**Hypoxia Red Staining**

**Reagents Used:**

|  |  |  |  |
| --- | --- | --- | --- |
| **Name** | **Catalog** | **Lot** | **Date Made** |
| Cyto-ID Hypoxia Red | ENZ-51042-0125 | 05051446 |  |
| HEPES | H0887 | RNBC9425 |  |
| MEM (1X) | 512000-038 | 1517552 |  |
| OGD Media |  |  | 11.19.14 |
| N2 | 17502-048 | 1621829 |  |
| HBSS |  |  | 1.7.15 |

**Procedure:**

*Dye Incubation*

1. Make up 50mL of NB/NS21 media
	1. Neurobasal 49 mL
	2. NS21 1 mL
	3. BME 50 µL
		1. Sterile Filter
2. Dilute original 1mM vial of Hypoxia Red to 10µM in HBSS
	1. Work with dye in the dark!!!
		1. Add 700µL of HBSS to the 7µL vial
		2. Mix well with a pipette
		3. Prepare 100µL aliquots of 10µM Hypoxia Red and store at -20oC
			1. DO NOT FREEZE THAW!!!
3. Dilute 10µM Hypoxia Red aliquots to a final working concentration of 0.5µM in HBSS
	1. You will need 250µL of 0.5µM Hypoxia Red per well
		1. To prepare 8mL (enough for 1 full 24 well plate):
			1. 7.6mL NB/NS21
			2. 400µL of 10µM Hypoxia Red (i.e. 4 aliquots)
4. *Control:* Add 250 microliters of 0.5µM Hypoxia Red in NB/NS21 to each well of a 24 well plate
	1. Transfer 1 coverslip to each well
	2. Incubate for 90 minutes at 37oC.
5. *90’ OGD:* Deoxygenate NB/NS21 by bubbling in mixed gas for 5 min.
	1. Prepare 0.5µM Hypoxia Red in deoxygenated NB/NS21
	2. Add 3 mL Hypoxia Red media to petri dishes
	3. Transfer 5 coverslips to each dish
	4. Close hypoxia chamber and flush with mixed gas for 5 min.
	5. Seal entrance and exit tubes and place into incubator for remaining 85 min.
6. After incubation, carefully aspirate the Hypoxia Red and wash twice with 1x PBS.

*Microscope Pics:*

1. Turn on fluorescence 🡪 for red dye fluorescence use the green fluorescence
	1. Box on the side
	2. When microscope is on fluorescence, turn brightness for halogen light to 0
	3. When you’re looking at bright field, turn brightness up
2. Take a picture of the same field of cells in bright-field mode, red florescent mode and one in between (so you can see all the cells as well as those that are stained) as follows:
	1. To setup computer:
		1. Select Acquisition 🡪 Multidimensional Acquisition 🡪 1 channel 🡪 set pseudo color for red to magenta 🡪 name the channel, “Hypoxia Red”
	2. For Fluorescent Images:
		1. Turn brightness knob for the halogen bulb to 0
		2. Live 🡪 Measure 🡪 Fine focus as needed 🡪 OK 🡪 Snap (in side window) 🡪 Save as a ZVI 🡪 Export (Or it will not retain the magenta pseudo color) 🡪 Select proper folder 🡪 OK to save
			1. Repeat save but this time as a JPEG
	3. For Bright-field Images:
		1. Turn brightness knob for the halogen bulb all the way up
		2. Live 🡪 Exposure 🡪 Snap 🡪 Save as both a ZVI and a JPEG
	4. For in between Images:
		1. While in the florescent cube, turn brightness knob for the halogen bulb all the way down. Then, while looking at the cells through the scope, begin increasing the halogen until you can still see red fluorescence but you can also begin to see all of the cells in the field
		2. Live 🡪 Measure 🡪 Fine focus as needed 🡪 OK 🡪 Snap (in side window) 🡪 Save as a ZVI 🡪 Export (Or it will not retain the magenta pseudo color) 🡪 Select proper folder 🡪 OK to save
			1. Repeat save but this time as a JPEG