Assay Methods

Protocol: Endothelial Cell Tube Formation Assay

CORNING

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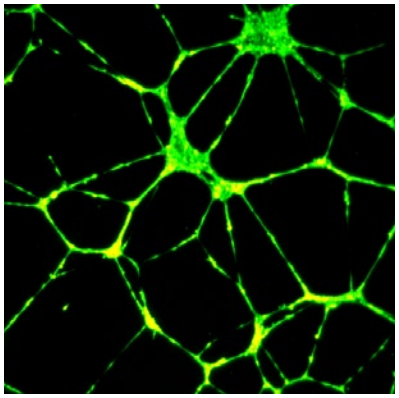

Introduction

Angiogenesis is characterized by a number of cellular events including endothelial cell migration, invasion and differentiation into capillaries. *In vitro* endothelial tube formation assays are used as a model for studying endothelial differentiation and modulation of endothelial tube formation by antiangiogenic agents. Image acquisition and quantification of fluorescently labeled cells can be achieved by using MetaMorph® software coupled with an automated imager. In the following procedure, assay conditions and cell labeling for quantification of endothelial cell tube formation have been optimized to maximize the fluorescent signal while minimizing the cytotoxic effects of calcein AM on human microvascular endothelial cell lines (HMEC-1 and HMVEC), and primary human umbilical vein endothelial cells (Corning HUVEC-2). Results may vary depending upon the cells, dye used, and the specific experimental conditions.

Materials

- Corning® Matrigel® Matrix, 10 mL (Corning Cat. No. 354234); recommended concentration of Corning Matrigel lot: 10 mg/mL or greater
- Falcon® 24 well flat-bottom standard tissue culture-treated plate (Corning Cat. No. 353047)
- Endothelial cell culture medium (e.g., MCDB131, Corning cellgro® Cat. No. 15-100-CV)
- Fetal bovine serum (e.g., Corning cellgro Cat. No. 35-010-CV) or appropriate growth factor as an angiogenesis stimulator
- Hanks' Balanced Salt Solution (HBSS) (e.g., Corning cellgro Cat. No. 21-023)
- Fluorophore (e.g., Corning Calcein AM Fluorescent Dye, 10 x 50 µg, Corning Cat. No. 354216)
- Dimethylsulfoxide (DMSO)
- Humidified tissue culture incubator, 37°C, 5% CO_2 atmosphere
- Endothelial cells such as HMEC-1, HMVEC, or Corning HUVEC-2 cells (Corning Cat. No. 354151)
- Laminar flow tissue culture hood
- Automated imager, fluorescence microscope, tube quantification software

Procedure

1.0 Reconstitution of Corning Matrigel Matrix

NOTE: Color variations may occur in frozen or thawed vials of Corning Matrigel Matrix, ranging from straw yellow to dark red due to the interaction of carbon dioxide with the bicarbonate buffer and phenol red. Variation in color is normal, does not affect product efficacy, and will disappear upon equilibration with 5% CO_2.

1.1 Thaw Corning® Matrigel® Matrix overnight on ice at 4°C and according to the recommendations provided in the product Guidelines for Use.

1.2 Once thawed, swirl vial to ensure that material is evenly dispersed.

1.3 Spray top of vial with 70% ethanol and air dry.

1.4 Keep product on ice and handle using sterile technique.

1.5 Dispense material into appropriate aliquots, using pre-cooled pipets, tips, and tubes, and refreeze immediately. Avoid multiple freeze thaws.

Precaution

Corning Matrigel Matrix will gel rapidly at 22°C to 35°C. Thaw overnight at 4°C on ice. Keep product on ice before use, and use pre-cooled pipets, tips, and tubes when preparing Corning Matrigel Matrix for use.

2.0 Coating Procedure

NOTE: Once gelled, Corning Matrigel Matrix should be used immediately. We recommend using Corning Matrigel Matrix lot with a protein concentration of at least 10 mg/mL. The concentration of Corning Matrigel Matrix is lot-specific and can be found on the Certificate of Analysis. You can pre-screen Matrigel lots and order lot-specific vials of Corning Matrigel Matrix with your preferred protein concentration by contacting Corning Customer Support.

2.1 Thaw Corning Matrigel Matrix as recommended in the product Guidelines for Use. Using cooled pipets, mix it to homogeneity.

2.2 Keeping 24 well culture plates on ice, add 0.289 mL of chilled Corning Matrigel Matrix (10 mg/mL) per well. This quantity should be sufficient to cover the entire growth surface easily. If Corning Matrigel matrix needs to be diluted to 10 mg/mL, dilute with serum free medium.

