Scan Manager (Section 3A) before proceeding with the physical installation of the Pixel Primo inside your incubator.

B. Installing the Primo in an Incubator

Place the Primo inside the incubator and plug in the power and ethernet cables. Cables can be either threaded through the front of the incubator through the glass inner door (as shown in the left image) or through an access port that may be available at the back of your incubator depending on the model (as shown in the right image).



Note: The surfaces of the Primo can be sterilized prior to placing it in an incubator. Contact with alcohol may degrade the coloration of the Primo housing but will not cause any structural damage.

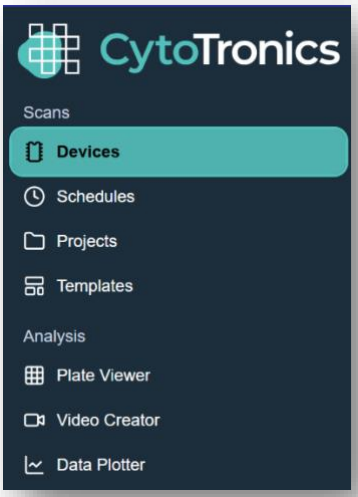
Note: To minimize edge effects cause by temperature fluctuations when opening and closing the incubator, Primo plates are designed to provide uniform heating across the entire plate. For optimal data acquisition, we recommend setting the incubator to 35°C and 85% humidity, which complements the internal heating of the Primo system and ensures consistent environmental conditions during measurements.

3. Data Acquisition

This section will describe the elements of the cloud-based Scan Manager, which includes steps necessary to prepare for an experiment.

A. Scan Manager

The Scan Manager is the cloud-based interface through which scans are defined and managed. It contains multiple tabs along the left-hand side of the page which will be described in detail in this section, specifically Devices, Schedules, Projects, and Templates. The Scan Manager can be accessed at cytotronics.io with customer logins.

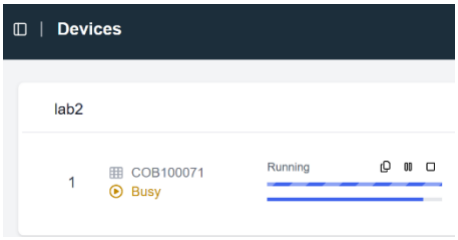


Note: The Analysis Dashboards (including Plate Viewer, Video Creator, and Data Plotter) will be discussed in Sections 5 and 6.

i. Devices

This tab provides a list of connected Pixel Primos, as well as which, if any, microplates are connected. This tab also allows for control of ongoing measurements, as well as initiating new measurements.

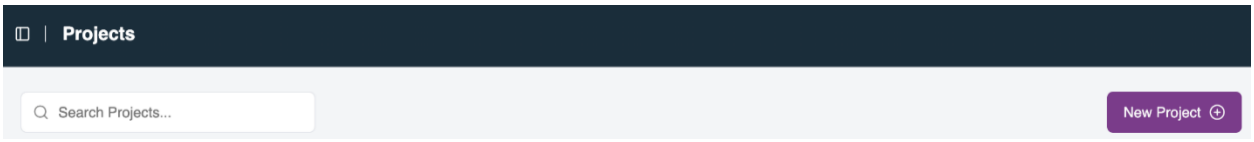
While an experiment is running this tab will display the Pixel Primo identification (lab2 in the example below) and the microplate identification (COB100071 in the example below). From this screen, a user may duplicate the current experimental settings, pause an experiment, and stop an experiment.



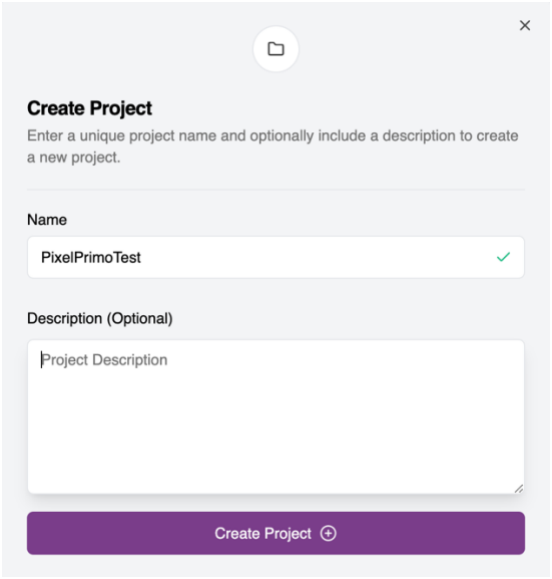
ii. Projects

This tab allows you to create and manage Projects and Experiments. The organization of this is entirely user defined. Experiments are nested within Projects.

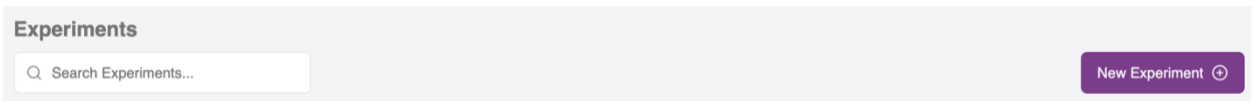
1. In order to create a new Project, click the 'New Project' button.



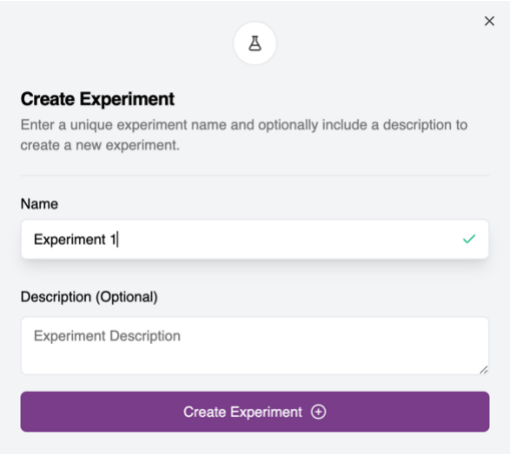
2. Name the Project and click 'Create'.



3. Each Project contains a grouping of user-defined Experiments. To create a new Experiment within a Project, click the 'New Experiment' button.



4. Name the Experiment and add a description (optional). Click ‘Create’.



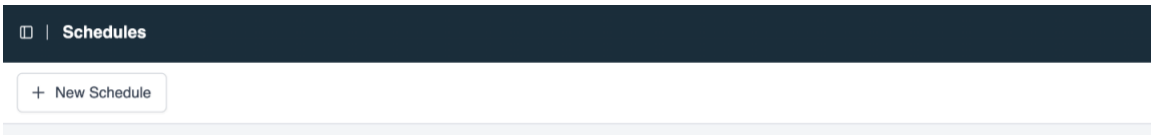
Note: An Experiment can contain data from multiple different microplates.

Note: If data has already been collected, measurements can be viewed by clicking on any given Experiment.

