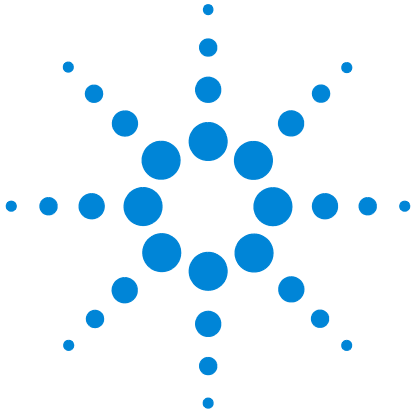

Agilent Seahorse XF Imaging and Cell Counting Software

Integrated Imaging and Metabolic Analysis Solution

User Guide

Agilent Technologies

Notices

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1 Introduction

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This manual provides step-by-step instructions for performing cell count based normalization of Seahorse XF data using Agilent's Seahorse XF Imaging and Cell Counting Software. This software automates the process of performing brightfield and fluorescence image acquisition as well as the calculation of cell counts in Seahorse XF microplates.

The Seahorse XF Imaging and Cell Counting Software is a required component of the XF Imaging and Normalization System.

Additionally, new features added to WAVE have been incorporated into this user manual so that researchers can use this as a guide for performing more successful cellular metabolism assays.

Requirements

Required Hardware

- XFe Controller Windows 10, 64-bit
- XFe96 or XFe24 Analyzer
- Handheld Barcode Wand
- BioTek Instrument's Cytation 1 Cell Imaging Multi-Mode Reader
  OR
  BioTek Instrument's Cytation 5 Cell Imaging Multi-Mode Reader

Required Software

- XFe Controller Software Version 2.6 (or greater)
- Wave Desktop Software Version 2.6 (or greater)
- Seahorse XF Imaging and Cell Counting Software Version 1.0
- Gen5 3.0.4 (Version 530407) Software

Support

Agilent Cell Analysis (Seahorse) Support

Email: seahorse.support@agilent.com

USA and Canada

1-800-227-9770; select option 3 then 8

Europe

UK: 0800 096 7632

Denmark: +45 8830 5083

Germany: 0800 180 66 78

Netherlands: 0800 022 7243

Other EU: +45 3136 9878

BioTek Instruments, Inc. Support

Email: TAC@biotek.com

Phone: (800)242-4685
(800)655-4740

Or see BioTek's support page, here.

1 Introduction

2 New Features in Wave

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This section gives an overview of new image integration and normalization features in Wave.

Well View/Import Images

Wave 2.6 supports the addition and association of well images through the Cell Imaging application. Upon completion of XF Assay, import and view collected images by adding the **Well View** from the **Add View** tab. In this tab, all image sets are visible on the left (**Set** refers to a single read of a cell plate. One plate's file can accommodate multiple sets of images). After selecting an image set, any images collected in the Cell Imaging application may be imported to a plate's Wave assay file. Select the entire plate or specific wells before clicking **Import Selected**.

NOTE Importing images may take several minutes. Images that have been imported are in a compressed format. Full resolution images can be exported from the Cell Imaging application.

Flagging

Upon review of images in Wave, individual wells can be flagged for further review. When one well is in view, simply click the star in the upper right-hand corner of the image. The flag will be applied to the well throughout the different review screens in Wave. Note that this will not remove or adjust the data for this well. Additionally, in the **Well View** tab, flags created in the Cell Imaging application environment can be imported by clicking **Import Flags**.

Heat Maps

Heat Maps allow a quick graphical view of cell count data. Color saturation of the well is indicative of the value of the cell count. A higher cell count will result in a darker shading of the well. Heat Maps may be viewed by group or across the entire plate. You can quickly identify if a few wells are significantly different from the rest of the group. The options are shown in the **Well View** tab. An example is shown below with the **Heat Map** selection menu highlighted.

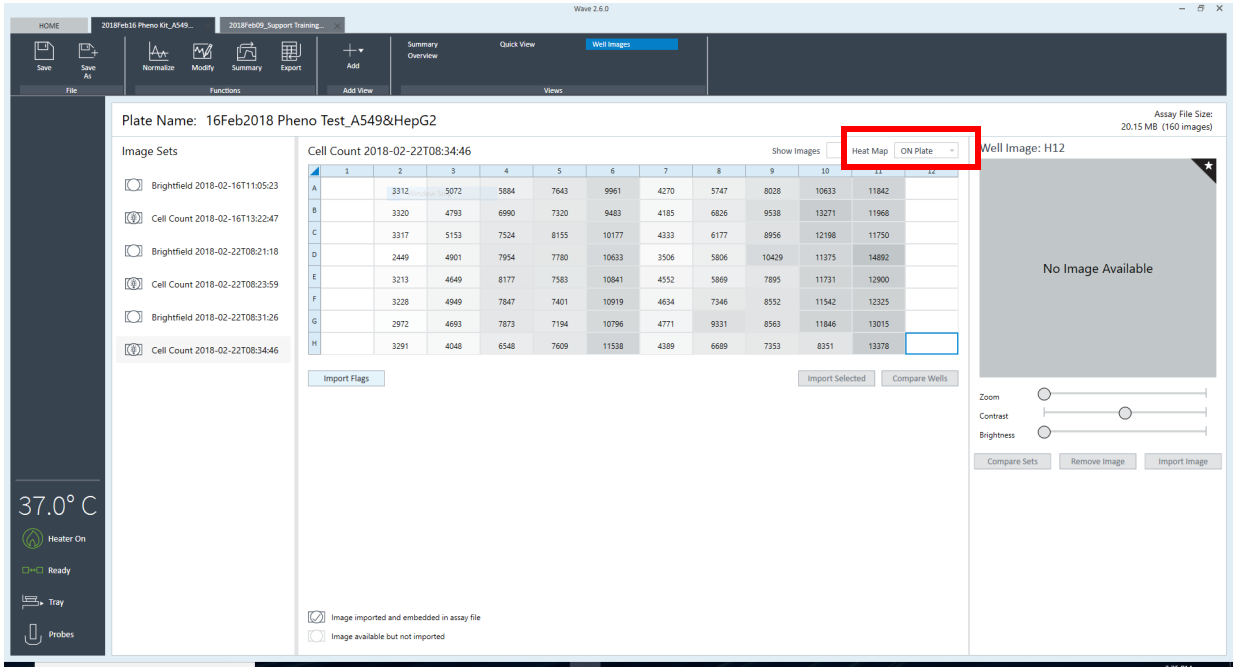

Figure 1 Well View tab with Heat Map menu

Compare Wells

To compare the images of two or more wells, simply select the wells you wish to view, and then click **Compare Wells**. This will open an image viewer with all selected images.

Use the options on the left-hand bar of the viewer to toggle to a more detailed view. Click on one well to view it with more details.

NOTE Wells can only be compared within the same image set.

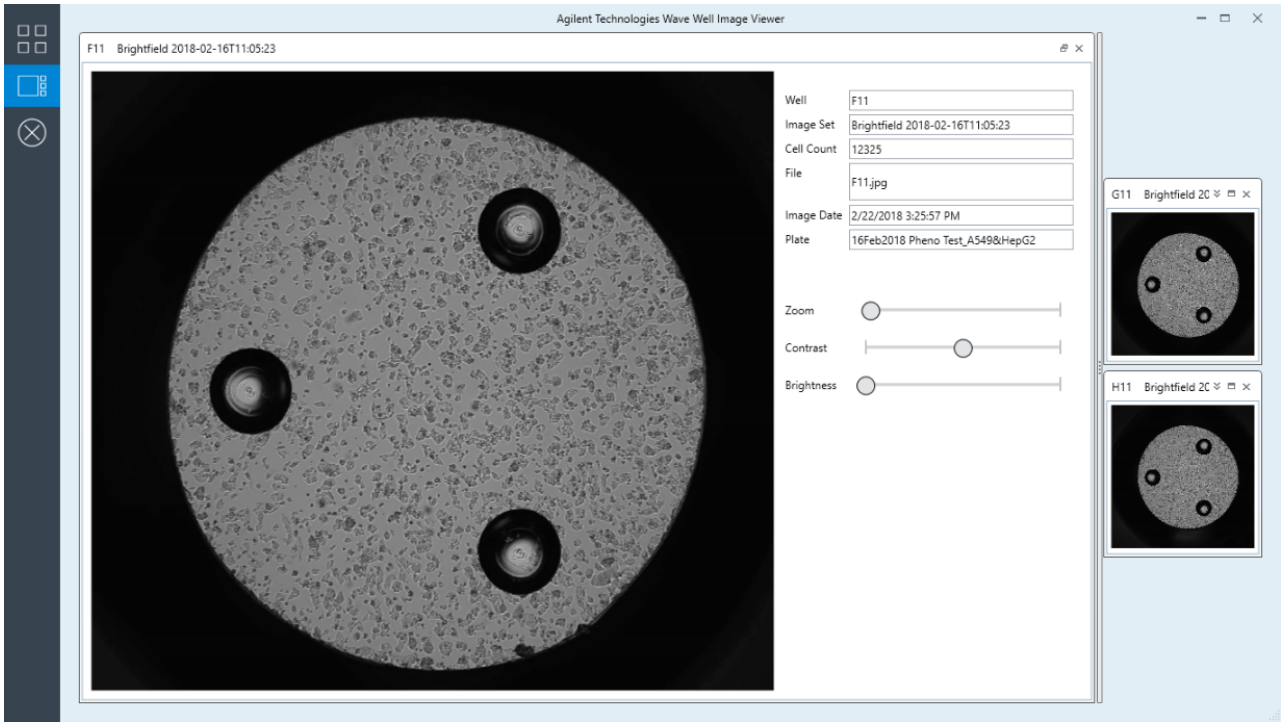

Figure 2 Compare Wells view

Compare Sets

To see all images of a single well that have been collected, select the well you wish to view, and then click **Compare Sets**. This will open an image viewer with all the collected images of that specific well.

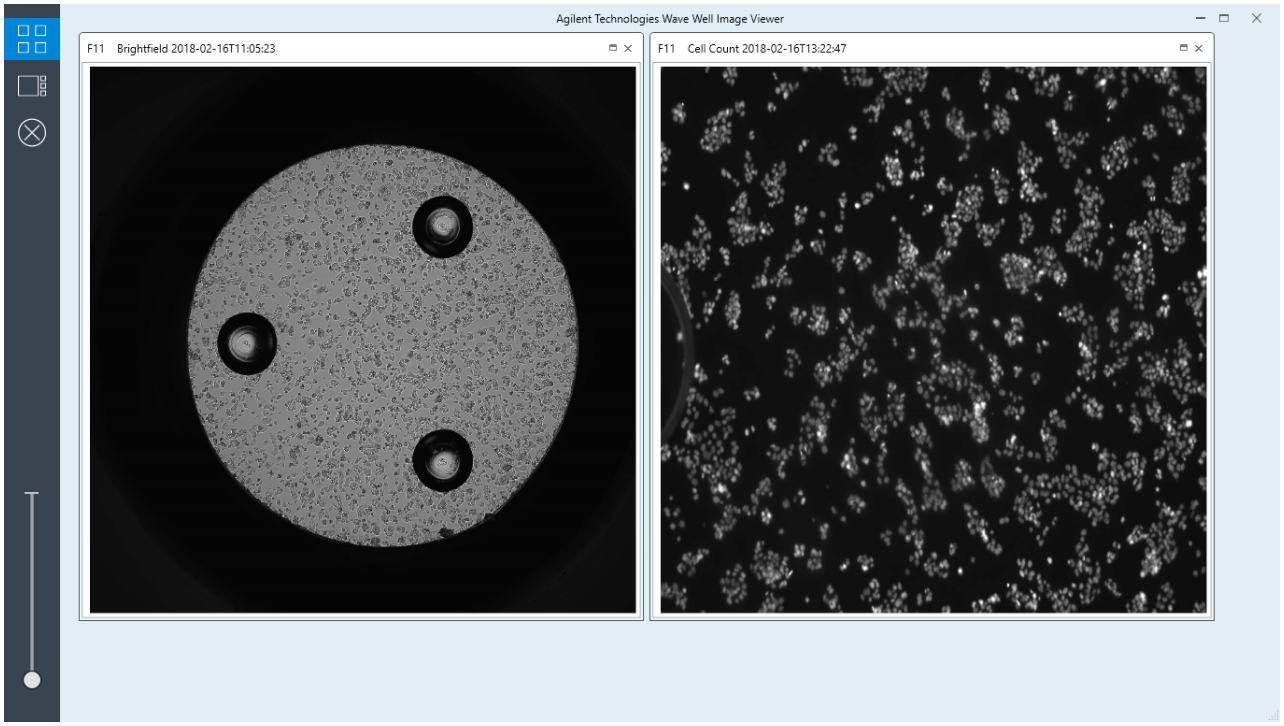

Figure 3 Compare Sets view

Import Cell Counts

Once fluorescent images and cell counts have been collected, the counts are available for normalization.

