

Guide to setting up hypoxic conditions on the EVOS FL Auto Imaging System with Onstage Incubator

Cellular responses to reduced oxygen—hypoxic conditions—have been linked to a wide range of human pathologies, including tumor development, atherosclerosis, inflammation, and abnormal angiogenesis. Although the importance of hypoxia in inducing these conditions is well known, creating model systems to accurately control the hypoxic conditions is extremely difficult for most researchers without access to elaborate systems that allow maintenance and precise control of temperature, humidity, and gases (CO₂ and O₂) during an experiment. The Invitrogen™ EVOS™ FL Auto Imaging System with Onstage Incubator provides an environmental chamber allowing for the precise control of oxygen levels, thereby delivering an effective system for researchers to evaluate cellular responses to hypoxia by long-term fluorescence live-cell imaging. Here we provide step-by-step instructions for setting up hypoxic conditions with the EVOS FL Auto Imaging System with Onstage Incubator.

In this particular study, response to cellular hypoxia was visualized using Invitrogen™ Image-iT™ Hypoxia Reagent. Prior to live-cell imaging, A549 cells were labeled using this fluorescent hypoxia indicator to visualize the cellular response to changing oxygen levels, and Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent was used to label nuclei in all cells. In normal conditions, the Image-iT Hypoxia Reagent is nonfluorescent but increases in fluorescence as oxygen levels are decreased in the cells. The NucBlue reagent, a form of Hoechst 33342, acts as a counterstain that is used for autofocusing throughout the experiment because its fluorescence remains relatively unchanged under hypoxic conditions.



I. Cell preparation (example)



1. Cells were plated to be at 70% confluency the following day and allowed to grow overnight at 37°C in an incubator set at normoxic conditions (~20% O₂).



2. A 1 mM stock solution of Image-iT Hypoxia Reagent (Cat. No. H10498) was prepared in DMSO by adding 1.45 mL of DMSO to the lyophilized powder.



3. Growth medium (MEM, 10% FBS) containing 10 mM Image-iT Hypoxia Reagent and 2 drops/mL of NucBlue Live ReadyProbes Reagent (Cat. No. R37605) was added to the cells.



4. Cells were placed on the EVOS FL Auto Imaging System (Cat. No. AMAFD1000) equipped with the EVOS Onstage Incubator (Cat. No. AMC1000) and incubated for 60 minutes to allow the system to reach the required temperature (37°C), humidity (>80%), and CO₂ level (5%).

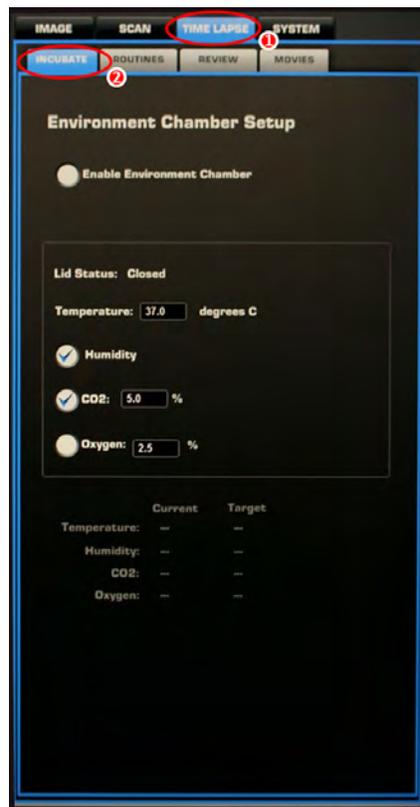


5. The EVOS FL Auto Imaging System was equipped with a DAPI Invitrogen™ EVOS™ Light Cube (Cat. No. AMEP4650) and a custom EVOS Light Cube to detect the NucBlue Live ReadyProbes Reagent and Image-iT Hypoxia Reagent, respectively, in the A549 cells. The custom EVOS Light Cube contained a GFP excitation source (488 nm) and an RFP emission filter (610 nm). In this experiment, NucBlue Live ReadyProbes Reagent was used as a counterstain for autofocusing on cells during the time-lapse experiment. If the use of a counterstain is not desired, the transmitted light channel can be used for autofocusing.
6. If you do not have an appropriate custom EVOS Light Cube, an RFP EVOS Light Cube (Cat. No. AMEP4652) can be used to capture a portion of the signal from the Image-iT Hypoxia Reagent and may be sufficient for data generation.

