Scanning Microscopy Vol. 12, No. 4, 1998 (Pages 631-639) 0891-7035/98$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

TRACERS IN VASCULAR CASTING RESINS ENHANCE BACKSCATTERING BRIGHTNESS

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(Received for publication March 28, 1996 and in revised form April 4, 1997)

Abstract

Studying cast microvasculature with scanning electron microscopy expands our knowledge of many circulations but need arises to determine the blood source of vascular beds that are supplied by two circulations. One way to do this is to mark the casting resin by adding a tracer compound that can be detected in the scanning electron microscope. A potential method of distinguishing different substances is to detect the backscattered electrons that are emitted from the tracer, if the tracer is a heavier element, as heavier elements backscatter more electrons. To explore different tracers, we tested lead, titanium, iron, osmium, and uranium as solutions of different polarity and powders. The tracers were added to 1 ml of methyl methacrylate in log concentrations. Shrinkage, hardness, cast quality, and change in brightness from the tracer were compared with multivariate analysis at scanning electron microscopic working distances of 15 and 39 mm on carbon coated and uncoated specimens. Several concentrations caused sedimentation of the tracer and prevented the resin from solidifying. Tetraethyl lead shortened the hardening time; uranyl acetate and osmium tetroxide prolonged it. Most tracers decreased shrinkage. When lead citrate and Reynold's solutions were removed, the brightness was correlated with increasing atomic number, concentration of the tracer, and mean atomic number of the specimen ($p < 0.0001$). The substances that increased contrast most were tetraethyl lead and uranium. Backscattering electron detection can distinguish methacrylate casts that have small amounts of heavier elements added to them, but an optimal tracer has not yet been established.

Key Words: Corrosion casting; microscopy, scanning electron; pulmonary circulation.

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Introduction

Studying cast microvasculature with scanning electron microscopy has greatly expanded our knowledge of many circulations [2, 11]. Methyl methacrylate, a synthetic polymer developed in the thirties, was first used by T. Murakami [12] as a casting material for scanning electron microscopy in 1971. Its relative sturdiness under the electron beam has given us many insights into the structure and function of microvascular beds [9]. The three-dimensional view and large sample areas allow the studying of many vascular circumstances, but a problem not yet resolved is defining flow patterns of vascular beds supplied by two circulations. Our problem arose from the desire to study the dual blood supply of the lung and distinguish casts of the bronchial and pulmonary circulations with the electron microscope. With gross and subgross anatomy, we can use different colored casting material [15] but the world of electron microscopy is black and white, so we sought to find a way to label different casting resins for this medium. We first wanted to determine what to add and how it would effect the casting. The method tested here was backscatter electron imaging of casts with traces of different elements.

As the atomic number of an element increases, the number of electrons that are backscattered by the incident electron beam proportionately increases. The backscattered electrons give a brighter electron image to the higher numbered elements, which was our rationale for adding heavier elements to the resin [1, 3]. We wanted a cast that would be distinguishable when filled by two connecting vascular beds. We wanted the tracer to be readily available, relatively inexpensive, and proficient at backscattering. We sought to determine if the tracer would enhance scanning electron microscopic brightness without altering the surface features, replicating ability, and hardening time (Table 1). We sought to select an element, a form, a concentration, a physical state, and an amount of the material that could be added to the methacrylate, which is the standard casting material. We also wanted to quantify the backscattered electron intensity to help determine which tracer was the best. The latter is not an easy task because several factors affect the brightness of the backscattered electron signal [7, 8].

Table 1. Desired properties of a tracer added to a casting resin.

1.	Casts with an ideal tracer should be easily differentiated from those without one.
2.	The tracer should not change physical properties of the resin, such as viscosity, hardness, or durability.
3.	The added chemical should mix completely and be distributed evenly throughout the resin.
4.	The tracer should not alter the polymerization process or produce shrinkage.
5.	The tracer should not alter the cast's ability to replicate structures, affect its surface, or leave sediments or precipitants.
7.	The tracer should be easy to prepare.
8.	The tracer should be inexpensive.
9.	The tracer should be nontoxic.
10.	The tracer should discriminate without regard to surface coating of the cast.