1 Navigate to the **Normalization** tab. There will be an icon to alert if cell counts are available for the plate.

2 Click **Import** and the normalization table will fill with cell counts.

Notice that any wells defined as background correction wells in the assay plate map do not show a cell count - the default count for any background wells is 0. The scale factor imports as a factor of 1,000 Cells.

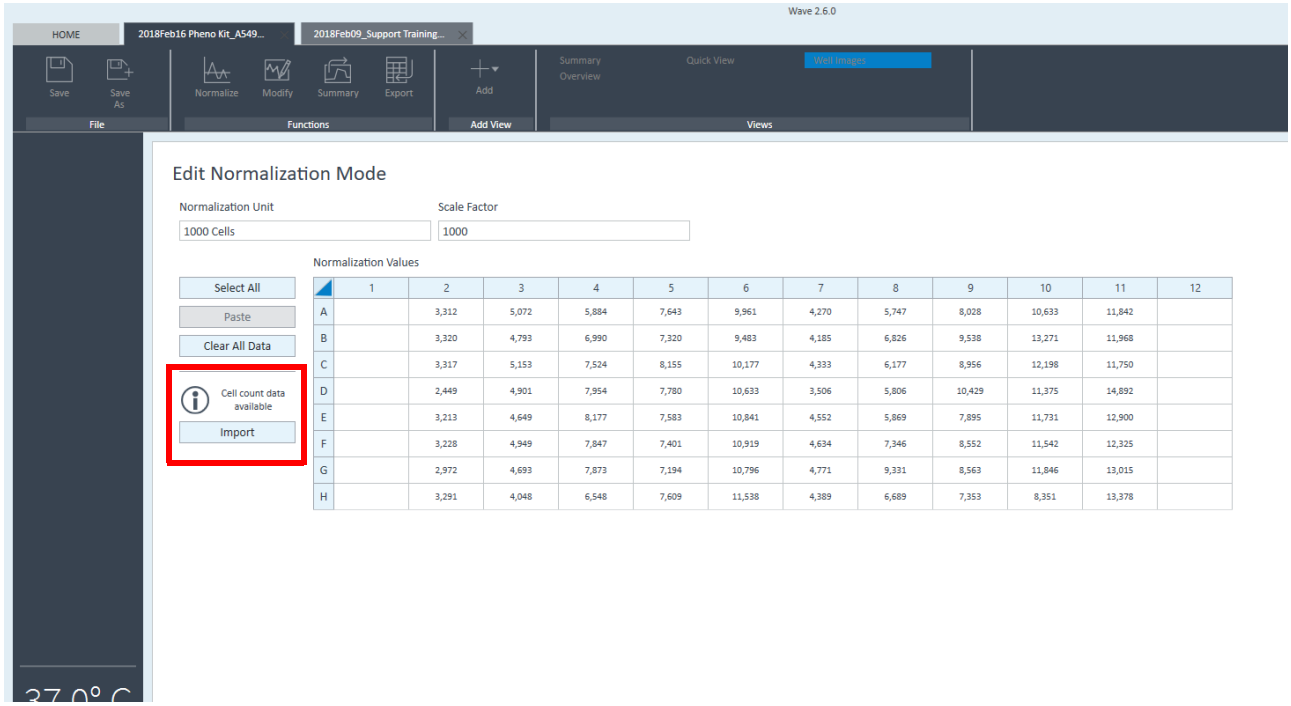

Figure 4 Normalization tab with cell counts imported

Save .asyr file with images

After importing images into Wave from the Cell Imaging application, notice that Wave will keep track of the images and file size in the **Well View** tab. The file must be saved after image import in order to export the data file with images. Note that upon importing the images to Wave, they are compressed from their original versions.

Figure 5 Well view tab with imported images

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This section reviews some useful features in the Cell Imaging application.

Software Introduction

Getting started

The Agilent Seahorse Integrated Imaging and Metabolic Analysis Solution requires the parallel use of both Agilent Seahorse XFe Analyzer and BioTek Cytation 1 or Cytation 5 instruments. This section assumes that the appropriate field specialists have installed the instruments and familiarized the users with their operation. Both instruments are operated using the same 64-bit, Windows 10 OS controller. This section outlines recommendations for powering on the systems and setting up save locations for files.

Powering the system

The following is a recommendation of the order in which each instrument should be powered on. Assuming all instruments and controller are powered off:

1. Ensure all components are connected: power cords, 1 USB for XFe, 2 USB for Cytation instrument, 1 serial cable for XFe, and 1 USB for external barcode wand. Additionally, if using a hardwired LAN connection, connect an ethernet cable.
2. Power on the controller, and log into the Windows system.
3. Power on the XFe Analyzer.
   a. After switching the analyzer on, wait a few moments before opening Wave.
   b. While the software is initializing, listen for the motor to move, and check for connectivity status in the status bar. This may take a few minutes.
   c. If there are problems with connectivity, contact Agilent Support.

NOTE The XFe Analyzer requires time to reach operating temperature and equilibrate. It is recommended that the instrument be turned on the day before an assay is planned to allow for adequate equilibration.

4. Power on the Cytation 1 multimode plate reader.
   a. Open XF Imaging and Cell Counting. The camera will initialize, the instrument may chirp and open the tray.
   b. To close the tray, press the button next to the door.
   c. Watch for connectivity in the status bar of the XF Imaging & Cell Counting software.
   d. If there are problems with connectivity, close XF Imaging & Cell Counting and try connecting the instrument with Gen5. For further problems, contact BioTek Support.

Image storage location

Image Library (This contains all raw images collected by the Cytation)

Because of the number of large image files that are created using this system, both Agilent and BioTek require that users move the image library off the controller's hard drive and either onto a multi-terabyte external hard drive, or to a network location. If the hard drive runs out of space, it will affect software performance. The Image Library must be set up through Gen5. To change the image library file path, go to: **System > Preferences > Image Save Options** in Gen5.

See Figure 6 for help navigating this menu. For further assistance, contact BioTek.

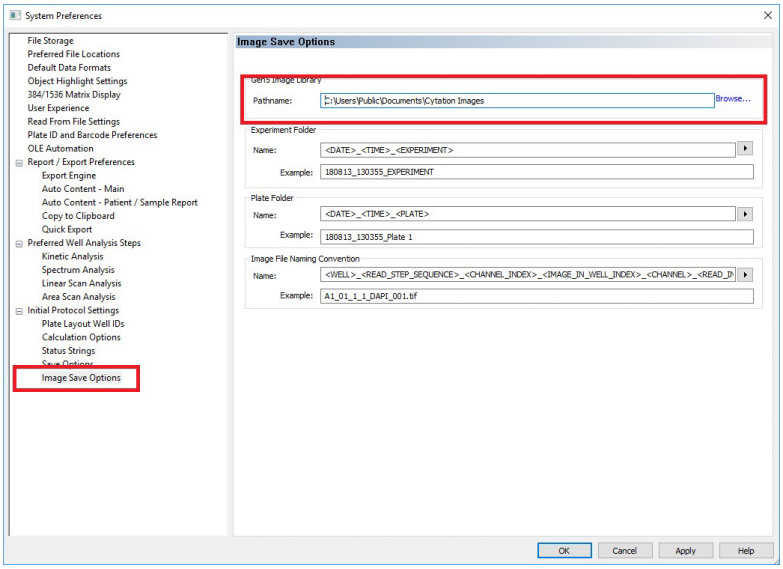


Figure 6 Image Save Options

XF Imaging & Cell Counting Image Database

Upon installation of XF Imaging & Cell Counting Software and Wave, an image database will be created. This database allows Wave to identify the correct image files for plates that have been imaged, and import those images and cell counts into the assay file. The default location for the database is on the computer's hard drive. If assay review and image import are completed away from the controller (for example, on a PC with Wave Desktop), the database must be moved to either a network location or a portable hard drive. Use the help menu in XF Imaging & Cell Counting and the Options menu in Wave to change the image database location, then restart both software programs. If a new file path is chosen, a new database will be created once the images are collected using XF Imaging & Cell Counting software. Figure 7 shows a screenshot of the Wave Options menu.

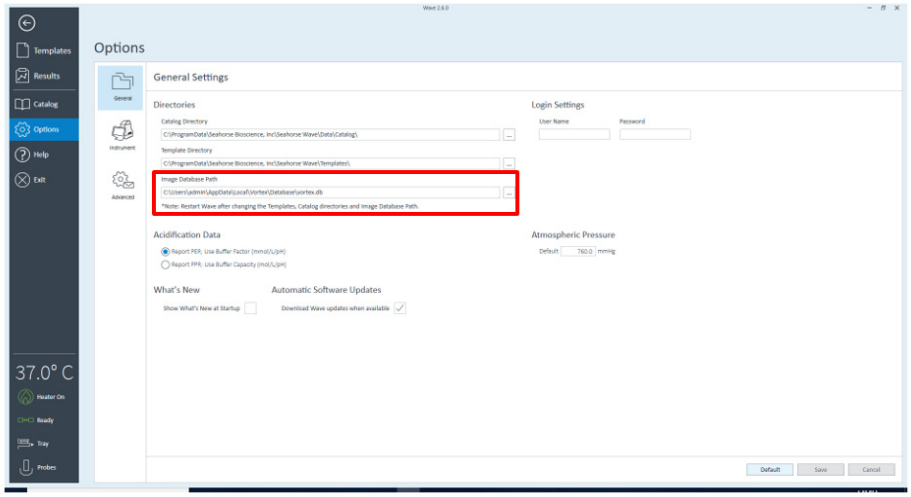


Figure 7 Wave Options Menu

NOTE If the database is changed after images were collected, import the images to .asyr files prior to moving the database. Alternatively, find the existing database and move it to the new file path.