II. Setting up the EVOS FL Auto Imaging System and Onstage Incubator

1. Connect the EVOS Onstage Incubator to separate CO₂, nitrogen, and compressed air lines. Set the CO₂ tank between 10 and 20 psi, and the compressed air and nitrogen tanks to 40 psi.
2. Fill the humidification chamber in the front of the onstage incubator with deionized water.
3. Apply the fluorescent labels to your cells (Section I) and place the cells on the stage of the EVOS FL Auto Imaging System, using the appropriate vessel holder inserted into the EVOS Onstage Incubator master stage plate (thermofisher.com/evosvesselholder).
4. Place the Stagetop Environmental Chamber over the cells.
5. On the main screen of the EVOS FL Auto Imaging System software, select the "TIME LAPSE" tab (1) and then select the "INCUBATE" tab (2).
6. Under "Environmental Chamber Setup", select the "Enable Environment Chamber" button.

Step 5



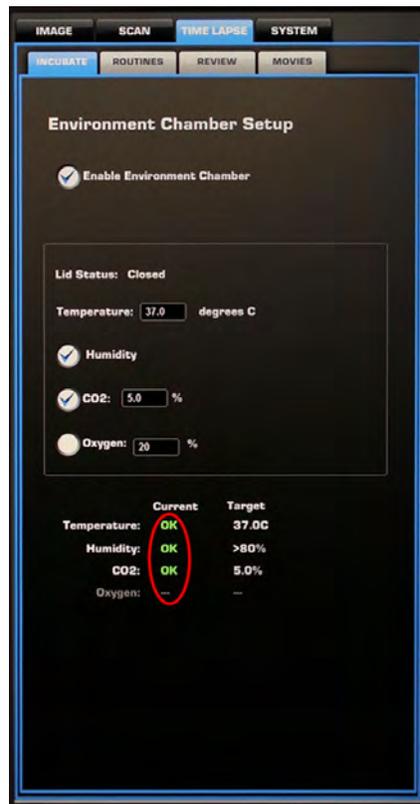
Step 6



II. Setting up the EVOS FL Auto Imaging System and Onstage Incubator (continued)

- Following equilibration of the humidity, temperature, and CO₂ levels (approximately 60 minutes), a green “OK” will be shown in each of these categories.
- Once the chamber in the EVOS Onstage Incubator is at equilibrium, adjust the light intensity and focal plane in the DAPI channel. Select the DAPI channel and then select “Capture focus nominal” to make this the channel used to autofocus before image acquisition throughout the experiment.
- To determine the best level of light intensity for capture of an increase in hypoxia signal, select the channel for the hypoxia marker (“GFP” in this case) and bring up the light intensity levels until an outline of cells are seen, then turn down the light intensity until the screen is black to create a baseline for the normoxic (20%) time point.

Step 7

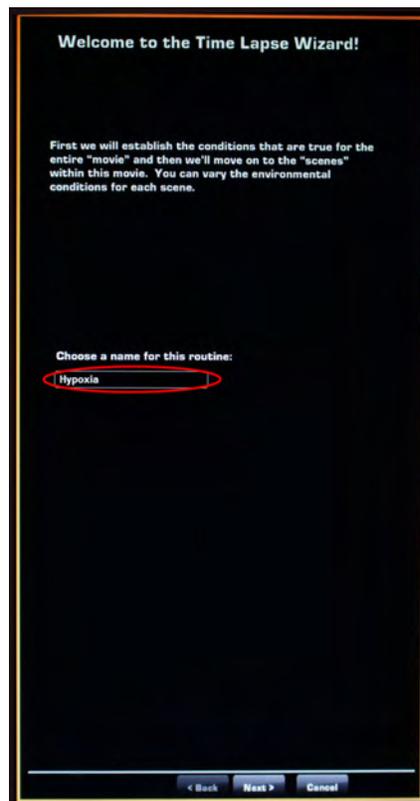


Step 10



- To set up the program for automatic image acquisition, select “TIME LAPSE” (1), then “ROUTINES” (2).
- Select “Create New Routine”, name the routine, and touch the “Next” button.
- Select the objective (1) and camera (2). Choose DAPI and the custom filter (3) for channels to acquire data. Select “Auto Find Sample” (4) and the DAPI channel (5) for autofocus on the cells, then touch “Next” (6).