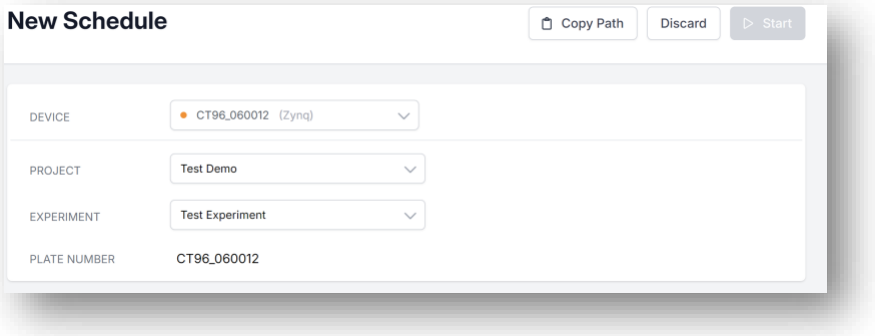
iii. Schedules

This tab provides an overview of measurement schedules where you can view a list of completed and ongoing measurements.

1. In order to create a new measurement schedule, click the ‘New Schedule’ button.



2. Select Well Plate (includes Primo and microplate), Project, Experiment, and microplate number to add a measurement template or manually create a new schedule.



Note: In order for a new schedule to be created, a microplate should be plugged into the Primo.

iv. Templates

This tab provides a list of all the available scan templates for experiments.

Note: Several scan templates will be preloaded into your Primo. One will be called *Impedance Calibration* and allow for the Calibration Scan described in Section 4C to be acquired. The other will be called *Impedance* and will allow for the standard set of impedance measurements (Barrier-Vertical Field, Vertical Field, Lateral Field, Lateral Field D2, and Radial Field) to be collected once every 30 minutes.

1. In order to create a new Scan Template, click the 'New Template' button.

2. Specify the following user inputs:

    a. Name: specify a name for the scan template

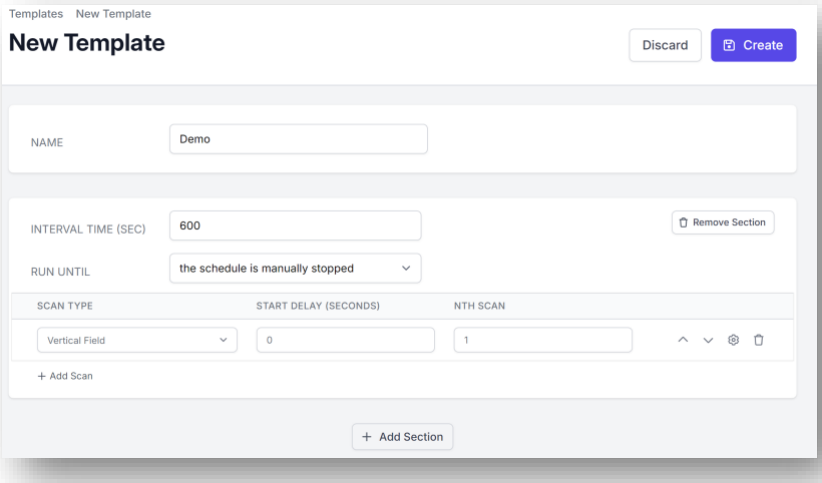
    b. Interval Time: frequency at which measurements are acquired in seconds
        i. At this time, the shortest recommended scan interval is 30 minutes (or 1,800 seconds).

    c. Run Until: determines when a set of measurements will be ended
        i. The schedule is manually stopped
        ii. A number of iterations have passed
        iii. A set amount of time has passed
        iv. A specified date and time

d. Scan Type: measurement which includes Barrier-Vertical Field, Vertical Field, Lateral Field, Lateral Field D2, Radial Field
    i. Click Add Scan to include additional measurements.

e. Start Delay: sets a time interval in seconds before a scan is performed

f. Nth Scan: specifies if a particular measurement is only to be run at longer intervals (i.e. every 3rd scan). Default is 1 (i.e. measurement runs at every interval specified in Interval Time).



4. Wet Lab Protocols

This section will describe how to run an experiment after the Pixel Primo has been properly connected to the internet and installed in your incubator.

A. Microplate Rehydration

Pixel microplates are shipped sterile and dry. To preserve sterility, all handling should be performed in a biosafety cabinet using aseptic technique. Before experimental use, the electrode arrays at the bottom of each well must be rehydrated with a 1.5% PBS-Tween solution to improve wettability and ensure reliable performance.

Note: During all solution steps, liquids (e.g., PBS, media) must fully cover the bottom of each well. Pipette carefully to avoid creating bubbles and use centrifugation or orbital shaking when indicated throughout the protocol. Bubbles interfere with electrical imaging and appear as high-impedance (yellow) regions when viewing scans. Bubbles can fill the entire bottom of a well or appear as diffuse microbubbles scattered throughout a well.

1. In a biosafety cabinet, unpack the Pixel microplate.

2. Remove the plastic connector cap.

Note: Retain the connector cap for use after the experiment is complete.

3. Prepare 15 mL of 1.5% PBS-Tween solution for one plate by adding 2.25 mL of 10% Tween-20 (Thermo Fisher, Cat No. 85113) to 12.75 mL of 1x PBS in a 15 mL conical. Mix gently by inverting the tube several times, taking care to minimize bubble formation.

Note: Ensure that the 1.5% PBS-Tween solution is well mixed prior to adding to the microplate.

4. Add 100 µL of 1.5% PBS-Tween to each well using a multichannel pipette.

Note: Dispense solution slowly and uniformly against the edge of each well to encourage even spreading and prevent bubble formation at the bottom of the well.

5. Centrifuge the plate at 30 rcf for 1 minute.

6. Shake the plate on an orbital shaker at 400 rpm for 5 minutes.

Note: Inspect wells for bubbles. If bubbles remain after centrifugation and shaking, gently dislodge them with a pipette tip and/or repeat the centrifugation and shaking steps. Bubbles must be removed, as they prevent proper electrode rehydration and can compromise downstream electrical signals.

7. Incubate the plate for 4-24 hours at 37°C. Do not exceed 24 hours for rehydration for optimal cell attachment.

Note: Once the wells have been rehydrated, take care to not let the wells dry out – all subsequent solution changes should be performed promptly to maintain electrode integrity.

8. After the plate has rehydrated for at least 4 hours, shake the plate on an orbital shaker at 400 rpm for 5 minutes.

9. Centrifuge the plate at 30 rcf for 1 minute.

10. Perform a Calibration Scan.

11. Assess results in the Plate Viewer Dashboard to determine whether plate has fully rehydrated.

    a. If wells have rehydrated fully, proceed to step 12. If plate needs additional rehydration time, return plate to 37°C and continue rehydration. Following additional rehydration time, repeat step 10.