Image library and database location setup examples

the following examples to help set up the image library and database locations:

Removable USB Storage Device Configuration:

Wave -> Options -> Database:
C:\Users\Admin\AppData\Local\Vortex\Database

Imaging & Cell Counting -> Help -> Database:
C:\Users\Admin\AppData\Local\Vortex\Database

Gen5 (Image Library)-> Settings -> Image Save Options:
D:\Gen5 Images

Networked Devices

If you are networking your Controller so that you can import cell counts and images remotely, the Imaging & Cell Counting database and Gen5 image library must be in a shared directory. For example, set up two shared directory locations:

\\shared server\shared directory\Vortex\Database

\\shared server\shared directory\Images

The settings in the software applications must reflect these directories:

Wave -> Options -> Database:

\\shared server\shared directory\Vortex\Database

Imaging & Cell Counting -> Help -> Database:

\\shared server\shared directory\Vortex\Database

Gen5 (Image Library)-> Settings -> Image Save Options:

\\shared server\shared directory\Images

Home screen

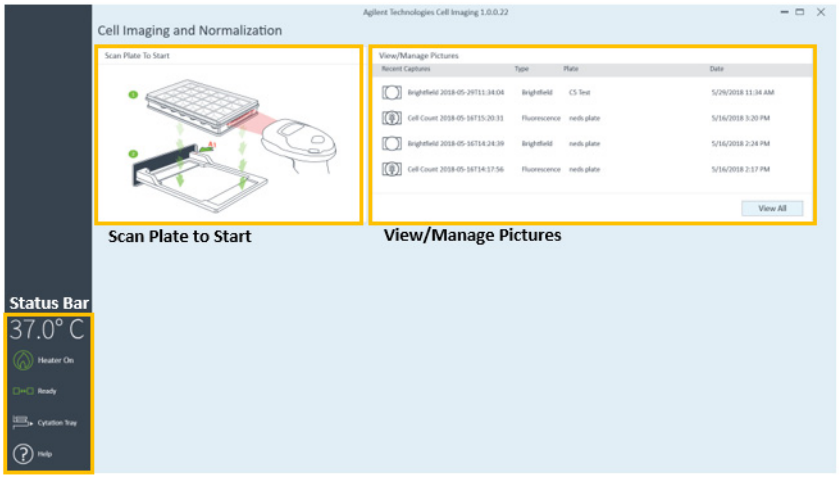


Figure 8 XF Imaging and Cell Counting Home Screen

Figure 8 on page 21 shows the main menu of the XF Imaging and Cell Counting Software. In this view, there are three sections of the screen that are interactive (each highlighted in yellow boxes).

Status bar

The bottom left section of the screen reveals the status of the instrument and software. This bar is available through all data collection and review screens as well. It is displayed from top to bottom as follows:

Current temperature

The internal incubation temperature of the Cytation instrument is shown.

Temperature control

The instrument heater status is shown (On or Off). To open options for turning the heater on/off, and setting the temperature, click the icon or text.

NOTE Note that the imaging protocols require the Cytation 1 temperature to be 37 °C during image collection.

Connection status

The connection status continually checks connections to both the handheld Barcode Wand and BioTek's Gen5 software. If either of these is disconnected, the icon turns red, and the software will be "Not Ready."

Cytation tray control

To manually open and close the Cytation tray, click the Cytation tray icon.

Help menu

Open the help menu for support and software information. For more details, see "Help menu" on page 23.

Scan plate to start

This section shows how to scan the plate barcode and place the plate on the tray holder to begin imaging protocols. This section is not interactive from the controller, a barcode must be scanned.

View/Manage pictures

This menu displays the four most recent image sets. To review previously imaged cell plates, click one of the sets shown or the **View All** button.

Help menu

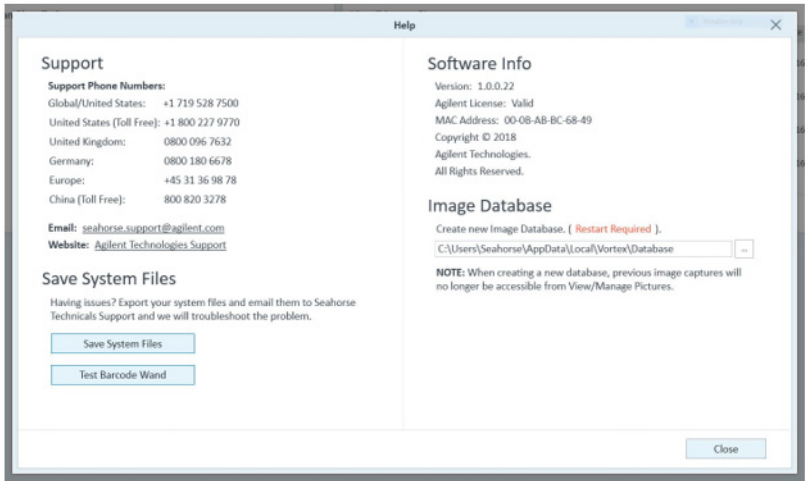


Figure 9 Help menu

The following paragraphs define the sections of the help menu.

Support

Use this section to find the phone number and email address to contact a member of our support team to assist you. Additionally, use the link to the Agilent Support website to find some technical resources.

Save system files

If the Technical Support team requests system files, use this button to find and send the appropriate files. The **Test Barcode Wand** button reveals an image of a barcode - use this to test the handheld barcode wand. Simply use the barcode wand to read the barcode displayed on the Controller's screen.

Software information

Details in this section display software and license information. This is where the MAC address, which is needed during XF Imaging and Cell Counting Software licensing, is found.

Image database

This section of the help menu displays the file path for the image database. This database must be the same in Wave (this can be found in Wave's Options menu). If the database location is changed, both XF Imaging and Cell Counting and Wave software should be restarted.

Features

Flagging

Upon review of both brightfield and fluorescently stained images, individual wells can be flagged for further review. For example, a brightfield image reveals one well is not as confluent as another; the well may be flagged in Cell Imaging by clicking the upper right-hand corner of the image during review, and the flags can then be imported into Wave.

Masked Images

Upon completion of fluorescent image scans, images may be reviewed for cell counting accuracy in Cell Imaging software. In this view, the option to **View Counted Cells** is available by clicking the checkbox in the lower right-hand section of the screen. (This feature is only available immediately after fluorescent images are collected; these are not saved, so they cannot be reviewed later). The overlay highlights the counted objects in the images to show efficacy of the counting algorithm.

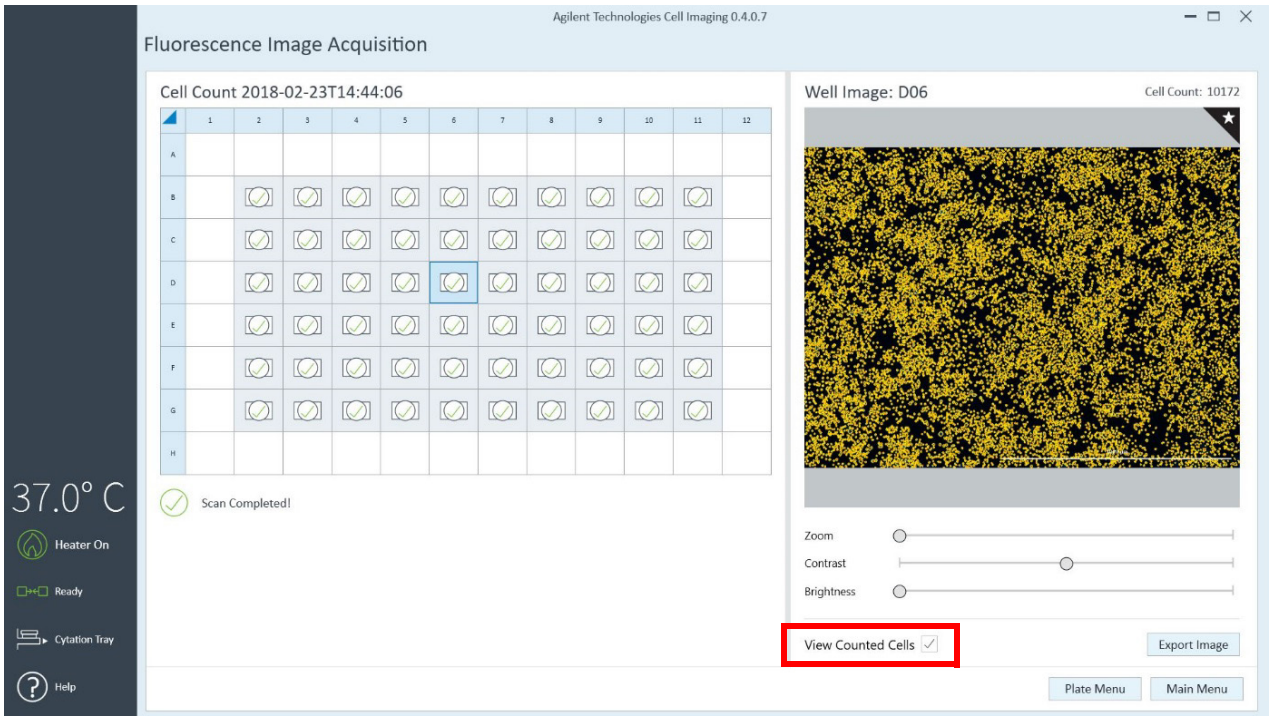


Figure 10 Viewing counted cells

Workflow

The procedure outlined in this guide assumes working knowledge of the XFe Analyzer and XF Assay Kits. This procedure can be adopted for use with any XF Assay Kit. For instructions on XFe Analyzers, Wave, and XF Assay kits, refer to each item's user manual. For more information on basic procedures needed to run an XF Assay, see the "Procedures to Run an XF Assay" on the Agilent Technologies, Inc. website, here.

The flowcharts in Figure 11, Figure 12, and Figure 13 display the XF Assay workflow with the Imaging and Cell Counting Application for Normalization.

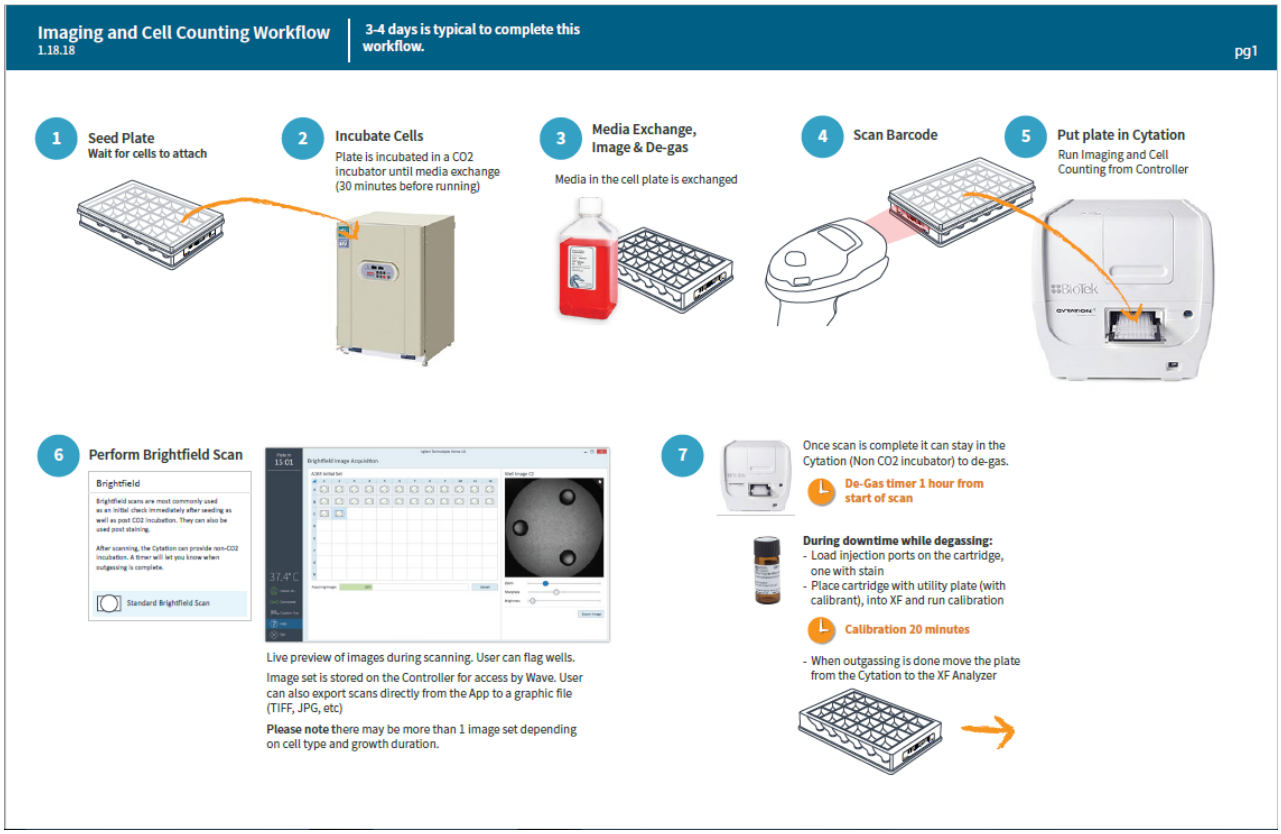


Figure 11 Imaging and cell counting workflow (1 of 3)