2.3 Avoid air bubbles in Corning Matrigel Matrix while pipetting the liquid into each well. If air bubbles get trapped in the wells, centrifuge the plate at 300 xg for 10 minutes in a centrifuge that has been pre-cooled to 4°C.

2.4 Incubate plates at 37°C for 30 to 60 minutes. Carefully remove the remaining liquid (medium) from the cultureware without disturbing the layer of Corning Matrigel Matrix just before use.

2.5 The plates are now ready to use.

3.0 Endothelial Cell Tube Formation Assay

3.1 Prepare the endothelial cell tube formation assay plate as directed in section 2.0.

3.2 Culture endothelial cells with desired endothelial cell medium to desired confluence. For Corning HUVEC-2, HMVEC, and HMEC-1, 70 to 80% confluence is recommended.

NOTE: Primary cells should be low passage number (e.g., HUVECs should not be passaged more than 5 times).

3.3 Prepare endothelial cell suspensions by trypsinizing the cell monolayers and resuspending the cells in culture medium with 5 to 10% serum or with your desired angiogenesis promoters at 4 x 10^5 cells/mL when using Corning HUVEC-2, HMVEC or HMEC-1 cells. Additional testing agents, such as inhibitory agents, can be included at this step as well.

NOTE: Most endothelial cell media does not contain a sufficient concentration of serum to deactivate trypsin. The use of a trypsin neutralizing solution is recommended.

3.4 Add 300 µL of the cell suspension (1.2 x 10^5 cells of Corning HUVEC-2, HMVEC, or HMEC-1) to each well.

3.5 Incubate the angiogenesis assay plate for 16 to 18 hours at 37°C, 5% CO_2 atmosphere.

4.0 Measurement of Tube Formation – Labeling with Corning® Calcein AM Fluorescent Dye

NOTE: Each well in the 24 well plate requires 300 µl of Corning Calcein AM dye at 8 µg/mL in Hanks Balanced Salt Solution (HBSS). We recommend preparing 9.0 mL of dye solution per plate to account for pipetting losses. If using 50 µg vials of Corning Calcein AM, two vials will be needed. HBSS is recommended since the use of culture medium results in auto-hydrolysis of the label, giving an unacceptably high backgrounds. This section may be performed under non-aseptic conditions.

4.1 Prepare Corning Calcein AM solution at 8 µg/mL. For each plate, 9.0 mL of Corning Calcein AM dye solution will be needed. If using two 50 µg vials, measure 12.5 mL of HBSS and warm to 37°C. Add 20 µL of DMSO to each 50 µg vial of Corning Calcein AM and then transfer both vials' contents to total HBSS volume of 12.5 mL resulting in a solution of 8 µg/mL.

4.2 Following incubation (step 3.5), carefully remove medium from the plates. Be careful not to disturb tubes that may have formed in the Corning Matrigel® Matrix. This can be accomplished by gently aspirating the medium using a Pasteur pipet.

4.3 Wash the plate with HBSS by adding 750 µL of HBSS to each well. Remove HBSS as described in 4.2.

4.4 Repeat the wash once.

4.5 Label cells by adding 300 µL/well of 8 µg/mL Corning Calcein AM in HBSS and incubate plates for 30 to 40 minutes at 37°C, 5% CO_2.

4.6 Remove the labeling solution as in 4.2.

4.7 Wash the plates twice with HBSS as in 4.3.

4.8 The plate is now ready for image acquisition using an automated imager or for taking pictures using a fluorescent microscope.

NOTE: Once hydrolysis occurs, Corning Calcein AM leaks out of cells resulting in a higher background. Labeled plates can be stored at 4°C for 1 to 2 hours with minimum increase in background.

4.9 Process the acquired images with the desired hardware and software. We use Gen-1 Cell-based Screening System and MetaMorph® software to automatically acquire images and measure tube length.

4.10 If an automated image acquisition instrument is not available, it is possible to use a fluorescent microscope that is capable of taking pictures manually, and process images using either MetaMorph or another equivalent software.

NOTE: Various researchers have measured a number of parameters such as tube length, tube areas, or branch points. In the Corning BioCoat™ Angiogenesis System: Endothelial Cell Tube Formation (Corning Cat. No. 354149 and 354150), tube formation is measured using the MetaMorph Software system. Some other commonly used imaging software packages for measuring the extent of tube formation include BD Pathway™ 855 Bioimager with sophisticated image and data analysis algorithms; Image-Pro® Plus (Media Cybernetics, www.mediacy.com) and NIH Image (http://rsb.info.nih.gov/nih-image/index.htmL).

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