We added lead (atomic number 82, weight 207), titanium (atomic number 22, weight 48), iron (atomic number 26, weight 56), osmium (atomic number 76, weight 190), and uranium (atomic number 92, weight 238), as solutions and powders to methyl methacrylate in log concentrations (Table 2). In addition, we sampled the specimens at an electron microscopic working distance of 15 and 39 mm and with carbon coated and uncoated specimens, as we thought that these factors may make a difference in the backscatter electron intensity.

Materials and Methods

The resin tested was colorless, partially polymerized, methyl methacrylate (Mercox, Ladd Industries, Burlington, VT) for all studies. To test different physical states, we selected a solution, powder, and organic form of lead, as Reynold's solution, lead citrate crystals, and tetraethyl lead, respectively, in different log concentrations. Osmium tetroxide was used as a 1% aqueous solution. Titanium dioxide and iron oxide (Jensen Souders Associates, Itasca, IL) were tested in powder forms and uranium was tested as both a powder (uranyl magnesium acetate) and solution (2% aqueous uranyl acetate) (Ted Pella Inc, Redding, CA). The iron oxide was a paint pigment, called yellow oxide, which is at least 86% ferric oxide in a hydrated form. Osmium tetroxide was obtained from SPI supplies (West Chester, PA). The other chemicals were obtained from Sigma (St. Louis, MO).

To make Reynold's solution, 1.33 g of lead nitrate and 1.76 g of sodium citrate were dissolved in 30 ml of boiled, cooled, distilled water. We let the solution stand for 30 minutes to complete the formation of the lead citrate. The resulting cloudy solution was cleared by adding 8 ml of 1 N sodium hydroxide. We then diluted it with 50 ml of distilled water to complete the stock solution. This Reynold's stock solution was further diluted 1:10 and 1:100 with distilled water.

To each of three microcentrifuge tubes were added 1 ml of methyl methacrylate and one of the following: nothing (additional methacrylate control), Reynold's stock solution, 0.001 ml, 0.01 ml, 0.1 ml, 0.1 ml, or 1 ml or 0.1 ml of 1:100 or 1:10 of diluted stock solution, or lead citrate powder 0.001 mg, 0.01 mg, 0.1 mg, 0.1 mg, 1 mg, and 10 mg. For the 0.001 mg and 0.01 mg concentrations, 0.1 mg of lead citrate was diluted 10- and 100-fold with methacrylate. The amounts of liquid tetraethyl lead added were 0.001 ml, 0.01 ml, 0.1 ml, and 1 ml. The 1% aqueous osmium tetroxide used 0.001 ml, 0.01 ml, and 0.1 ml. The amounts of powdered titanium dioxide, iron oxide, and uranyl magnesium acetate were 10 mg each. The amounts of 2% aqueous uranyl acetate added was 0.001 ml, 0.01 ml, and 0.1 ml.

Each tube containing the methacrylate and tracer was vortexed for 15 seconds, and the polymerization initiator, 0.1 ml 55% benzoyl peroxide, was added. The solution was again vortexed for 15 seconds. We placed a drop of the mixture on an aluminum stud. For control, we placed a drop of methyl methacrylate (with initiator) next to it. We examined the drops with a dissecting and scanning electron microscope for surface abnormalities, such as particles and plasticizing defects.

To measure shrinkage, the material was drawn into the stem of a Pasteur pipette. Both ends of the fluid column were marked and measured. The column was measured again after 24 hours. The percentage of shrinkage was one, minus the final length divided by the initial length, times 100.

After adding the initiator, a stop watch was started and the solution in the microcentrifuge tube was mixed by continuous agitation with a wooden applicator. The time at which the mixture first separated from the wall of the tube was the **first hardening time**. The cast was then removed from the tube and probed with a sharp, wooden, round tooth pick until it could not penetrate the cast, which was the **second hardening time**.