Step 11



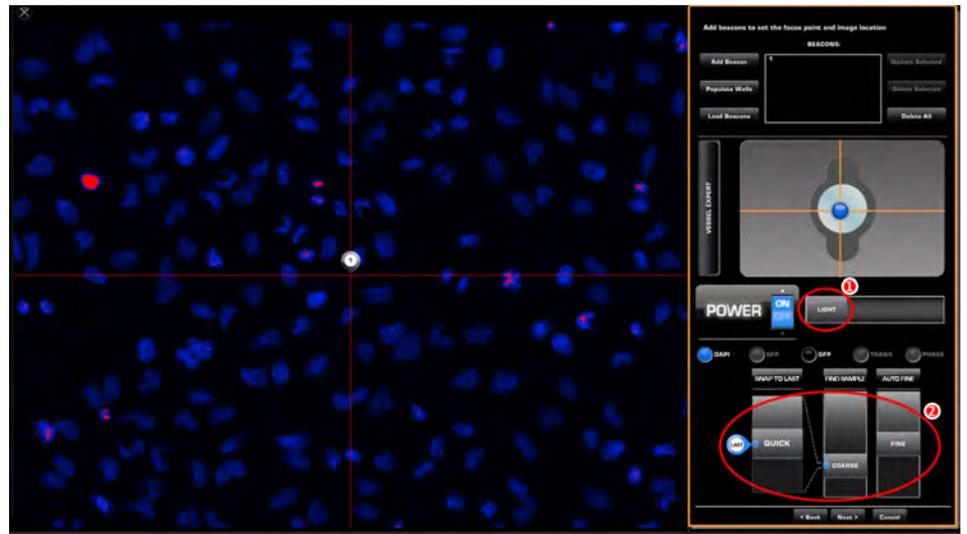
Step 12



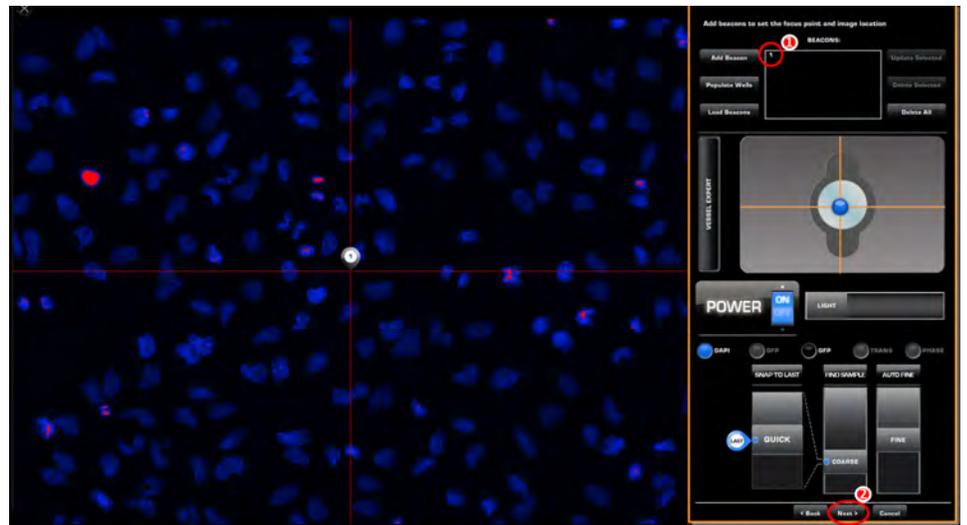
13. Check the light intensity (1) and focal plane in the DAPI channel (2), and adjust as necessary.

14. Add a “Beacon” (area to acquire data) by selecting “1” (1). Add as many beacons as desired. For this experiment, data were acquired at only one location. Select “Next” (2).

Step 13



Step 14



II. Setting up the EVOS FL Auto Imaging System and Onstage Incubator (continued)

15. Choose either “TIFF” or “PNG” file format for data acquisition and export (1). To have signals from each channel saved in addition to the overlay, select the “Save Individual Channels” button (2). Select “Next” (3).

16. Choose the number of scenes or time points in the routine. For the initial normoxic baseline data point, the oxygen level was set at 20%. Subsequent data points were established at the following oxygen levels: 5%, 2.5%, 1%, and 20%, for a total of 6 scenes or time points.