12. Aspirate the PBS-Tween solution using a multichannel vacuum aspirator.

Note: If a multichannel vacuum aspirator is not available, use a multichannel pipette to carefully aspirate all solution from each well. Proceed immediately to the next solution step to ensure the electrodes do not dry out.

13. Add 150 µL of 1x PBS to each well using a multichannel pipette.

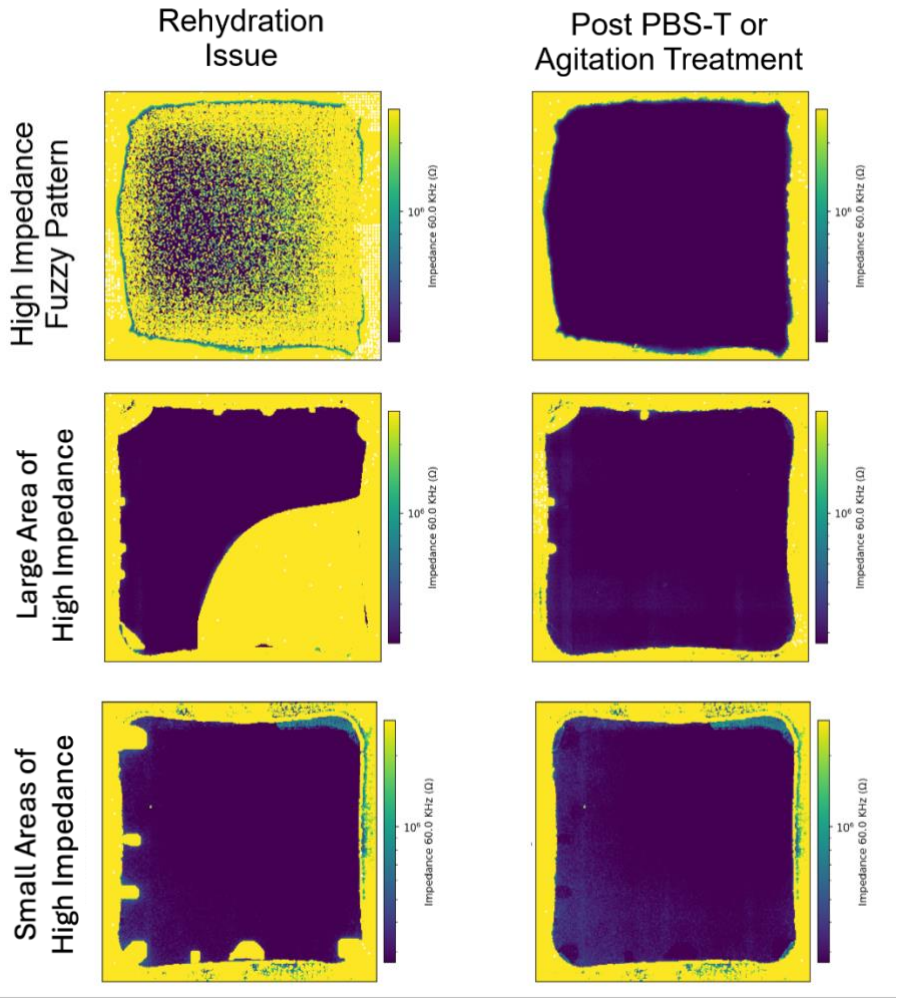
14. Mix by pipetting 75 µL (half volume) up and down 4 times taking care not to introduce any bubbles.

15. Aspirate the PBS and wash the wells twice more (for a total of three washes).

16. After the final wash, add 150 µl of fresh PBS to each well.

17. Centrifuge the plate at 30 rcf for 1 minute.

Examples of rehydration issues in the microplate displayed using the VF 60 kHz measurement. The left column displays the rehydration issue with problem areas in yellow and the right column displays the resulting impedance after the appropriate treatment with PBS-T or pipetting/centrifugation.

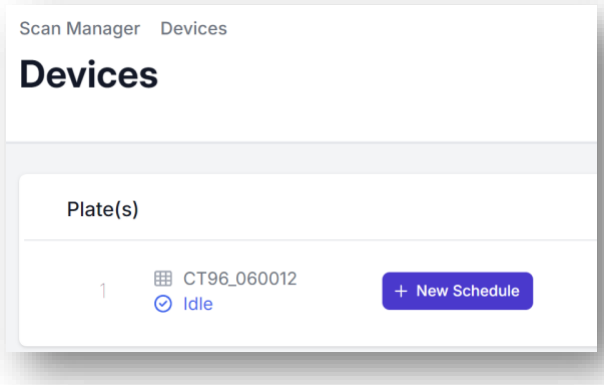


If areas of high impedance, such as those illustrated above, persist after rehydration, please contact CustomerSupport@cytotronics.com for additional guidance.

B. Acquiring a Calibration Scan

Prior to plating cells, a Calibration Scan must be taken in order to assess the overall quality of the plate and electrodes.

1. Connect the microplate to the Pixel Primo by plugging it into the plate connectors. When the plate is successfully connected the Scan Manager will display the microplate ID with an indication that it is 'Idle' (i.e. not currently being scanned).



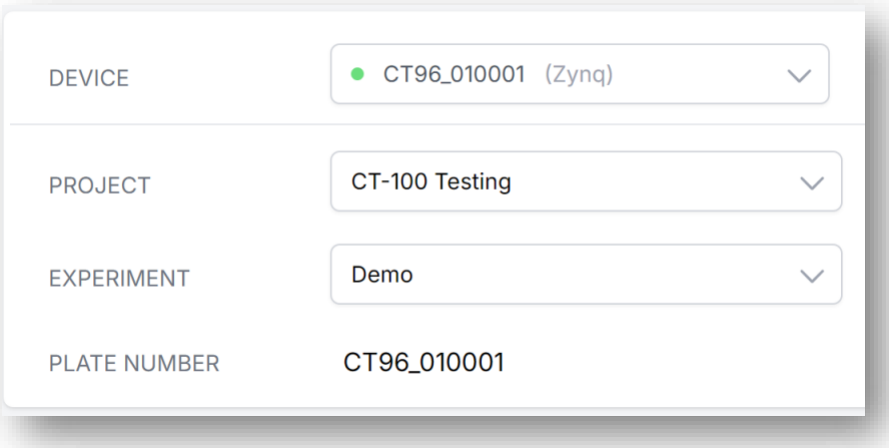
The front panel of the Primo will also indicate when a plate is connected, as well as its plate ID.

Note: The connectors on the plate are on the short edge along column 12. For proper alignment, position the connectors on the microplate with the connectors on the Pixel Primo using the metal guide on the base of the Primo. You will need to press firmly in order to make the connection so that the Primo recognizes the plate.

