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# Direct Measurement of Blood Flow in Microvessels Grown in Matrigel *In Vivo*

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***Background.*** **The Matrigel assay provides a versatile platform to examine vessel growth. Similarly, the microsphere method is used extensively in laboratory animals to measure tissue-specific blood flow. However, microsphere models have not been used with Matrigel to study angiogenesis in live animals. The goal of this study was to develop a novel technique to directly measure blood flow with microspheres in vessels grown in Matrigel *in vivo*.**

***Methods.*** **In calves ($n$ = 10, 110 ± 5 kg), 5 mL of Matrigel was injected subcutaneously. After 10 d, a percutaneous cardiac catheterization was performed. Fluorescent-labeled 15 μm microspheres were injected into the left ventricular chamber to distribute throughout the body based on systemic blood flow patterns. Afterwards, Matrigel plugs were removed, and animals were recovered. Flow cytometry was used to count microspheres and quantify blood flow within the plug. FITC-conjugated isolectin-B4 staining was performed to quantify Matrigel capillary density. Flow cytometry was performed to quantify circulating plasma CD34$^+$ cells. Linear regressions were used to determine relationships between Matrigel blood flow, Matrigel capillary density, and plasma CD34$^+$ cells.**

***Results.*** **Over 10 d, small-caliber vessels grew into subcutaneous Matrigel plugs. Microspheres lodged throughout the plug and indicated that newly grown vessels in the Matrigel were functional and able to accommodate blood flow. Modest associations between Matrigel blood flow, Matrigel capillary density, and circulating plasma CD34$^+$ cells were noted.**

***Conclusion.*** **This method provides a novel and cost-effective technique to measure blood flow within vessels grown in Matrigel *in vivo*.** 

***Key Words:*** **angiogenesis; neovascularization; Matrigel; fluorescent microspheres; isolectin-B4; capillary density; CD34$^+$.**

## INTRODUCTION

Angiogenesis, the growth of new vessels from resident endothelial cells, plays an important role in numerous (patho)physiologic processes [1]. Over the past two decades, the role of vascular proliferation in embryology, neoplasia, ischemia, tissue growth, wound healing, and exercise physiology has generated intense interest in novel methods to study angiogenesis. To quantify angiogenesis *in vivo*, the Matrigel plug assay provides a versatile platform to examine new microvessel growth as well as the effects of pro- and anti-angiogenic factors [1–3]. Matrigel, a basement membrane extract of the Engleberth-Holm-Swarm tumor, self-assembles from a liquid at 4° to a semi-solid at 37°C and thereby provides a scaffold for the proliferation of vascular structures. Standard (immuno)histologic techniques are regarded as the gold standard to quantify microvessel density in the Matrigel plug [4, 5]. However, determination of the function of experimentally grown vessels is difficult, and an *in vivo* technique to directly measure blood flow within vessels grown in Matrigel plugs has not been described. Such

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an assessment would provide important and complementary information to standard histologic techniques.

Over the past 40 y, the microsphere method has been used extensively in laboratory animals to quantify tissue-specific blood flow [6–16]. Small diameter microspheres injected into the left atrium mix with blood in the left ventricle distribute throughout the body according to systemic blood flow patterns and become trapped in capillaries of end organs. The quantity of microspheres lodged in the tissue of interest is proportional to a calibrated reference blood flow sample that allows the precise calculation of regional blood flow in mL/min/g of tissue [11, 14]. With this technique, radioactive-labeled [14, 15], fluorescent-labeled [6–10], and neutron-activated [13] microspheres have been used to determine regional tissue blood flow in acute and chronic preparations.

More recent reports have documented repeated microsphere injections in large-animal models to serially and accurately determine tissue-specific blood flows over an extended protocol of weeks or months in the same animal [7–9, 12]. These preparations facilitated the examination of both immediate and long-term effects of pathologic, pharmacologic, and environmental influences over blood flow in multiple vascular beds. However, chronic microsphere models have not been used concomitantly with Matrigel plugs to assay angiogenesis *in vivo*. Therefore, the goal of this study was to develop a microsphere-based technique to directly measure blood flow in neovascularized Matrigel plugs in live animals. Our results indicate that the combination of the microsphere technique with the Matrigel plug assay represents an important technical advance for the functional assessment of angiogenesis *in vivo*.