17. For each scene, set the time and the capture interval or frequency of image acquisition. For scene 1 and the all of the following scenes, oxygen levels were changed every 30 minutes (1) and two images were captured during that time period (at 15 min and 30 min) by setting the capture interval at 15 minutes (2).

18. Enter the appropriate oxygen level (20%, 5%, 2.5%, 1%, or 20%) for each scene or data point (1). After the information is entered for the scene or time point, touch “Next” (2).

Step 15

The screenshot shows the 'Time Lapse Wizard' interface. At the top, it asks 'What image file format would you like to use?' with two radio buttons: 'PNG' (1) and 'TIFF' (2). Below this, there is a checked checkbox for 'Save Individual Channels' (2). Further down, there are three radio buttons: 'Create video for each beacon', 'Include the following information as a watermark:', and 'Capture one frame only'. Under the watermark option, there are four checked checkboxes: 'Image', 'Time Lapse', 'Incubator', and 'Life Logo'. At the bottom, there are three buttons: '< Back', 'Next >' (3), and 'Cancel'.

Step 16

The screenshot shows the 'Time Lapse Wizard' interface. It asks 'Set the number of scenes for a movie.' and provides a definition: 'A "scene" is a convenient way to represent one set of environmental conditions.' Below this, there is a text input field for 'Number of scenes:' with the value '6' entered (1). At the bottom, there are three buttons: '< Back', 'Next >', and 'Cancel'.

Step 17

The screenshot shows the 'Time Lapse Wizard: Scene 1' interface. It asks 'How long is this scene?' with a 'Duration' field set to '30' and a dropdown menu set to 'Minutes' (1). Below this, it asks 'What is the capture interval?' with a 'Capture images every' field set to '15' and a dropdown menu set to 'Minutes' (2). At the bottom, there are three buttons: '< Back', 'Next >', and 'Cancel'.

Step 18

The screenshot shows the 'Time Lapse Wizard: Scene 1' interface. It displays '1080 minutes, 55 images' and a checked checkbox for 'Use Environment Chamber'. Below this, there are several environmental parameters: 'Temperature: 37.0 degrees C', 'Humidity' (checked), 'CO2: 5.00 %', and 'Oxygen: 20.00 %' (1). At the bottom, there are three buttons: '< Back', 'Next >' (2), and 'Cancel'.

19. Enter the duration and capture interval for the second scene or time point. The same values were used for all of the scenes in this routine. Continue this process until all scenes are completed.

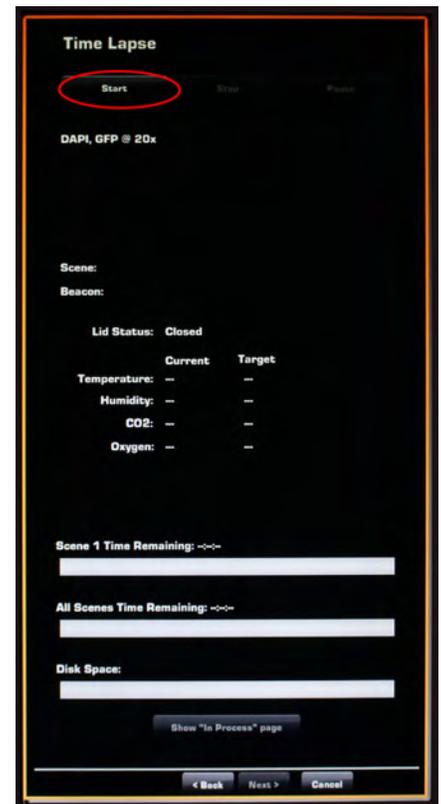
20. Once you have set up the conditions for all of the scenes, select a location to save the routine, and then touch “Next”.

21. Begin the time-lapse experiment by touching “Start”.

Step 20



Step 21



III. Analysis of hypoxia data

By providing precise control of temperature, oxygen, and humidity levels, the EVOS FL Auto Imaging System with Onstage Incubator delivers a long-term live-cell imaging environment for detailed analysis of cells under hypoxic conditions. In this demonstration, hypoxia was analyzed in A549 cells labeled with Image-iT Hypoxia Reagent and NucBlue Live ReadyProbes Reagent. Under normoxic conditions (20%) there was no signal from the Image-iT Hypoxia Reagent, but in response to the decrease in oxygen levels the signal from the Image-iT Hypoxia Reagent increased, as shown by red punctate staining (Figure 1). Hypoxic cells were first observed at 5% O₂ levels, with nearly all the cells being hypoxic at 1% O₂ levels. The signal from Image-iT Hypoxia Reagent is reversible: when oxygen levels return to normal, the signal decreases back to baseline (data not shown).