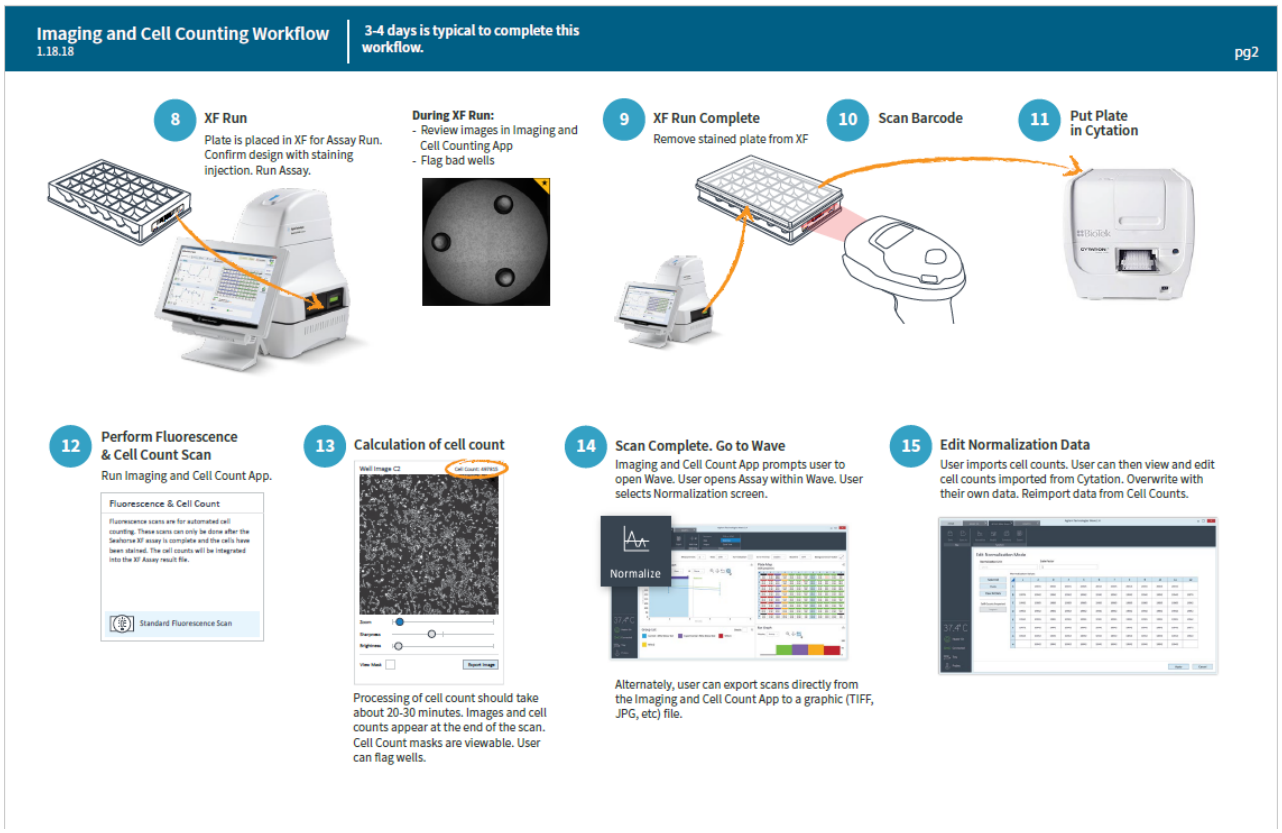


Figure 12 Imaging and cell counting workflow (2 of 3)

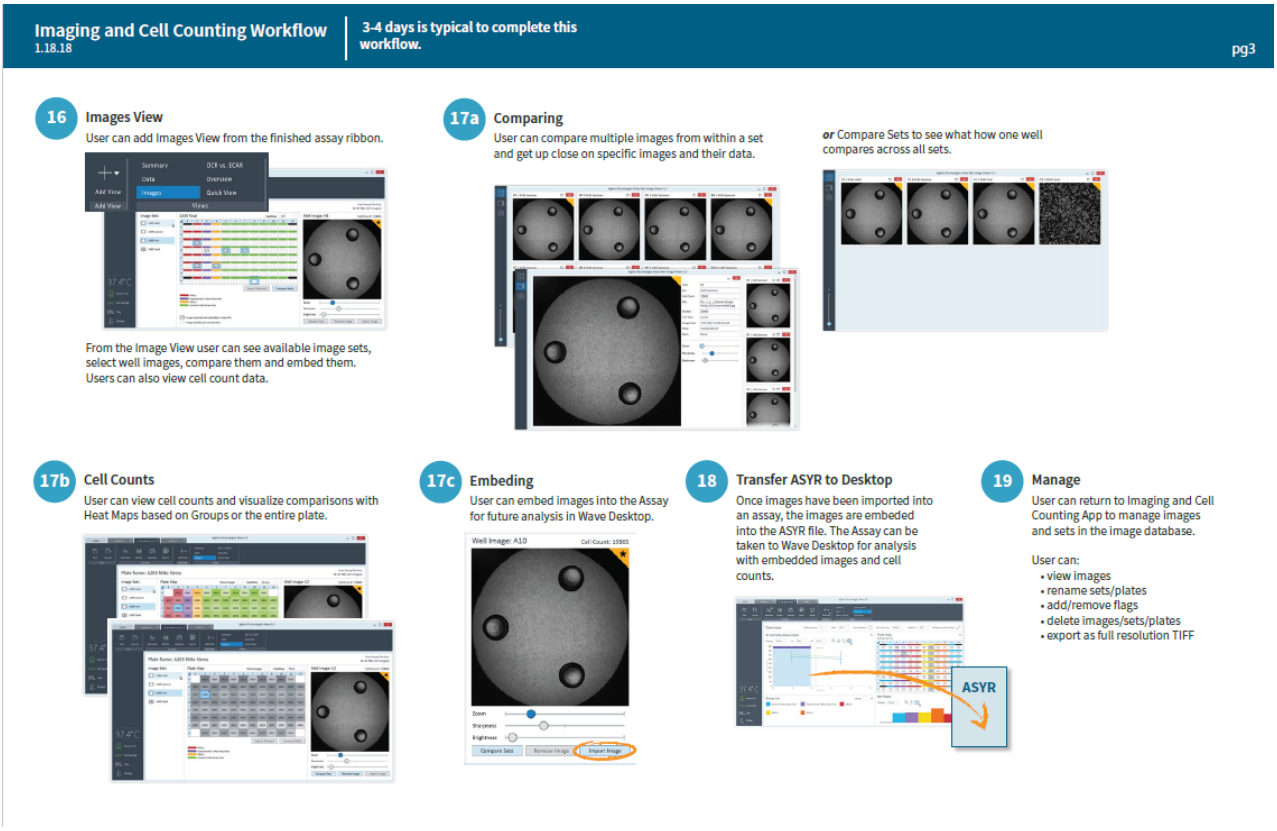


Figure 13 Imaging and cell counting workflow (3 of 3)

Important Notes

- XF software application updates may occasionally become available. Upon opening or restarting Wave or Imaging and Cell Counting applications, you may be prompted to update the software. For the most accurate and up-to-date features, update the applications when prompted.
- Images and the image directory should not be stored on the XFe Controller as it can cause instability in Wave and Cell Imaging if the disk becomes full. BioTek and Agilent recommend using a network location or external hard drive for image storage. (Directions for networking can be found here.)
- Gen5 software for the Cytation 1 must be licensed from BioTek Instruments, Inc. For help with the Cytation, Gen5 software and licensing, refer to BioTek.
- This is the recommended order of operations for running an XF Assay with Cell Imaging software. Downtime has been minimized or noted where applicable.

Day(s) Prior to Assay

1. Turn on the XFe Analyzer to allow it to warm up and equilibrate overnight.
2. Plate cells in Seahorse XF Cell Culture Microplates using cell culture growth medium.
3. Hydrate sensor cartridge in Seahorse XF Calibrant at 37 °C in a non-CO_2 incubator overnight.
4. Design experiment in Wave. See the instrument user manual for additional details.

Day of the Assay

Preparing and Running an XF Assay

Refer to the online guides in the links below for assistance in running XF Assays and for more information on Seahorse XFe Consumables and Kits:

- Procedures to Run an XF Assay
- Seahorse XFe Consumables

XF Cell Culture Microplate Preparation and Bright Field Imaging

1 Open the Cell Imaging application on the controller and ensure connectivity; wait for the **Ready** status in the lower left of the screen.

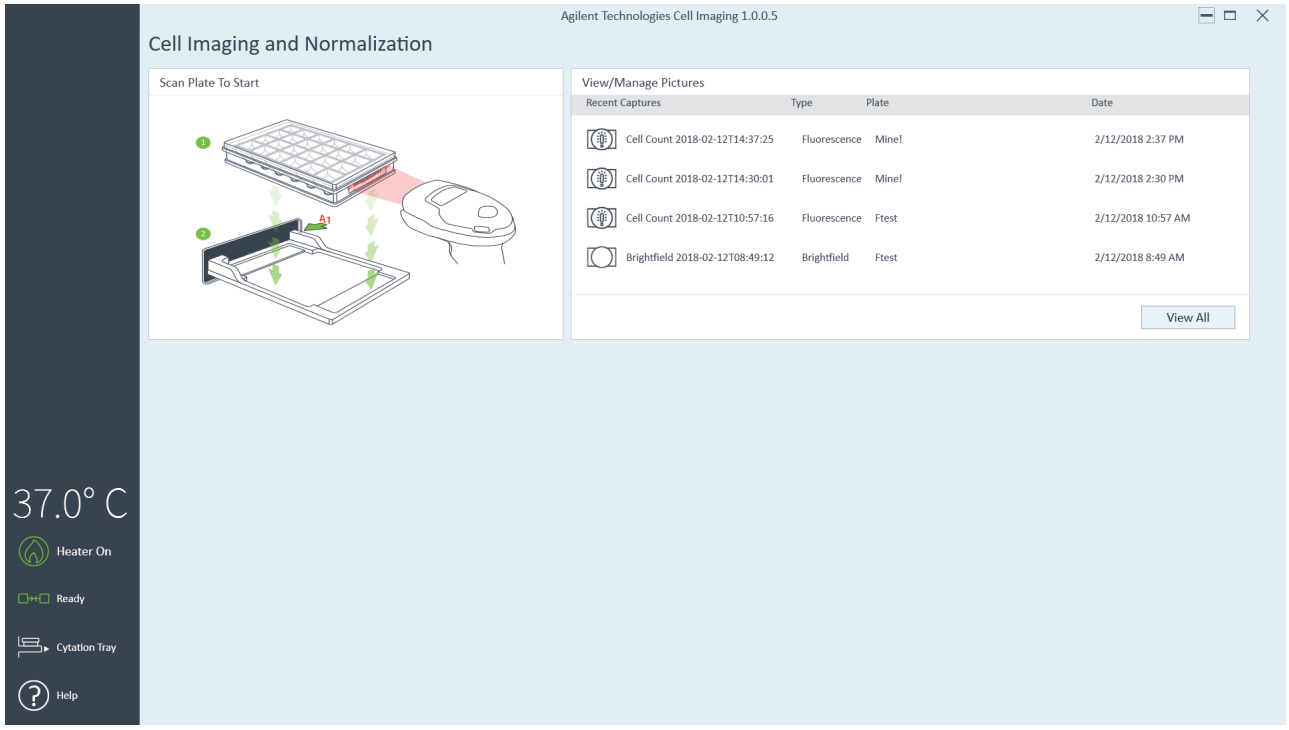


Figure 14 Imaging and Cell Counting application main menu

2 Prepare assay medium by supplementing Seahorse XF Base Medium. Recommendations for each XF Assay Kit can be found in their respective user guides.