To determine the difference in electron backscattering between the test and control samples, we placed the studs in a JEOL (Tokyo, Japan) JSM 35C scanning electron microscope with an accelerating voltage of 39 kV, final aperture of 200 μm, 0° tilt, and load current of about 100 μA. The backscatter electron detecting device was an overhead scintillator photomultiplier detector designed for the JEOL JSM35-C by Dr. Robert Becker, Department of Anatomy and Cell Biology, University of Illinois at Chicago

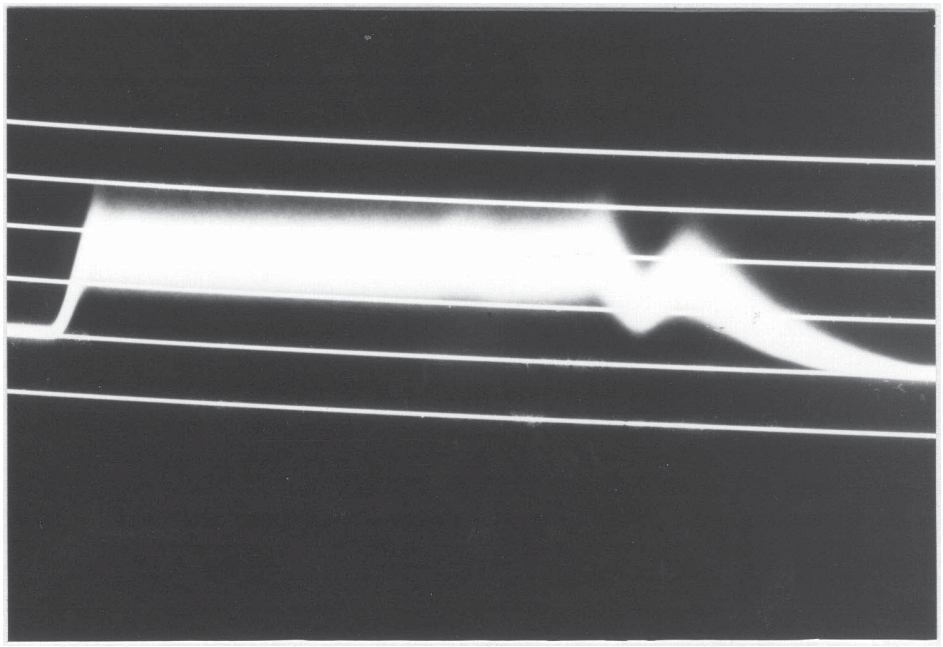

Figure 1. The brightness was measured from the amplitude-modulation line scan with the Rapid-2 setting. It has six horizontal parallel lines each 7 mm apart. With the electron beam on methacrylate control, we fixed the bottom of the broad wave (which ignored spikes) on the bottom line of the scale. By turning the contrast adjustment, we set the upper part of the broad wave (again ignoring spikes) 2 lines above the base, giving a range of 14 mm. We then shifted the stage so that the test drop came under the beam without altering any setting and recorded similar measurements

[4]. We used low magnification (100 X) to obtain a large field and avoided areas where electron charging occurred. We measured the brightness from the amplitude-modulation line scan with the Rapid-2 setting (Fig. 1). This scale has six horizontal parallel lines each 7 mm apart. With the electron beam on the control methacrylate, we fixed the bottom of the broad wave (which ignored spikes) on the bottom line of the scale. By turning the contrast and brightness adjustments, we set the upper part of the broad wave (again ignoring spikes) two lines above the base giving a range of 14 mm. We also measured the range of spikes that went above the broad waves. The range of both the waves and spikes were measured in millimeters. We then shifted the stage so that the test drop came under the beam without altering any setting and recorded similar measurements. Three measurements from different locations on each drop were taken. If charging occurred, another area was sampled.

To test different working distances, all lead samples were examined, both coated and uncoated, at 39 mm, and half were examined again at the 15 mm working distance. The other specimens were measured only at 15 mm. The specimens were coated with carbon, about 10 nm thick (Edwards E306A Coating Unit, Edwards High Vacuum International, Wilmington, MA). We measured the brightness first with the control close to the backscattered electron detector (position 1) and then rotated the stud 180° so that the test sample was closer to the backscatter detector (position 2).