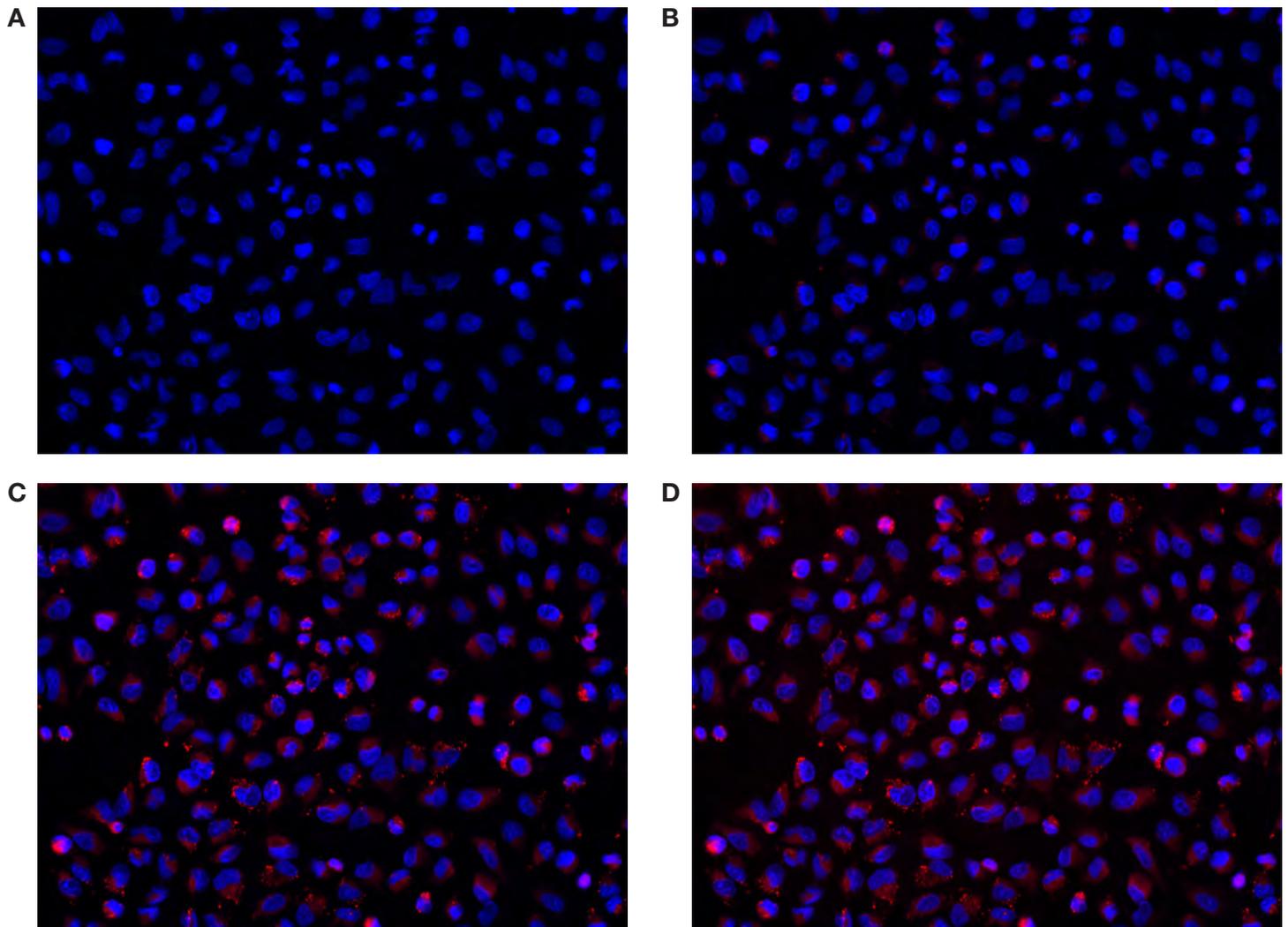


Figure 1. A549 cells stained with Image-iT Hypoxia Reagent and exposed to different oxygen levels. (A) 20% O₂. (B) 5% O₂. (C) 2.5% O₂. (D) 1% O₂.

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