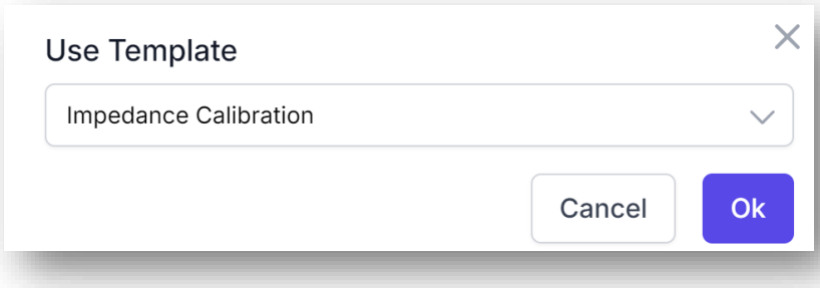
Note: When a microplate is plugged into the Primo, the plate should be securely attached to the reader and may require the use of both hands to pull out.

2. In the Scan Manager, under the Projects tab, set up a Project and Experiment. See Section 3Ai for more details.

3. In the Scan Manager, under the Device tab, click the 'New Schedule' button to perform the calibration scan. Assign the Project and Experiment from the relevant dropdown menus.

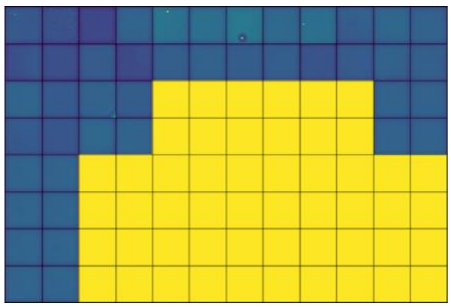


4. Press the 'Use Template' button, select the Impedance Calibration template, and press Start.



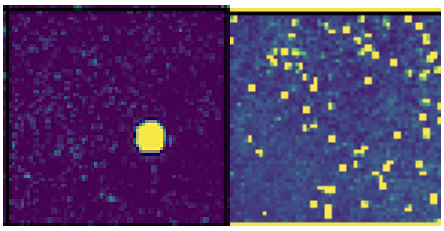
5. After the Calibration Scan has completed, use the Plate Viewer Dashboard (described in Section 5) to view the impedance measurements.

   In particular, examine each measurement for signal uniformity across the microplate. If the signal is yellow on large sections of the microplate, it may indicate that the microplate is not plugged in properly. If this is the case, unplug and re-plug in the microplate and run the Calibration Scan (Steps 3-4) again. Here is an example of a microplate that was not plugged into the Primo properly:



Note: Contact Cytotronics' Customer Support (CustomerSupport@cytotronics.com) if you experience issues with any impedance measurements during a Calibration Scan.

If round yellow spots are observed in any wells, this means that there are likely bubbles. In this case, gently pipet the media in each well up and down 2-3 times and re-run the Calibration Scan. Here are two examples of bubbles in a single well:



***Tip*:** If you have access to a plate centrifuge, spinning the plate for 1-2 minutes at 30 rcf can also help to eliminate bubbles.

Note: If the plate has been properly rehydrated with 0.5% PBS-Tween (as described in Section 4A) bubbles are less likely to appear during the Calibration Scan and subsequent experimental scans.

6. Once a satisfactory Calibration Scan has been acquired, the microplate is ready for cells.

C. Coating Microplates with Extracellular Matrix (ECM)- optional

A variety of ECM coatings, including collagen, fibronectin, PDL, laminin, and gelatin, have been used in conjunction with the microplates. It is important to note that any coating applied to the microplates must be thin enough to ensure that cells are within a distance of 20-30 μm of the electrodes for measurements.

Note: ECM coatings should be applied prior to plating cells. The inclusion of ECM proteins in the plating media only (and not on the well surface) has been found to not be as effective with regards to cell attachment to the surface of the microplate.

1. Dilute the desired ECM to the appropriate concentration for a given assay according to the manufacturer's instructions or previous optimizations.

2. Add ECM solution to each well using a multi-channel pipette, taking care that the minimum volume in each well is 50 μL.

   ***Note*:** Avoid introducing air bubbles while pipetting and be sure that the bottom surface of the well is fully covered in solution.

3. As per manufacturer's instructions or prior optimization, incubate at the proper time and temperature.

   Note: Microplates can be stored at 4°C, room temperature, or 37°C for this step.

   Note: Microplates can be coated with ECM overnight if desired.

4. Remove ECM solution using a multi-channel pipette or vacuum aspirator and perform washes with PBS, if necessary. To do so, add 100 μL of PBS to each well using a multi-channel pipette. Set the pipette to half the total volume (50 μL) and mix each well by pipetting up and down 4 times.

   ***Note*:** Tilt the plate towards yourself and aspirate from the proximal (i.e. closest to you) edge of the wells to ensure the entire contents of the well are removed.

   ***Note*:** Avoid introducing air bubbles while pipetting.

5. After the final wash, aspirate PBS or ECM using a multichannel pipette or vacuum aspirator and immediately add 75 μL of cell media using a multi-channel pipette.

D. Plating Cells and Starting an Experiment

1. Prepare a cell suspension for plating in 75 µL media per well. Recommended starting plating densities are 5-40k per well for optimization purposes.

   Note: Each well in a 96-well Pixel microplate has an average plating area of ~0.132cm^2 and a working volume of 150 µL.

   Note: For extended cultures (i.e. > 96 hours), we recommend plating cells only in the center 60 wells in order to avoid edge effects. Be sure to fill outer wells with media or PBS and refill as necessary.

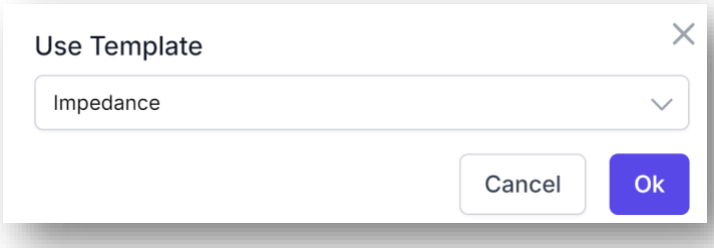
2. Add diluted cell suspension (at least 75 µL) to each well of the microplate, taking care to add the cell suspension into the center of the wells. This will result in a total volume of 150 µL per well.

3. Plug in the plate as described in Section C.

4. In the Scan Manager, under the Devices section, start the experiment by pressing the 'New Schedule' button. Assign the Project and Experiment from the dropdown menus.

5. Select your desired scan template from the dropdown menu and then press Start.

   Note: Templates can include the pre-programmed Impedance template or custom-generated templates for your specific application as described in Section 3Aiii.