Each tracer and concentration was evaluated in random order determined by a random-number table. The following factors were analyzed: the element, the atomic number and weight of the element, the physical form, the amount of the solvent used, the molar concentration of the tracer per unit of methacrylate, and the mean atomic number of the specimen (Table 2). Brightness was determined by subtracting the range of the broad wave for the control samples from the test samples with the stud in both positions. Results from the two positions were totaled to give a final brightness score. We did not use the wave spikes because they often resulted from electron charging artifact. We ran a multivariate analysis on a Dell Pentium microcomputer with SAS for Windows software, version 6.03 (SAS Inst., Cary, NC).

Results

Macroscopic cast appearance, hardening times, and shrinkage

The undiluted Reynold's solution at 1.0 ml precipitated and separated from the rest of the resin. The fluid column in the pipette showed alternating areas of water and methacrylate. It could not be used for casting. Precipitation occurred in all Reynold's mixtures, although those in the lower concentrations could only be detected with electron microscopy. The Reynold's solution at the 1:1 dilution and the 0.1 ml tetraethyl lead did not solidify well. The crystals of uranyl magnesium acetate sedimented and significantly affected the casting. The lead citrate crystals did not dissolve in the methacrylate even in the lowest concentrations. When 0.1 ml tetraethyl lead was mixed with 1.0 ml of the methacrylate, the tetraethyl lead appeared to form a gradient at the bottom despite mixing.

Table 2. The different groups tested and the mean atomic of the final product.

Specimen	Group	Mean atomic number
Methyl methacrylate alone*	1	6.41
Reynold's solution (Pb)		
Undiluted		
0.001 ml	2	6.41
0.01 ml	3	6.42
0.1 ml	4	6.55
1 ml	5	7.23
Diluted		
1: 100	6	6.48
1: 10	7	6.49
Lead citrate (Pb)		
0.001 mg	8	6.45
0.01 mg	9	6.79
0.1 mg	10	10.02
1.0 mg	11	26.2
10.0 mg	12	42.5
Tetraethyl lead (Pb)		
0.001 ml	13	6.65
0.01 ml	14	7.76
0.1 ml	15	18.78
Osmium tetroxide (Os)		
0.001 ml	16	6.41
0.01 ml	17	6.42
0.1 ml	18	6.54**
Ferrous oxide (Fe)		
10 mg	19	19.28
Titanium dioxide (Ti)		
10 mg	20	15.48
Uranyl acetate (U)		
0.001 ml	21	6.46
0.01 ml	22	6.93
0.1 ml	23	11.20
Uranyl magnesium acetate		
10 mg	24	25.89

*All mixtures had 0.1 ml 55% benzoyl peroxide.
**This solution did not mix.

The methacrylate at the top appeared to polymerize first, with secondary polymerization occurring later in the lower part of the methacrylate.

The 1% aqueous osmium tetroxide mixed with methyl methacrylate and changed its color to a dark brown for the less concentrated and black for the more concentrated solutions. The 0.1 ml concentration of osmium tetroxide did not solidify. Titanium dioxide changed the solution to white and iron oxide changed the solution to yellow. Both mixed satisfactorily with the resin.

Adding the tracers to the methacrylate resin changed the polymerization time. Table 3 shows that tetraethyl lead shortened the hardening time whereas uranium and osmium tetroxide prolonged it. These were greatest at the highest concentrations. Diluting the tracer was correlated with increased hardening time ($r = 0.28$, $p < 0.01$) and shrinkage ($r = 0.16$, $p < 0.05$). Adding tracers generally decreased shrinkage, but not in a dose-dependent manner (Table 3). The mean atomic number was not correlated with any parameters of polymerization; it was only correlated with the millimolar concentration of the tracer ($r = 0.31$, $p < 0.0001$).

Surface changes

The specimens appeared smooth under the dissecting and scanning electron microscopes, but the plastic made with the Reynold's solutions had brightly backscattered particles 15 μm to 25 μm in diameter. The margins of the particles blended into the surrounding methacrylate. The size of the crystals of lead citrate and uranyl magnesium acetate varied from 15 μm to more than 100 μm in diameter and had sharply defined margins by scanning electron microscopy. The other agents were not associated with particles or other surface changes.

