Video Article

Optimized Fibrin Gel Bead Assay for the Study of Angiogenesis

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Abstract

Angiogenesis is a complex multi-step process, where, in response to angiogenic stimuli, new vessels are created from the existing vasculature. These steps include: degradation of the basement membrane, proliferation and migration (sprouting) of endothelial cells (EC) into the extracellular matrix, alignment of EC into cords, branching, lumen formation, anastomosis, and formation of a new basement membrane. Many in vitro assays have been developed to study this process, but most only mimic certain stages of angiogenesis, and morphologically the vessels within the assays often do not resemble vessels in vivo. Based on earlier work by Nehls and Drenckhahn, we have optimized an in vitro angiogenesis assay that utilizes human umbilical vein EC and fibroblasts. This model recapitulates all of the key early stages of angiogenesis and, importantly, the vessels display patent intercellular lumens surrounded by polarized EC. EC are coated onto cytodex microcarriers and embedded into a fibrin gel. Fibroblasts are layered on top of the gel where they provide necessary soluble factors that promote EC sprouting from the surface of the beads. After several days, numerous vessels are present that can easily be observed under phase-contrast and time-lapse microscopy. This video demonstrates the key steps in setting up these cultures.

Protocol

PREPARING CELLS

1. Bring up HUVEC and fibroblasts in M199/10% FBS/Pen-Strep (1:100) 1-2 days before beading.
2. Switch medium to EGM-2 (Clonetics) the day before beading for HUVEC and the day before embedding for fibroblasts.
3. A concentration of ~ 400 HUVEC per bead is needed.
4. 20,000 fibroblasts per well is needed.

COATING THE BEADS WITH HUVEC - DAY -1

1. Trypsinize HUVEC.
2. Allow beads to settle (DO NOT CENTRIFUGE!). Aspirate the supernatant and wash the beads briefly in 1 mL of warm EGM-2 medium.
3. Mix 2500 beads w/ 1×10^6 HUVEC in 1.5 mL of warm EGM-2 medium in a FACS tube. Place it vertically in the incubator. (This will be enough for ~10 wells. Scale up if needed)
4. Incubate for 4 hours at 37°C, shaking the tube every 20 min. (Good coating is crucial for sprouting.)
5. After 4 hours, transfer the coated beads to a T25 flask in 5mL of EGM-2 and leave O/N.

EMBEDDING COATED BEADS IN FIBRIN GEL - DAY 0

1. Prepare the 2.0 mg/mL fibrinogen solution (See recipe section).
2. Add 0.15 Units/mL of aprotinin to the fibrinogen solution.
3. Transfer coated beads to a 15mL conical tube and let beads settle. Resuspend beads in 1mL of EGM-2 and transfer to a 1.5mL centrifuge tube.
4. Wash the beads 3X with 1mL of EGM-2 by pipeting up and down SLOWLY.
5. Count beads on a coverslip and resuspend in fibrinogen solution at a concentration of ~500 beads/mL.
6. Add 0.625 Units/mL of thrombin to each well.
7. Add 0.5 mL of the fibrinogen/bead suspension to each well of a 24-well plate.
Change the pipette tip for each well !!!
8. Mix the thrombin and the fibrinogen by going up and down gently with the pipette tip ~ 4 to 5 times. Be careful not to make large bubbles.
9. Leave the plate for 5 min in the hood, then place it in the 37°C-incubator for 10-15 min to generate a clot.
10. While waiting for the clot, trypsinize fibroblasts.
11. Add 1 mL of EGM-2 per well drop wise.
12. Seed fibroblasts on top of fibrin gel at a concentration of 20,000 cells per well.

NOTES:

Usually, when the fibrin gel is formed, you will see tiny bubbles in the gel. Don't worry, they will disappear in 3-4 days.

Change the media every other day, i.e., Day 2, 4, 6, etc...

By day 3 or 4 you should start to see sprouting.

Discussion

There is a growing consensus that three-dimensional (3D) in vitro angiogenesis assays offer a model which is much closer to the actual environment in vivo than can be achieved using 2D cultures. It is apparent that superior 3D systems should be reproducible, and be able to mimic

several of the major steps of angiogenesis. While several previous 3D assays have been developed, many of these either use hard-to-obtain microvascular cells, or only recapitulate some of the stages. In this video, we describe and perform an optimized in vitro angiogenesis assay that utilizes human umbilical vein EC, which are easily obtainable and the most commonly used EC in vascular research. The assay, over the course of several days, consistently reproduces long vessels with clear, patent intercellular lumens surrounded by polarized EC. Later stages of EC branching and fusion of vessels (anastomosis) are also observed. Importantly, in these cultures the HUVEC undergo all of the morphological changes that are seen with microvascular EC, either in vivo or in vitro, including sprouting, migration, alignment, proliferation, tube formation, branching and anastomosis. The gene expression profile of the HUVEC changes, in parallel, to more closely match that of microvascular EC. In conclusion, we present an optimized protocol for an in vitro angiogenesis model that recapitulates several important stages of this process.

References

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