E. Pausing an Experiment

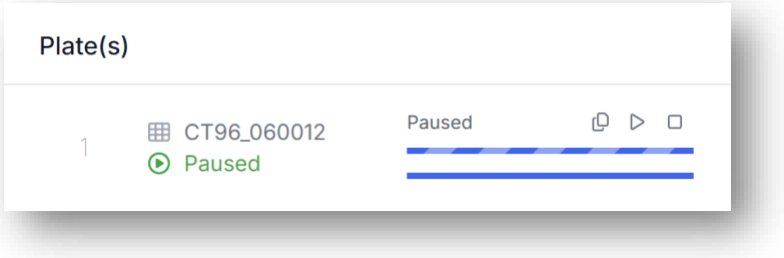
This section will describe the process for pausing and resuming experiments in the event that any interventions (i.e. media changes or compound additions) need to be performed during an ongoing experiment.

Note: Always ensure that a minimum of 20 µL of media remains in each well to prevent drying of the electrodes and cell detachment.

1. In the Scan Manager, under the Devices tab, press Pause to pause the experiment. If a Scan is underway, the Primo will finish the ongoing scan before pausing.

2. Physically remove the microplate from the Primo.

   Note: Removing the plate while a scan is being performed may cause an error in the Scan Manager and with data acquisition. Ensure that the plate Scan Manager is showing that measurements have paused before you remove the microplate from the Primo. The image below shows what a properly paused experiment looks like.



3. Perform the cell culture intervention.

4. Plug the microplate back into the Pixel Primo.

5. In the Scan Manager, under the Devices tab, press Resume to restart the experiment.

Alternately, an ongoing experiment may be paused using the front panel of Primo itself.

While an experiment is running the front panel will display the plate number, name of the experiment, and plate status ('Running' or 'Awaiting next steps').

1. Press the Pause ⏸ button to change the plate status to 'Paused'.

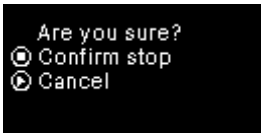
2. Once an intervention has been completed and the microplate has been reinserted on the Primo, press the play ⏵ button to resume measurements. At this point the plate status will update to 'Running' or 'Awaiting next steps'.

F. Ending an Experiment

An experiment can be ended from either the Scan Manager or the Primo itself.

1. To end an experiment from the Scan Manager, press the stop experiment button in the Scan Manager under the Devices tab. By stopping an experiment, any ongoing scans will be ended.

2. To end an experiment from the Primo, press the stop ⦿ button on the front panel once which will bring up a confirmation window.



   To confirm that the experiment will be ended, press the stop ⦿ button a second time.

   Press play ⦿ to resume the experiment if the stop button was pressed in error.

3. Remove the CytoTronics microplate from the reader.

G. Harvesting Cells and Preparing Plates for Refurbishment/Shipping

After completing an experiment, Pixel microplates should be cleaned to remove any biological material and then sent back to CytoTronics for refurbishment. At this stage, cells can also be harvested for downstream applications such as qPCR or protein assays. If you are planning to harvest the cells, follow **Protocol A**; if you are not harvesting the cells, follow **Protocol B**.

Protocol A: Harvesting cells for downstream applications

1. Remove all media from the well by careful aspiration with a multi-channel pipette.
2. Add 100µL of PBS gently to each well with a multi-channel pipette, being careful not to dislodge the cells during PBS addition.
3. Remove PBS by careful aspiration with a multi-channel pipette.
4. Add 50µL of Trypsin to each well and incubate at 37°C until the cells detach.

Note: Incubation time will vary depending on the cell type and will be similar to detachment times on standard plastic- or glass-bottomed cell culture dishes.

5. Once cells have detached, add 100µL of cell culture media to each well. Pipette up and down to ensure that all cells are removed from the surface.
6. Collect the cell suspension(s) and process as usual for downstream applications.
7. Proceed to **Plate Cleaning**.

Protocol B: Removing cells without harvesting for downstream applications

1. Remove all media from the well by aspirating with a vacuum aspirator.
2. Add 100µL of PBS to each well with a multi-channel pipette or with a squirt bottle.
3. Remove PBS using a vacuum aspirator or by dumping the liquid onto a paper towel.
4. Add 50µL of Trypsin to each well and incubate at 37°C for 60 minutes.
5. Proceed to **Plate Cleaning**.

H. Plate Cleaning

Note: All subsequent steps can be performed outside a biosafety hood, preferably near a sink. It is recommended to use a squirt bottle to ensure each well is fully filled with liquid during each wash step. If a squirt bottle is not available, perform each wash step twice using 150 µL of deionized water. Do not use tap water on the microplate.

1. Fill wells with 70% ethanol for 10 minutes to sanitize. In addition, spray all surfaces of the plate with 70% ethanol to sanitize the remainder of the plate.

2. After 10 minutes, remove the ethanol by dumping onto a clean paper towel or into the sink.

3. Using a squirt bottle, wash the wells with deionized water.

   Note: Make sure to squirt water directly into all the wells and let them overflow to ensure complete removal of all biological materials.

4. Remove the deionized water by dumping onto a clean paper towel or into the sink.

5. Using a squirt bottle, once again fill up all the wells with 70% ethanol.

6. Remove the ethanol by dumping onto a clean paper towel or into the sink.

7. Wipe the outside surface of the microplate dry with a clean paper towel.

8. Allow the wells to air-dry at room temperature on the bench for 15-30 minutes.

9. Once the microplate is completely dry, place the plate in the biohazard bag provided in your original shipment of plates.

10. Fill out the Hazardous Substances form and return the plate to CytoTronics using the original packaging at the following address:

    CytoTronics
    12 Farnsworth St
    Suite 600
    Boston, MA 02210

5. Real-Time Monitoring of Experiments

This section describes how to monitor scans from an ongoing experiment using the Plate Viewer Dashboard.

A. Plate Viewer Dashboard

This Dashboard compares multiple scans from the same Experiment and microplate.