Brightness

Quantifying the brightness was complicated by the position of the test and control sample with respect to the backscatter electron detector. The uncoated measurements were taken in only one position but were similar to the carbon-coated readings shown in Figure 2. The brightness was not uniformly distributed, especially in the lead citrate and Reynold's solution. Lead citrate crystals were bright but areas around them were dark and the brightness of the lead citrate was haphazard because of the crystals. The tetraethyl lead was brightest near the edge of the droplet. Because of the heterogenous appearance of the brightness in the lead citrate and Reynold's plastics, the analysis was done with and without these groups. With lead citrate and Reynold's in the analysis only the atomic number ($p < 0.05$) and atomic weight ($p < 0.05$) of the tracers predicted the backscattered intensity. Without these samples, brightness was highly correlated with atomic number ($p < 0.0001$) and mean atomic number of the entire specimen ($p < 0.0001$).

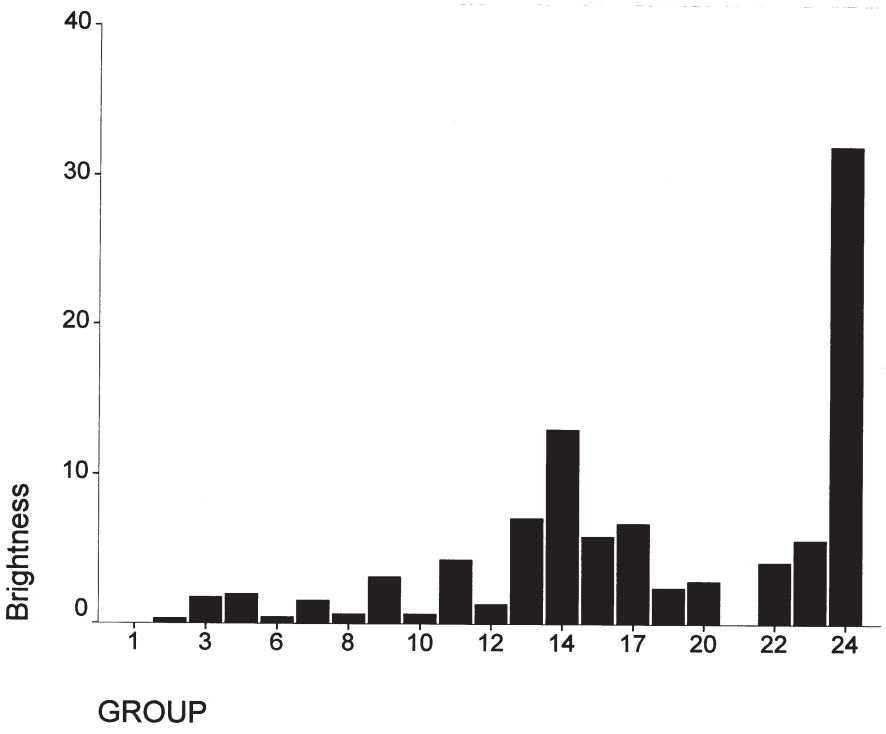

Figure 2. This graph shows the mean brightness of the different groups, which are listed in Table 2.

Physical forms, coating, and working distance

The uranyl magnesium acetate did not dissolve well. The lead citrate dissolved poorly and left crystals. The fine iron and titanium powder mixed well but small particles were detected with the scanning electron microscope. Reynold's solution precipitated when added to the methacrylate. The amount of fluid used had no affect on the brightness, although the casts made with Reynold's solution had vacuoles caused by trapped water droplets. The vacuoles affected the strength of the cast. The solid tracers tended to give a higher brightness score (8.1) than did the liquid (4.9) ($p < 0.06$), when the Reynold's and lead citrate were not in the analysis. Carbon coating the specimens had no substantive effect on the brightness but decreased the electron charging. The brightness from the two working distances was not significantly different.