3 Wash XF Cell Culture plate and change media to prepared assay medium.

4 Bring washed cell culture microplate to the Cytation instrument and navigate to the Cell Imaging application. Scan the barcode with the hand wand to initiate imaging procedure.

5 You will be prompted to name the cell plate. This name will be used for any set of images that is associated with that plate (both Bright Field and Fluorescent image sets). Then, click **Save**.

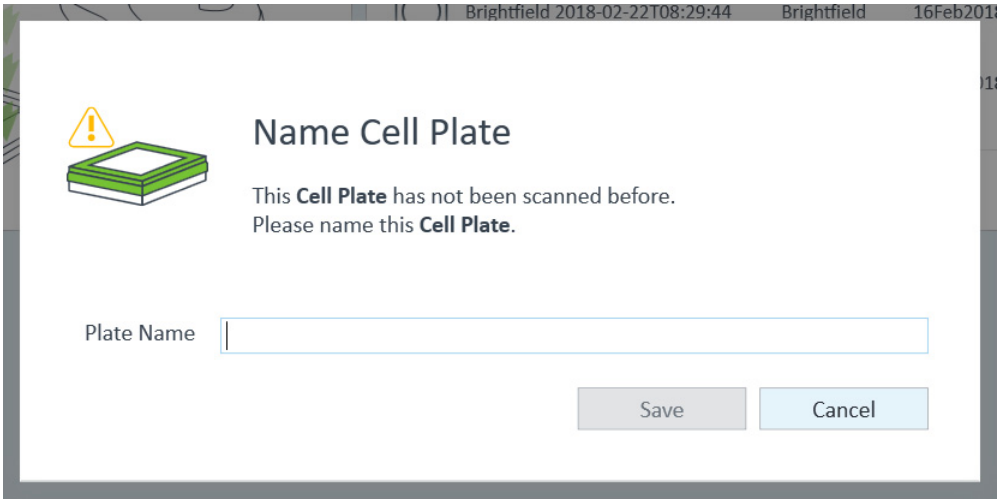


Figure 15 Prompt requesting cell plate name

6 The next screen shows the options for imaging protocols. Prior to the XF Assay, select **Start Brightfield Scan**. This page will also display any image sets that have previously been collected with this cell plate in the **Previous Scan for this Plate** menu.

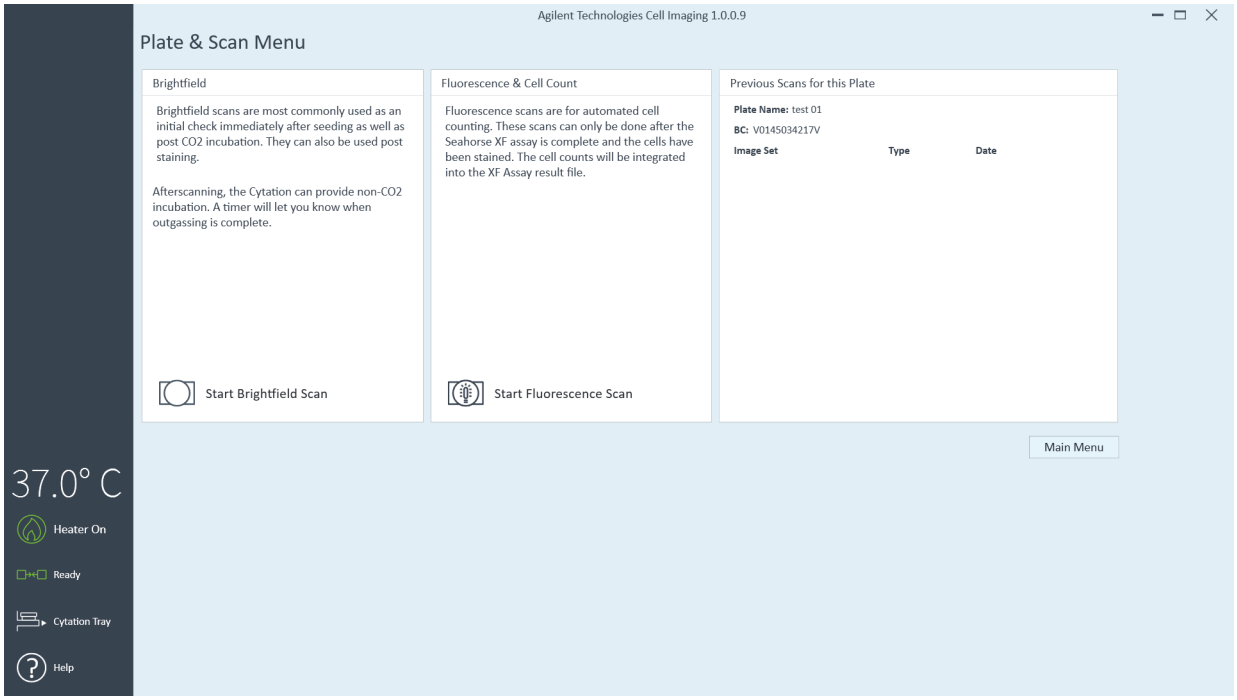


Figure 16 Plate & Scan menu

7 Place the cell culture microplate on the holder of the Cytation instrument. Keep the cell plate lid ON. Use the image in the dialog box to check if the orientation of the microplate is correct, then click **Close Tray**.

NOTE Agilent highly recommends a standard practice of wiping the bottom surface of the microplate with ethanol/isopropyl for better quality images.

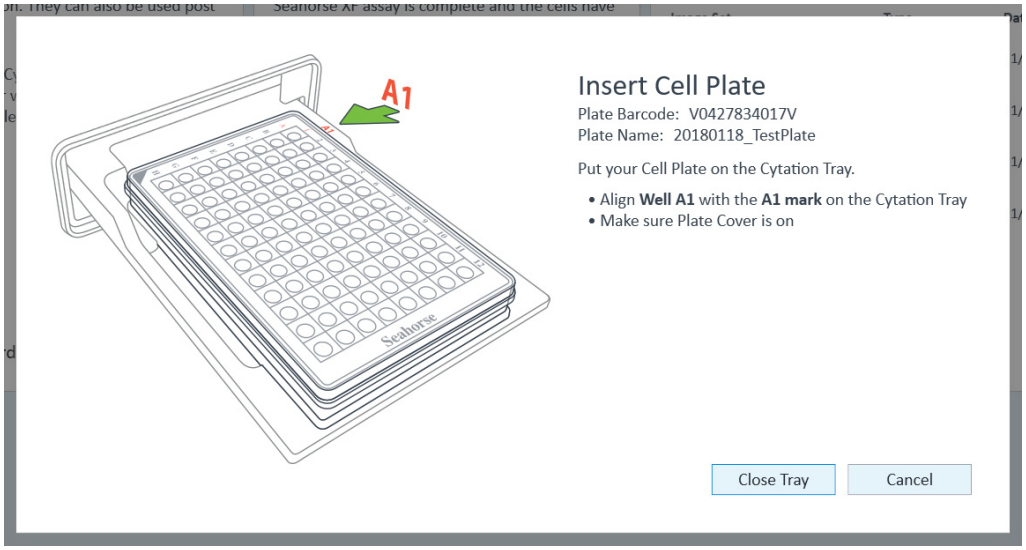


Figure 17 Insert cell plate dialog box

8 The next screen shows the image acquisition screen. On the plate map, you may select specific wells to be imaged, as is shown, or click **Scan All Wells** to image the entire plate.

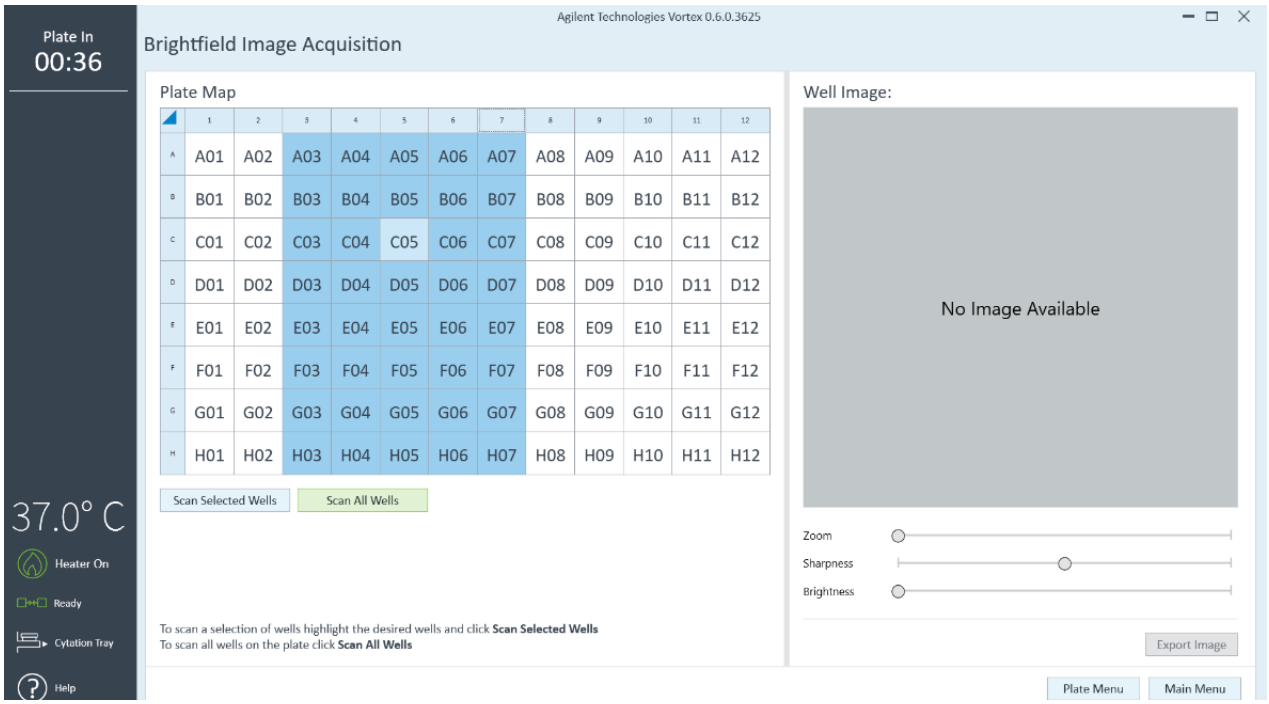


Figure 18 Well selection screen

9 Before images are collected, there are a few initialization steps that may take up to 2 minutes. (Temperature must reach 37 °C inside the Cytation and there are auto-exposure and white-balance steps).