1. Navigate to cytotronics.io and select the Plate Viewer.

2. Select a Project, Experiment, and Well Plate.

3. In the Scan Loops box, select up to two time points for comparison and the measurements.

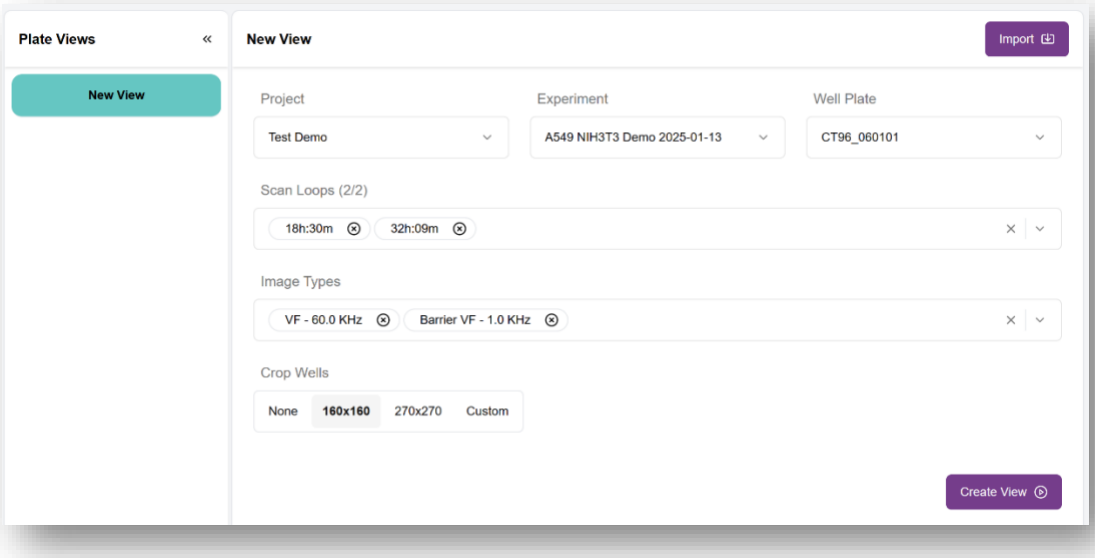
4. In the Image Types box, select which measurement(s) will be displayed.

5. *Optional:* determine whether to crop wells. Determine whether to crop wells. Cropping removes the gasket from view, but slightly reduces the visible well area. The default crop sizes are 160x160 pixels and 270x270 pixels, though a custom size can be specified if needed.

   Note: The gasket refers to the silicone-based well divider that is sandwiched between the black plastic top plate and the chip containing the electrodes. Due to the pressure required to hold the plate together, part of the gasket does "bleed" into the electrode array. This will be seen as a ring of yellow impedance around the outside of each well.

6. Click Create View.

   Note: When two timepoints are selected, in addition to displaying the impedance scans at each timepoint, a third image will be generated that is the difference between the two scans. This visualization can be helpful in viewing cell growth, as red areas will be representative of new cell coverage from one image to the next. On the other hand, blue areas will be representative of areas that are no longer covered by cells, either due to detachment, death, or movement.



As an example, this set of options will generate an image of the Barrier-VF 1.0 kHz and VF-60 kHz at timepoints 18h:30m and 32h:9m for Project: Test Demo; Experiment: A549 NIH3T3 Demo 2025-01-13; and Well Plate: CT96_060101. The resulting images will be cropped to 160x160 to remove the gasket.

6. Data Analysis

Note: These Dashboards can also be utilized at any point during an ongoing experiment to monitor real-time data.

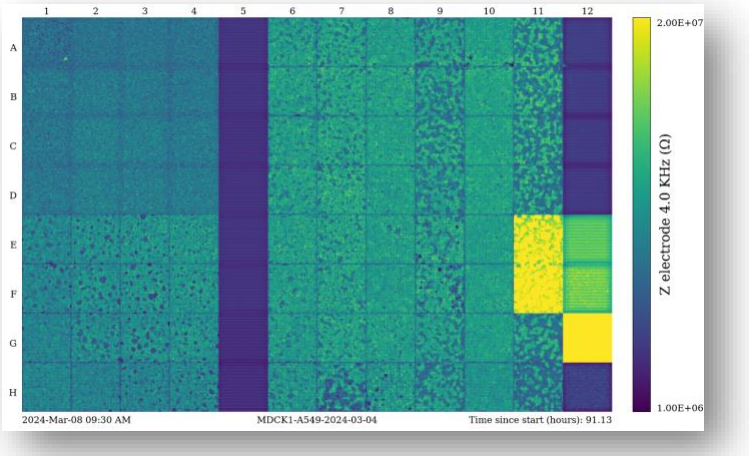
A. Video Creator Dashboard

This Dashboard generates .mp4 videos of impedance measurements.

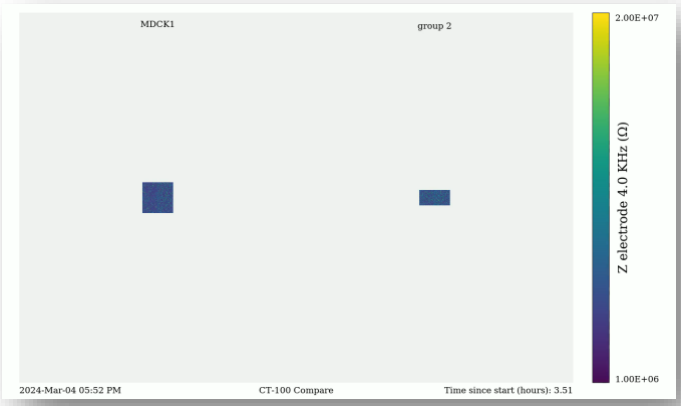
1. Navigate to cytotronics.io and select the Video Creator.
2. Select a Project, Experiment, and Well Plate.
3. Select a measurement or multiple measurements.

   Note: An independent movie will be generated for each measurement selected.
4. Define the Time Range for which the video should be generated.
5. Specify the number of frames per second (FPS). Default is 12 FPS.
6. Choose the desired output style of the video.

   a. 'Full Plate' will generate a video of the entire plate with time stamps, plate labels, and a measurement scale bar.



   b. 'Selected Wells' will generate a video of a user-defined subset of wells in a grid with time stamps, plate labels, and a measurement scale bar.

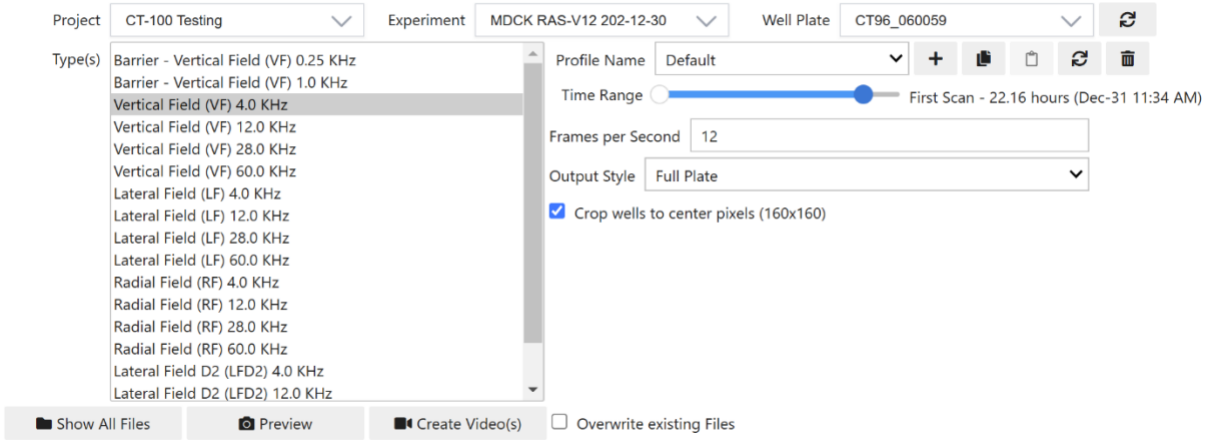


7. Choose whether to crop the gasket out of wells with the ‘Crop wells to center pixels (160x160)’ toggle.

8. Toggle whether to overwrite any existing videos.

9. Click ‘Create Video(s)’ to generate desired videos.

   Note: All previously created videos for a given microplate within the specified Project and Experiment can be viewed with the ‘Show All Files’ button.