## METHODS

### Animals

Male, mixed-breed calves ($n = 10$, 110 ± 5 kg) were used. All animals received humane care and were handled in accordance with National Institutes of Health and University of Louisville animal care committee guidelines. All experimental procedures were approved by the University of Louisville Institutional Animal Care and Usage Committee.

### Matrigel Injection

The animal's left rump was shaved. Temporary sedation was induced with intramuscular dexmedetomidine (0.02 mg/kg). The injection site was disinfected with alternating 2% chlorhexidene gluconate and 70% isopropyl alcohol. Matrigel (BD Biosciences, Bedford, MA) and endothelial media (DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin, 150 μg/mL VEGF, 60 units/mL heparin) were mixed 1:1 and stored on ice. A 1 cm full-thickness skin incision was made. Through the incision, a 5 mL volume of 1:1 Matrigel/endothelial media was injected subcutaneously into the rump with a 20-gauge needle. Two skin staples closed the incision. Topical neosporin was applied.

At the completion of the procedure, sedation was reversed with atipamezole (equal volume to dexmedetomidine). At the time of anesthetic induction and once every 24 h for 48 h after the procedure, flunixin meglumine (1.5 mg/kg) was administered for analgesia. To allow for adequate vessel in-growth and neovascularization, the Matrigel plug remained in the animal for 10 d prior to surgical removal.

### Cardiac Catheterization, Microsphere Injection, and Matrigel Excision

After 10 d, the animal was pre-anesthetized with atropine (30 mg i.v.) and prepared for strict aseptic surgery. In the catheterization laboratory, general anesthesia was administered with isoflurane (3%–5%) and room air. Tidal volume and respiratory rate were adjusted to maintain arterial oxygen saturation above 90%. *Via* modified Seldinger technique, a 7-French introducer sheath was inserted into the left common carotid artery. The animal was anticoagulated with a single bolus of intravenous heparin (100 units/kg).

Under fluoroscopic guidance, a 6-French pigtail catheter was advanced from the carotid artery into the left ventricle. A single color of 15 μm fluorescent-labeled microspheres (5.25 million NuFlow Microspheres; Interactive Medical Technology, Irvine, CA) was injected into the left ventricular chamber. Simultaneously, a reference blood sample was withdrawn from the carotid sheath side-port at a rate of 15 mL/min for 100 s with a calibrated syringe pump (Harvard Apparatus, Holliston, MA). The withdrawal sample acted as a reference to determine organ specific flows in mL/min/g tissue [9, 14]. The number of counted microspheres in the reference blood sample (known–counted with flow cytometry) was compared with the number of microspheres that lodged and were counted in a tissue sample of interest (known–counted with flow cytometry). The ratio between the two sphere counts was equal to the ratio between the calibrated rate of aortic withdrawal (known–15 mL/min) and flow in the tissue of interest (unknown).

Within approximately 30 min of microsphere injection and with the animal still under general anesthesia, a 1 to 2 cm skin incision was made, and the Matrigel plug was excised. The incision was closed. At the end of the procedure, the catheter and sheath were removed. The percutaneous access site was closed and compressed for 30 min until hemostasis was achieved. The animal was recovered.

### Quantification of Matrigel Blood Flow

The Matrigel plug was photographed and sectioned. One to two gram sections of Matrigel and the reference blood sample were sent to IMT/Stason Laboratories (Irvine, CA) for automated digestion, counting of microspheres with flow cytometry, and calculation of blood flow within each Matrigel plug.

### Histology

Histology was performed on Matrigel fixed in 10% buffered formalin. To visualize microspheres, 500 μm sections were cut, and the fixed specimen was viewed with an epifluorescence microscope (Nikon TE2000, Melville, NY, USA). Microspheres were identified as small, spherical spots of intense fluorescence.

To quantify capillary density, Matrigel plugs were embedded in paraffin, sectioned at 4 μm, deparaffinized, rehydrated, and stained. For each Matrigel plug, a minimum of three high-power fields was analyzed. Capillary density was determined by staining endothelium with fluorescein isothiocyanate (FITC)-conjugated isolectin-B4 (Vector Laboratories, Burlingame, CA) and expressed as the ratio of area occupied by FITC to the area of Matrigel sampled [17]. Images were viewed with epifluorescence microscopy and analyzed with Metamorph Imaging Software (Molecular Devices, Sunnyvale, CA, USA).