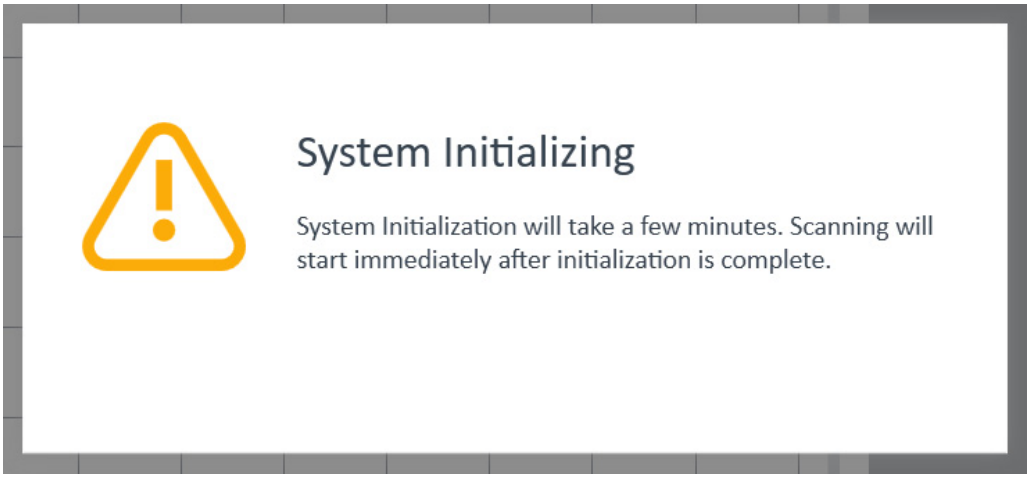


Figure 19 System initialization

10 An entire XF96 well plate may take up to 35 minutes to image. During this time, prepare the XF Assay Kit and load the sensor cartridge. While the cell plate is in the Cytation instrument, the plate is heated at 37 °C in a non-CO_2 environment. Cell Imaging will keep a timer for cell plate outgassing, and the plate will stay in the Cytation until the user removes it for the XF Assay. Total outgassing time is 1 hour.

XF Assay Preparation

1. Prepare XF Assay Kit Compounds as directed in the appropriate XF Assay Kit user guide.
2. Add Hoechst stain to the final reagent in the injection scheme for your XF Assay. Alternatively, add an injection to the assay for the Hoechst dye.
   a. We recommend optimizing the concentration of Hoechst dye needed for your cells. Our testing was completed with Hoechst 33342 from Thermo Scientific; Catalog Number PI62249.
   b. Create a titration of Hoechst dye over a plate seeded with the same number of cells in each well; we recommend a starting point of 20 µM injection solution concentration.

   The dye is most active and best imaged within 20-45 minutes of injection. By default, you may need 3 to 5 measurement cycles after the final injection. This equates to 18-30 minutes on the XFe96 and 24-40 minutes on the XFe24 after the injection, but before the plate may be removed to be imaged.
3. Load prepared reagents in XF Sensor Cartridge drug ports. Ensure reagents including Hoechst dye are included in the final injection of the series. Alternatively, designate an additional port after any reagents have been injected to add the Hoechst dye. Use the table below for injection port volume recommendations:

	XFe96 Starting Well Volume (180 µL)	**XFe24 Starting Well Volume (500 µL)**
Port	**Injection Volume (µL)**	**Injection Volume (µL)**
A	20	56
B	22	62
C	25	69
D	27	75

4. Begin XFe Assay calibration: using an existing template or create your own design, load the cartridge and begin calibration. It will take about 20 minutes before the cartridge is calibrated - this can be performed while the cell plate is in the Cytation instrument.
5. Once outgassing and cartridge calibration have both completed, remove the cell culture plate from the Cytation instrument, click **Load Cell Plate** in Wave, and move the cell plate to the XFe instrument for reading.

After XF Assay Completion

NOTE The following wells must be seeded and stained to ensure successful initialization of autofocus and autoexposure:

XF96: D5 and E8

XF24: B3 and C4

1. Upon completion of the XF Assay, remove the plate from the XFe Analyzer.
2. Carefully remove the sensor cartridge and replace the cell plate lid. The cells are ready for fluorescent imaging and cell counting.
3. It is helpful to check the cartridge or the XF Assay data for any injection failures. If the reagent containing Hoechst dye failed to inject, the cell count will not be appropriate.
4. Scan the plate barcode with the handheld barcode reader. If the plate has already been imaged, it will not require a new name.
5. Select **Fluorescence & Cell Count**,
6. Place the cell plate on the Cytation tray holder.
7. Wipe the bottom of the plate with ethanol/isopropyl.
8. Click **Close Tray**.
9. In the image acquisition window, select the wells to be imaged, or select **Scan All Wells** to begin imaging. Fluorescence imaging takes about 15-20 minutes to scan the entire plate.

NOTE Images are not available for review while they are being collected. Once the Cytation 1 has collected all images and counted each well, the images will be available for review.

10 Review fluorescent images and cell counts in the Imaging and Cell Imaging application. Notice that there is an option to **View Counted Cells** (highlighted in the figure below). This option shows a 'masked' image, highlighting the objects that were counted.

NOTE

Note that the algorithm uses the brightness of the stained nuclei to determine whether it is an 'object' or not. Some common counting problems include variation in intensity and failed dye injections.

Be sure to optimize the concentration and incubation time for Hoechst dye and check for injection failures.

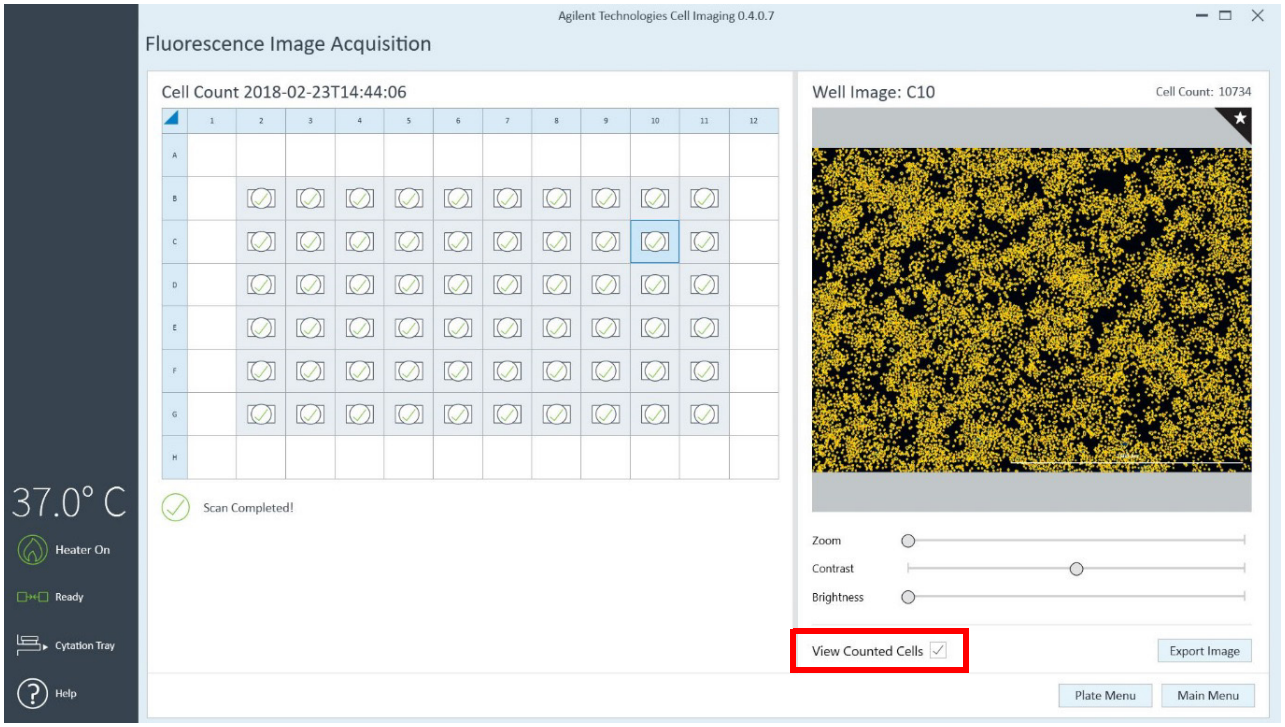


Figure 20 Reviewing fluorescent images

11 If the images are satisfactory and the cell plate and sensor cartridge are no longer needed, they may be discarded appropriately.

XF Data Normalization

1 After image collection is complete, image review and data normalization can be performed in Wave. From the Wave application, open the appropriate assay file.

2 To review images, use the **Add** button and choose **Well Images**. This adds the images to your available Views.

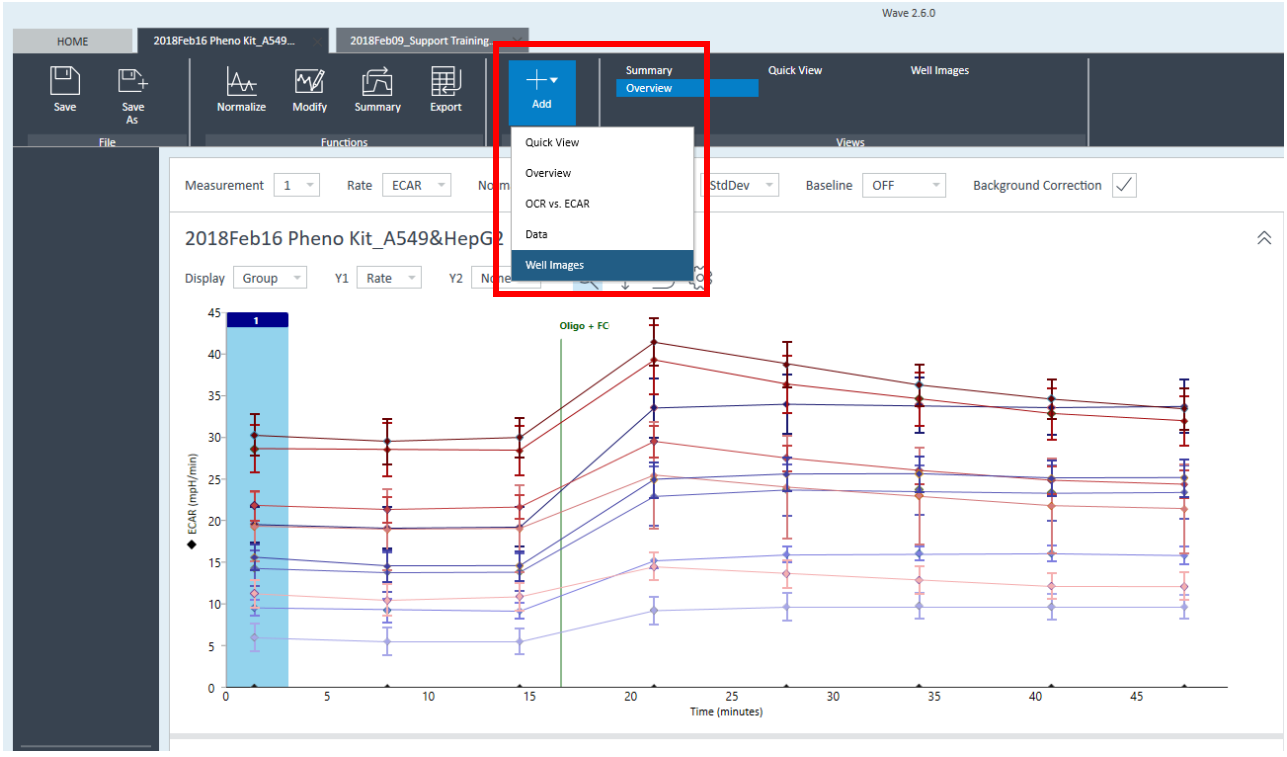


Figure 21 Adding images to your available views

3 On the **Well Images** tab, choose Bright Field or Fluorescent images from the **Image Sets** menu. Click on each well to review the images. You may also compare image sets and flag wells using the star in the upper right-hand corner of the images (highlighted in yellow).