Discussion

Anatomists studying vascular corrosion casts with scanning electron microscopy need to identify injection sources under the microscope. Our interest has been in distinguishing the contributions of the bronchial and pulmonary circulations at the capillary level. The bronchial circulation is important in neoplasm, inflammation, and lung transplantation [6]. Verifying which circulation provides which part of the lung could be important in designing drug-delivery systems for the airway. For example, lung neoplasms appeared to be supplied by the bronchial circulation. If this is true, chemotherapy could be directed through this circulation to avoid systemic toxicity. Being able to trace the relative contributions of different sources may be important in studying the physiology of the lung and other vascular beds, such at the pituitary and liver, that have a dual blood supply.

In 1979, G.M. Roomans gave a list of ideal properties for quantitative analysis standards of thin embedded sections [13]. Many of the same features are true of tracers in casts that we listed in Table 1. Roomans stated the compounds should be organic or organometallic compounds because mineral salts do not dissolve well in resins. They should be nonpolar because aqueous solutions are insoluble. They should be stable under the electron beam and should not react chemically with the resin or have volatile components - which is also good advice for workers trying to establish tracers in casts. We can conclude from our work that aqueous material is unlikely to work well with the possible exception of small quantities of reactive species, such as OsO_4. As previous work has shown, the backscattered electron brightness is proportional to the mean atomic number of the sample [10]. The exception to this was the lead citrate and Reynold's solution, which, if included, added so much variation that the correlation of mean atomic number and backscattered brightness was obscured. Although this study shows that the concept is valid, it does not clearly identify the tracer ideal for

Table 3. Hardening time and shrinkage (standard deviation). Time 1 was the number of minutes required for the mixture to separate from the wall of the microcentrifuge tube. Time 2 was the number of minutes at which the wooden probe could no longer penetrate the cast. Shrinkage was the percentage of length change in a glass cylinder between the fresh resin and the solidified plastic at 24 hours.

Specimen	Hardening		Shrinkage
	Time 1	Time 2	(%)
Methacrylate			
	2.99 (0.30)	7.35 (0.19)	5.68 (2.45)
Reynold's solution			
Undiluted			
0.001 ml	2.88 (0.51)	7.33 (0.13)	4.21 (2.61)
0.01 ml	2.74 (0.35)	6.63 (0.58)	3.33 (0.67)
0.1 ml	2.91 (0.43)	7.60 (0.45)	6.25 (1.56)
1 ml	Did not harden		
0.1 ml diluted			
1: 100	3.24 (0.26)	9.52 (2.67)	2.84 (0.15)
1: 10	2.99 (0.35)	7.71 (0.44)	5.56 (3.90)
Lead Citrate			
0.001 mg	2.78 (0.50)	7.27 (0.93)	3.44 (0.70)
0.01 mg	2.81 (0.39)	6.57 (1.10)	3.69 (0.80)
0.1 mg	2.68 (0.35)	6.97 (0.39)	2.50 (1.21)
1.0 mg	2.61 (0.29)	7.27 (0.05)	2.70 (0.33)
10.0 mg	2.95 (0.39)	6.97 (0.59)	4.89 (2.52)
Tetraethyl lead			
0.001 ml	1.96 (0.34)	6.30 (0.73)	1.76 (0.21)
0.01 ml	1.49 (0.07)	4.29 (0.16)	2.23 (0.16)
0.1 ml	Did not harden		
Osmium tetroxide			
0.001 ml	4.87 (0.43)	8.53 (0.35)	2.90 (0.73)
0.01 ml	7.77 (0.93)	14.00 (1.50)	3.72 (2.99)
Ferrous oxide			
10 mg	4.51 (0.37)	8.43 (0.74)	2.92 (0.72)
Titanium dioxide			
10 mg	4.76 (0.58)	8.49 (0.61)	3.03 (4.50)
Uranyl acetate			
0.001 ml	4.60 (0.53)	7.67 (0.48)	6.28 (1.09)
0.01 ml	5.03 (1.10)	9.20 (1.46)	5.86 (0.92)
0.1 ml	9.08 (2.69)	12.35 (2.32)	9.73 (1.56)

distinguishing different methacrylate casts.