As an example, this set of options will generate a Vertical Field 4.0 KHz video from Project: CT-100 Testing; Experiment: MDCK RAS-V12 202-12-30; Microplate: CT96_060059 for the scan range from the beginning of the experiment to 22.16 hours at 12 FPS. The generated video will include a time stamp, plate labels, and a measurement scale bar (‘Full Plate’). The resulting video will have cropped wells.

B. Data Plotter Dashboard

This Dashboard generates longitudinal plots of measurements and is useful for analyzing impedance trends over the course of an experiment. It can be used at any point during or after an ongoing experiment in order to monitor cells.

1. Navigate to cytotronics.io and select the Data Plotter.

2. Select a Project, Experiment, and Well Plate.

3. Load a previously-defined Profile using the dropdown menu or add a new Profile by clicking (+).

   Note: A Profile will allow the user to save settings from Data Plotter so as to enable easier re-plotting of data. Any changes made to a Profile will overwrite the previous settings upon saving or plotting.

   Note: If a Profile is not specified, the Data Plotter settings will be automatically saved under the Default profile.

   Note: Profiles can also be copied and pasted into the analysis for another microplate by utilizing the 'Copy Profile Settings' and 'Paste Profile Settings'.

4. Define the Time Range over which the data should be plotted.

5. <u>Masks</u>

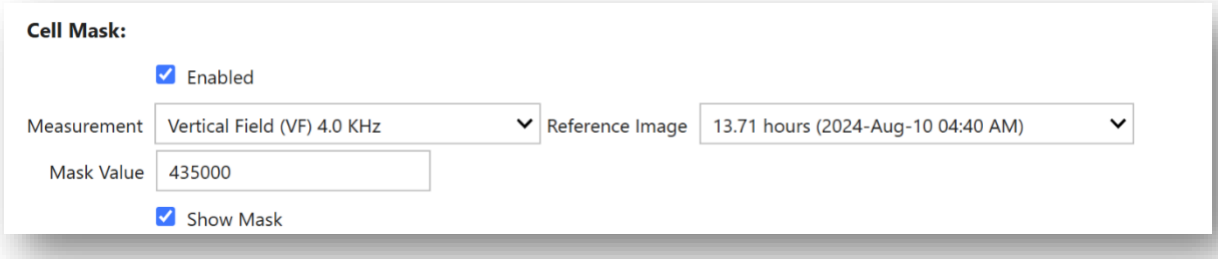
   The Cell Mask will further aid in determining which electrodes are occupied by cells in a well. Subsequent analyses will ignore data from empty electrodes across all measurements.

   a. Wells can be cropped to center pixels (160x160) to eliminate the gasket.

   b. Choose a 'Measurement' and time point ('Reference Image') from which to calculate the values of the cell mask. Using the histogram and plate map, adjust the Mask Value such that only electrodes with cells as identified as being above background.

      Note: For 'Measurement', we currently recommend using Vertical Field (VF) 60kHz to establish the cell mask.

Note: For the 'Reference Image,' choose a time point where both cells and empty electrodes without cells are visible. This typically occurs *6–8 hours after cell plating*, when the cells have attached but have not yet spread out or formed a confluent monolayer.



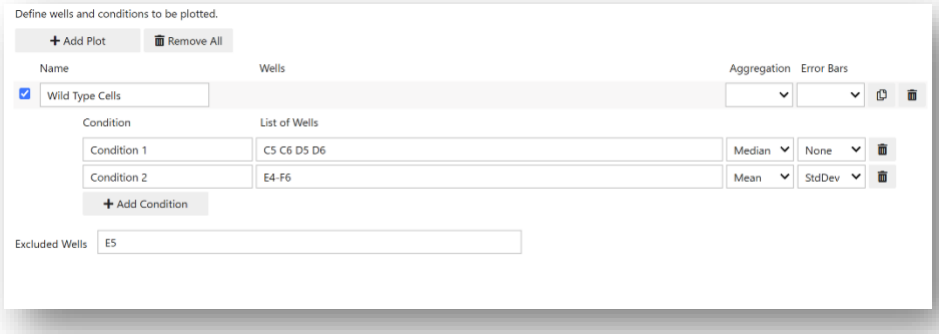
6. <u>Define Wells</u>

   a. Each Plot (viewed by clicking 'Add Plot') will generate a new set of plots for the selected measurements (on the next tab).

   b. Conditions (specified within each Plot) will be separate traces on a plot aggregated as Mean, Median, or Individual Wells. Error Bars can be displayed as Standard Deviation or Standard Error.

      Note: There is also the option to hide a selected condition, as necessary, for visualization purposes.

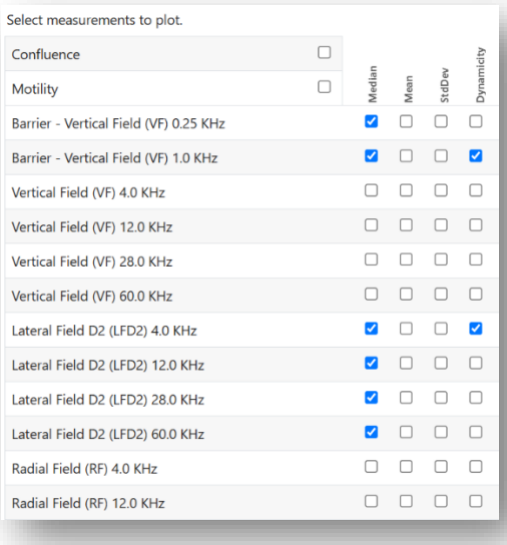
      Note: Wells can be specified individually with one space in between (i.e. 'C5 C6 D5 D6') or as a group with a dash (i.e. 'E4-F6') which will plot the rectangle of wells (i.e. E4, E5, E6, F4, F5, and F6).



7. <u>Measurements</u>

   a. Choose measurement types to plot and desired aggregations (Median, Mean, StdDev, Dynamicity) to be plotted.

Note: Dynamicity is calculated using the frame-to-frame difference between the specified impedance measurement and can be broadly defined as a change in a measurement over time.