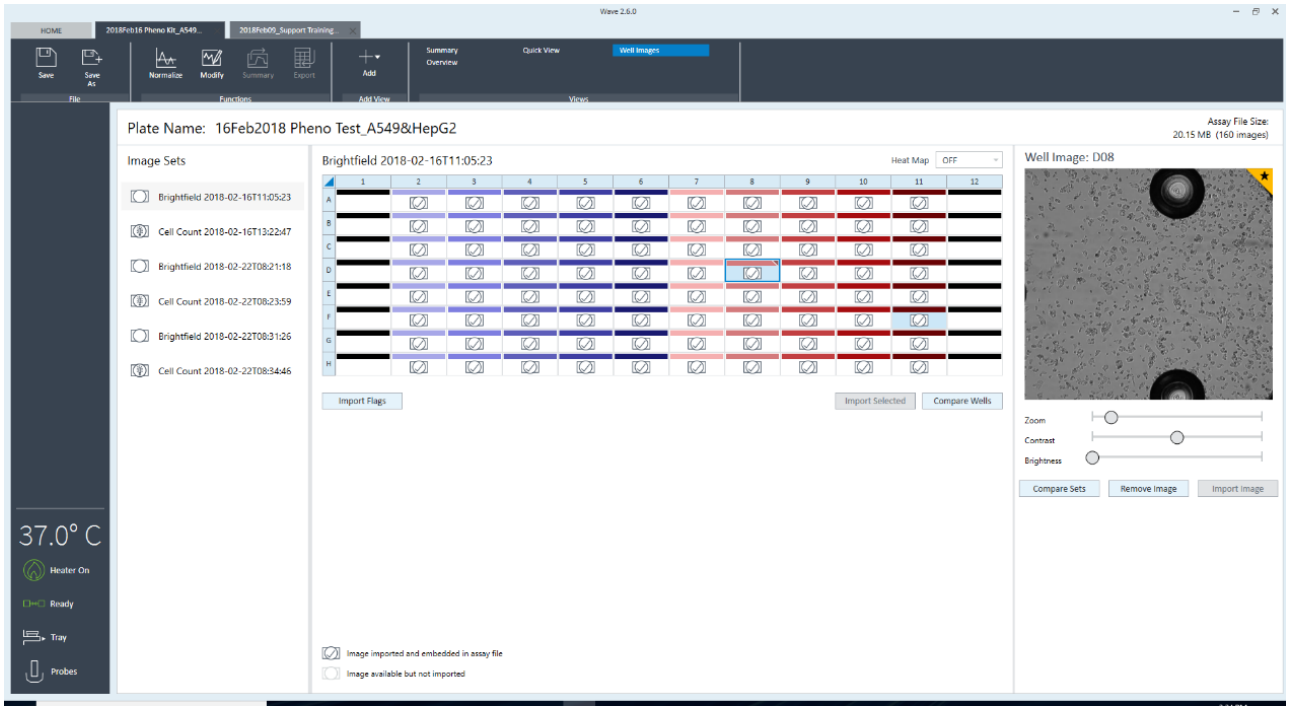


Figure 22 Flagging an image for comparison

4 Heat Maps are also available in this view. The heat map uses cell count values to visually display cell counts. Click the **Heat Map** drop-down list box and choose to either view by group, or across the entire plate:

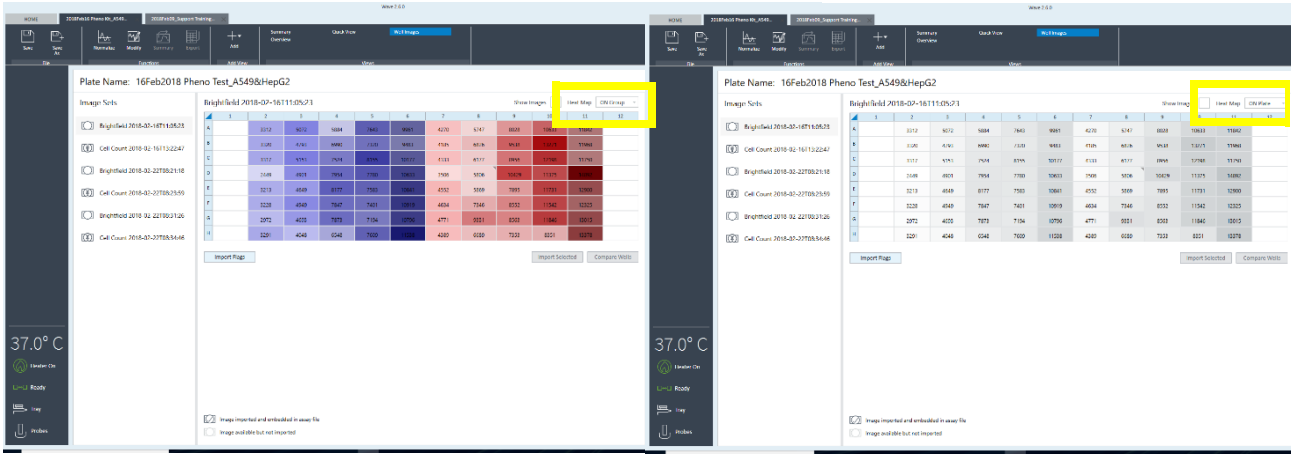
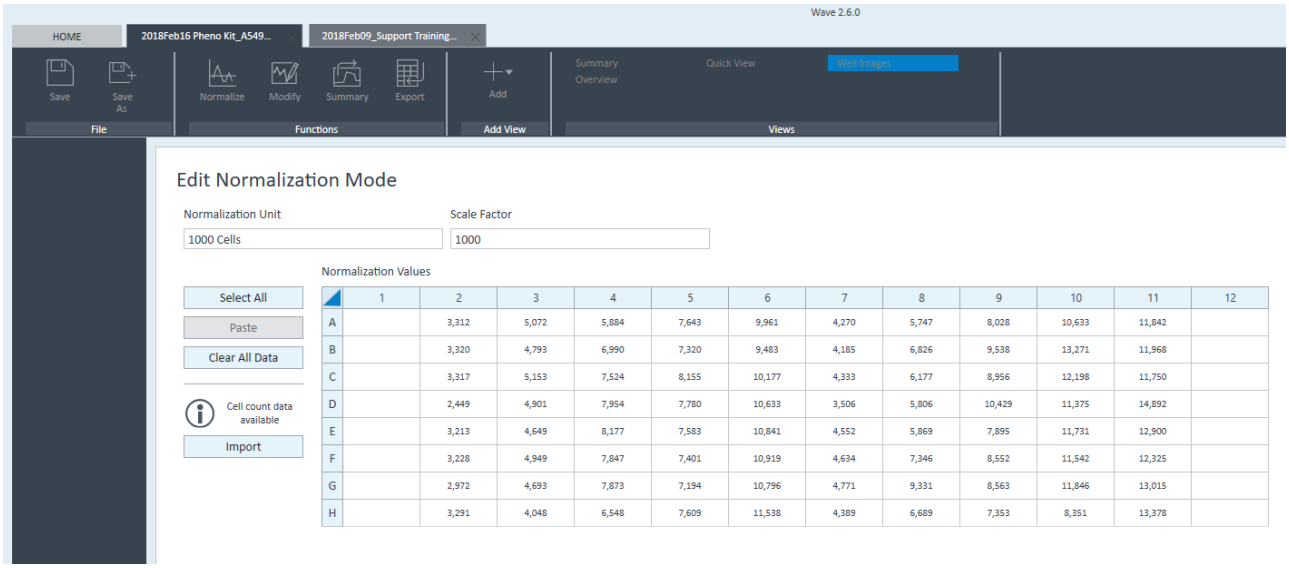


Figure 23 Viewing heat maps

5 To Normalize XF Data using the cell counts collected, navigate to the **Normalize** tab of the **Functions** options. Notice that there should be an ⓘ symbol noting that **Cell count data available**.

6 Click **Import** to fill in the table with cell counts. Note that wells defined as background will remain empty (there are no cells in these wells).



	1	2	3	4	5	6	7	8	9	10	11	12
A		3,312	5,072	5,884	7,643	9,961	4,270	5,747	8,028	10,633	11,842	
B		3,320	4,793	6,990	7,320	9,483	4,185	6,826	9,538	13,271	11,968	
C		3,317	5,153	7,524	8,155	10,177	4,333	6,177	8,956	12,198	11,750	
D		2,449	4,901	7,954	7,780	10,633	3,506	5,806	10,429	11,375	14,892	
E		3,213	4,649	8,177	7,583	10,841	4,552	5,869	7,895	11,731	12,900	
F		3,228	4,949	7,847	7,401	10,919	4,634	7,346	8,552	11,542	12,325	
G		2,972	4,693	7,873	7,194	10,796	4,771	9,331	8,563	11,846	13,015	
H		3,291	4,048	6,548	7,609	11,538	4,389	6,689	7,353	8,351	13,378	

Figure 24 Normalizing XF Data using the cell counts collected

7 Upon cell count import, the default scale factor is 1000 and the normalization unit is 1000 cells. Ensure scaling is appropriate, then click **Apply** to view normalized XF Data.

NOTE Please note that importing images and cell counts with Wave Desktop (on a PC) is not currently supported with the use of the Cytation 5. Images and cell counts must be imported to the .asyr file using the XFe Analyzer's Controller.

4 Licensing Seahorse XF Imaging and Cell Counting Software

Logging into SubscribeNet 42
Registering the Software 43

The Cell Imaging software allows a 30-day window for initial registration. If the controller cannot be networked, registration can be completed on a PC or laptop. An authorization code required for activating the XF Imaging and Cell Counting software license is delivered along with the rest of the normalization solution components.

Logging into SubscribeNet

Agilent SubscribeNet is an Electronic Software Delivery service provided by the Global Software Distribution Center for Agilent employees and Non-Agilent workers.

For customers of Agilent log in here:

https://agilent.subscribenet.com

For Agilent employees log in here:

http://softserv.cos.agilent.com/register.html

Registering the Software

1 To connect the controller to a local network, refer to the Networking guide.

2 Open SubscribeNet and select **CLICK HERE** at the bottom of the screen

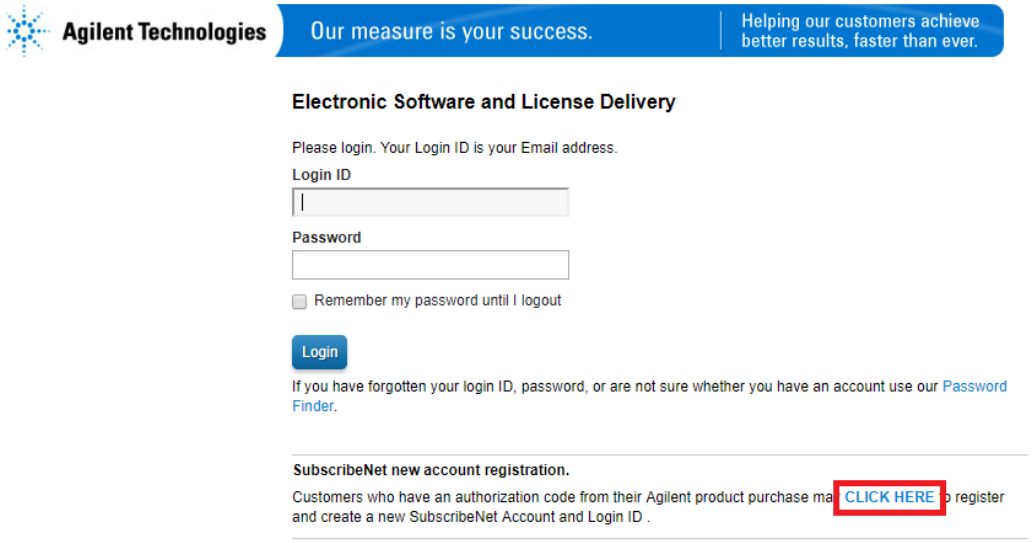


Figure 25 Registering a new account

3 Fill in the required fields and click **Submit**.

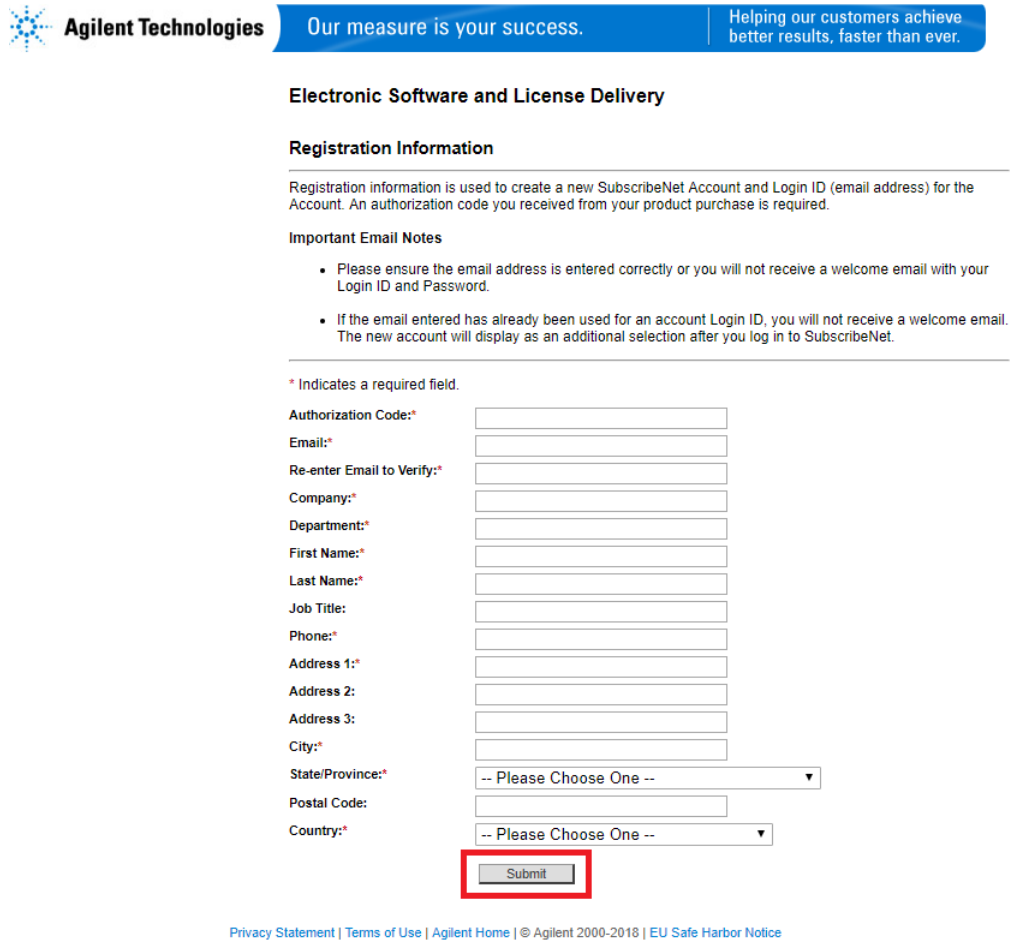


Figure 26 Registration information

After registration has been submitted, a new screen will appear with the license.

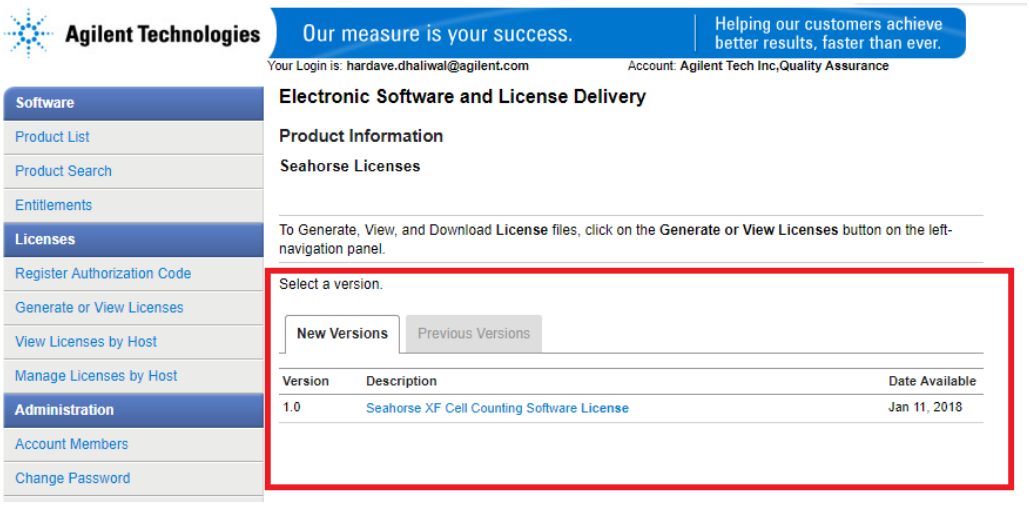


Figure 27 Selecting the license

4 Select the **Seahorse XF Cell Counting Software License**. A screen will appear, prompting for an Authorization Code. This is found on the sticker received with the upgrade.

5 Enter the code and click **Submit**.

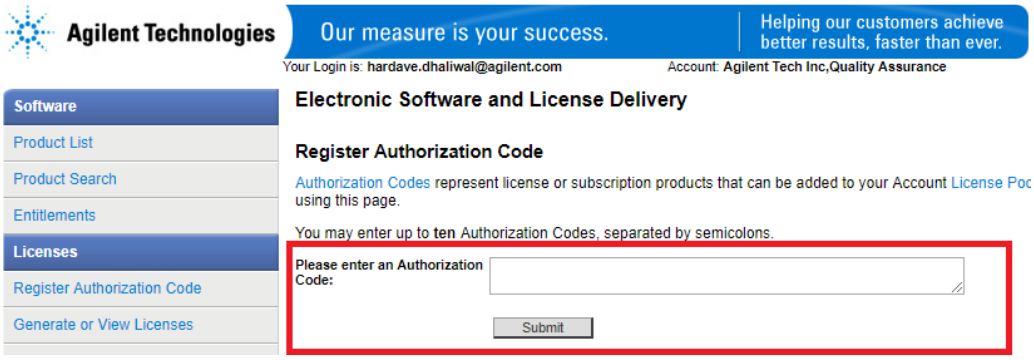


Figure 28 Entering the authorization code

6 The next dialog prompts the generation of the license. Choose the check box next to the desired license and then click **Generate**.

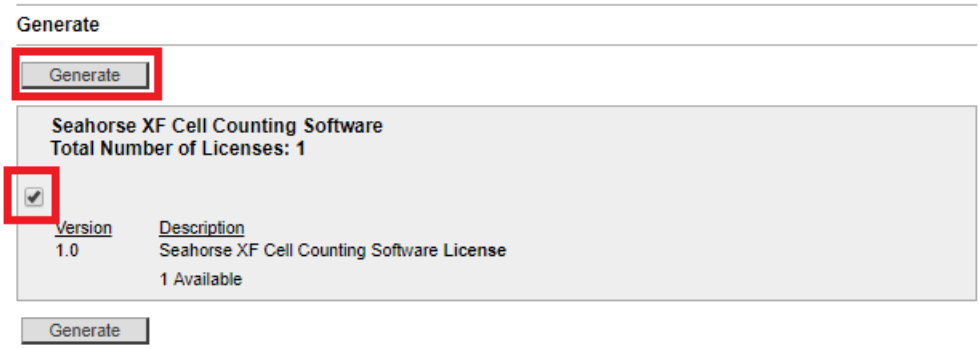


Figure 29 Generating the license

7 The next screen requires the MAC address. Find the MAC address on the controller.

a Open the Cell Imaging application.

b Navigate to the Help menu to find and copy the MAC address.

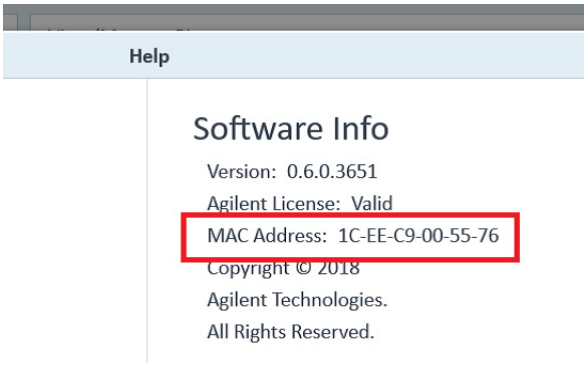


Figure 30 Finding the MAC address

8 Paste the MAC address into the **Host ID MAC Address** bar.

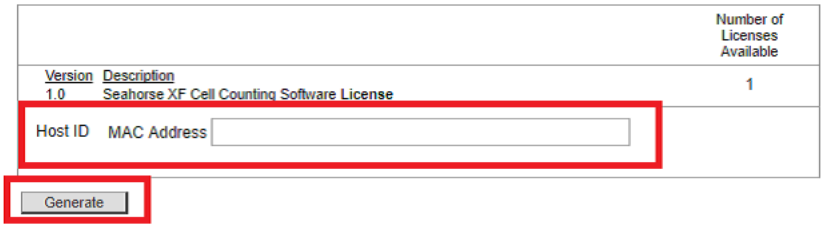


Figure 31 Adding the MAC address

9 Choose the check box next to the desired license and then click **Generate**.

10 Click **Download License File**. This file will be saved in the "Download" folder. The title of the file will be the MAC address.

11 Copy this license file to the folder path shown below:

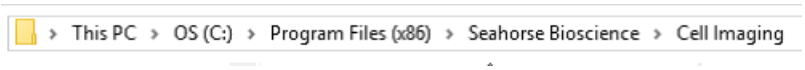


Figure 32 License path

12 Rename the file to "vortex.lic"

13 Return to the **Help** menu in the Cell Imaging application to verify that the Agilent License is valid. The software may need to be closed and reopened to show the status.

14 If a controller needs to be replaced, the license may be returned for another and setup on the new controller. In SubscribeNet, click **Return** and follow the instructions.

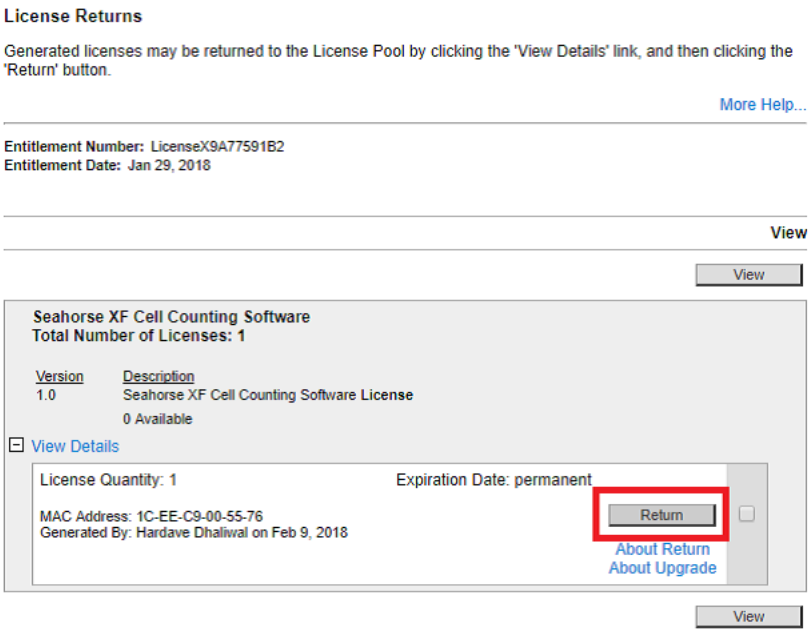


Figure 33 License returns

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