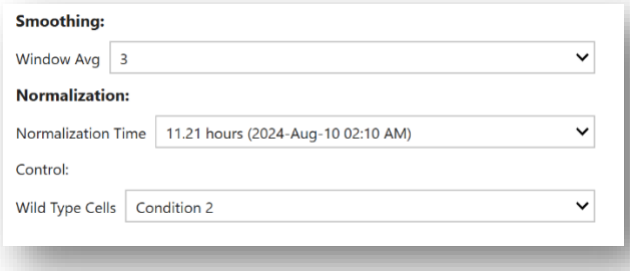
8. <u>Post-Processing (optional)</u>

    a. Specify how many frames to window average the data over, if desired.

    b. Specify a Normalization Time for the plots, if desired.

    Note: For example, this option can be used to normalize all conditions before compound treatment, ensuring that the compound effects are measured relative to the same starting point.

    c. Specify a condition from ‘Define Wells’, to use as a normalization control, if desired.



9. Plot Settings

    a. Specify whether to include a legend on the generated plots.

10. Click ‘Plot and Save Settings’ to plot data as specified.

11. Click on ‘Download raw data (CSV)’ or ‘Download plot data (Excel)’ to generate data files for custom plotting and analysis outside of CytoTronics’ Dashboards.

12. Once plots have been generated in the Data Plotter Dashboard, they can be saved and downloaded by hovering over the desired plot and clicking ‘Save’.

7. System Configuration

This section is geared towards use by IT professionals, if needed, while configuring the network for the Pixel Primo.

For additional IT support, please contact the CytoTronics team at IT@cytotronics.com.

A. Network Configuration

The Ethernet connection on the Pixel is configured by default to use DHCP.

To modify the network configuration, create a plain text file named `network.conf` using the syntax and directives described here:

https://www.freedesktop.org/software/systemd/man/251/systemd.network.html

In addition to the systemd network configuration directives, the following additional directives are available to configure an HTTP proxy:

- `http_proxy`
- `https_proxy`
- `no_proxy`

For example, to use a proxy server, a static IP address, two DNS servers and a custom NTP server:

```
http_proxy=http://proxy.example.com:8080
https_proxy=http://proxy.example.com:8080

[Network]
DHCP=no
Address=192.168.100.2/24
Gateway=192.168.100.1
DNS=8.8.8.8
DNS=8.8.4.4
NTP=time.google.com
```

To apply the settings to your Pixel Primo device, use the following steps:

1. Place the `network.conf` file at the top level of a FAT32-formatted USB drive.
2. Plug the USB drive into the port at the back of the Pixel.
3. The configuration will be copied to the Pixel Primo device and then restart automatically to apply the settings.

To revert to default DHCP settings, use the same steps as above but with an empty `network.conf` file.

8. Appendix

A. Solvent Optimization

The Pixel microplate is optimized for aqueous and low % v/v DMSO solutions. When adding compounds, avoid DMSO concentrations exceeding 0.5% v/v, as these may affect electrode performance.

To optimize solvent concentration, capture impedance images at least 1 hour post solvent addition and compare them with the microplate's Calibration Scan. The addition of a solvent should cause minimal changes in the impedance of bare electrodes. If significant changes are observed, re-optimize by reducing the solvent concentration.

B. Cell Line Optimization

Prior to initiating any experiment using a specific cell line, it is crucial to optimize plating conditions. For this optimization, test coatings (if necessary), as well as various cell densities.

Note: The average area of a well in a microplate is 0.132cm^2 and the plate surface most closely mimics that of a glass-bottomed dish.

Note: We recommend culturing cells at equivalent plating densities and coating conditions in sister glass-bottomed 96-well plates as a benchmark for the interpretation of the impedance-based morphological signatures. This is especially important in the culture of potentially more sensitive cell types (e.g. neurons and primary cells).

1. Plate cells at different densities, both with and without ECM coatings (if necessary), allowing them to grow undisturbed for the desired duration of your assay.

2. Determine the optimal cell density for your specific experimental conditions and biological questions during this period by observing impedance measurements using Video Creator and Data Plotter. For example, if a biological question involves development of a barrier, track changes in Barrier-VF 0.25 and 1 kHz.

3. Utilize the identified optimal coating condition and cell density for all subsequent experiments.

9. Troubleshooting Guide

CytoTronics Website

Issue	*Solution*
Unable to log in to cytotronics.io	Refresh and try again. If problem persists, contact customersupport@cytotronics.com

Devices

Issue	*Solution*
A microplate is plugged into the Primo, but the device shows 'Disconnected'	The microplate may not be fully plugged in. Plug it in again by pressing forcefully until it cannot be inserted any further. Alternatively, the connectors on the plate or Primo may be dirty or otherwise unable to form a complete connection. Clean the connectors with isopropanol and a cotton swab and then try again. If the problem persists, contact customersupport@cytotronics.com for additional guidance.
A microplate is plugged into the Primo, but the device shows 'Unassigned'	
The microplate number shown on the Scan Manager differs from the microplate's actual assigned ID	The Scan Manager may not have updated from a previous plate. Refresh and check again. Otherwise, the microplate lids may have been switched at some point. Ensure that you have the correct plate.
Information in the Schedules tab and the Devices tab are different	This may occur because the Devices tab has not been updated. Attempt to refresh the Devices tab to sync.
When creating a new Schedule the Start button is greyed out	This could be due to an improperly named Experiment. Ensure that no special characters are present in the Experiment name. Also ensure that no other experiments are currently running and then try again.
Changes can't be made to the Scan Template	Ensure that the template is not locked.
The Primo is dripping or soaked in water	This can occur due to the temperature difference between the incubator and the Primo cooling system. Wipe off the water with a paper towel and put a drip plate under the Primo.

Scans

Issue	*Solution*
Yellow spots appear in scans	These are likely bubbles. Proper rehydration of the electrodes with 0.4% PBS-Tween prior to plating cells is critical in mitigating this issue. If bubbles, persist pipet up and down several times and/or centrifuge the plate.
All white or all yellow wells across the microplate	This indicates some kind of connection issue or an issue with the electrodes in the plate. Try cleaning the connectors with isopropanol and a cotton swab. If the problem persists, contact customersupport@cytotronics.com for additional guidance.
Scans not appearing in Plate Viewer or Data Plotter dashboards	Scans take a few minutes to upload to the cloud. Wait five minutes and refresh. If the problem persists, check the version number of the dashboard you are using and contact customersupport@cytotronics.com to see if you need to run a version update.

High Impedance in Initial Scans

High Impedance Pattern	**Cause**	**Resolution**
Spotty or fuzzy patches	Incomplete rehydration - PBS-Tween prepared improperly - Incubation time too short - Incubation temperature too cold	Repeat rehydration with 0.4% PBS-Tween overnight at 37° C. Ensure percentage of PBS-Tween solution is accurate.
Circular	Air bubbles and/or incomplete hydration of gasket	Visually inspect for bubbles around gasket. Agitate solution via pipette or spin plate at 30xg for 1 minute