Although small particles could be a marker themselves, they may alter the appearance of the structure of which replication is desired, obscure microvascular filling, and detract from the electron microscopic image. Non-polar solutions combine better with methacrylate than polar compounds, but neither pure polar compounds, such as water, or pure nonpolar compounds, such as emersion oil, combine adequately in high proportions. With polar solutions, the sedimentation and precipitation appear to be related to the concentration of the tracer and the total amount of water added. Coating the specimen with carbon did not attenuate the backscatter electron detection and prevented electron charging that would frequently make certain fields unusable. We found no statistical difference in contrast between the test and control samples when measured at 15 mm and 39 mm working distances, but the comparison was done only for lead, which had considerable inconstancy, especially with the lead citrate.

Lead citrate mixed poorly with methacrylate and sedimented causing a poor relationship between brightness, which was erratic, and concentration. Precipitated lead citrate crystals were large enough to interfere with capillary filling. Although the precipitation that occurred with the Reynold's solution was dependent on the concentration, even the lower concentrations had inhomogeneous brightness, which makes it a poor candidate to be a tracer. Homogeneous distribution of the element throughout the resin is an essential quality of a good tracer [13]. Tetraethyl lead was the best form of lead tested, giving a good cast with no precipitation. The tetraethyl lead probably reacted with the benzoyl peroxide and methacrylate, because it shortened polymerization time and appeared to increase exothermicity. The backscattering brightness was the highest with tetraethyl lead, but there are several drawbacks to the use of the agent. The backscatter signal was inhomogeneous, perhaps reflecting an affect on the polymerization process. Tetraethyl lead is toxic and expensive. This toxicity and that of osmium may limit their usefulness. In addition to the hazards of preparing the samples, there may be volatilization of these elements under the electron beam, although we did not study this question. Another potential problem not addressed is that the backscattering signal may change with prolonged electron exposure [5, 16, 18].

Titanium and iron are lighter elements that gave weaker backscattering but satisfactory casts. Their affordability, availability, and lack of toxicity should earn them a place in *in vivo* studies, but they are unlikely to be the ideal agents. The backscattered brightness of uranyl acetate was good, but not as good as the tetraethyl lead, and uranyl magnesium acetate in the concentration we tried had casting problems. Osmium tetroxide enhanced

brightness. It is a reactive species that may chemically interact with the methacrylate and benzoyl peroxide. We used the 39 kV accelerating voltage to obtain maximal backscattering, which is a higher voltage than is commonly used for viewing methacrylate casts. With our system, this voltage has given the best backscatter electron discrimination [14].

Although our concern was not with the quantification of the tracer, there no doubt will be areas where both circulations contribute to the cast which will cause a gradient of the tracer. These areas may pose problems for backscatter electron imaging unless a quantification is obtained. For this reason, energy dispersive X-ray analysis may be a better tool than backscatter electron imaging. X-ray energy is easily measured and could give a quantification of the contribution of the blood flow to precise capillaries under certain conditions.

In summary, it appears that tetraethyl lead, at a concentration of between 0.001 and 0.01 ml per ml of methacrylate and 2% uranyl acetate at a concentration of 0.01 ml per ml of methacrylate were the best candidates to be added to methyl methacrylate to enhance backscattering electron imaging contrast, but much more must be done to develop the standard. The oxides of osmium, iron, and titanium were also reasonable choices. Reynold's lead and lead citrate powder are unsuitable. The working distance does not appear to matter. The specimens should be coated with carbon to decrease charging. Other methods, both physical and chemical, may be superior to backscattering and should be tested. Energy dispersive X-ray analysis might differentiate casts if the appropriate elements are added. It has the advantage of identifying lighter elements but it is slower.

Acknowledgment

We thank Messrs. Phil Smith of Jensen Souders Associates and Jose Domingo of Federated Paint for their advice and titanium and yellow oxide, Drs. Robert Becker, Suman Goel, and George Gould from the University of Illinois at Chicago and Dr. Godfried Roomans, for advice and assistance, and the Electron Microscopy Facility of the Research Resources Center (Misses L. Vedegys and K. Jarosius), University of Illinois at Chicago for assistance and use of the equipment.

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Discussion with Reviewers

A. Lametschwandter: For practical work and obtaining good casting of vascular beds the viscosity of the medium is important. It would be interesting to know how the different additives and their various concentrations affect the viscosity of the Mercox.
Authors: We agree that it would be interesting to know, but we did not measure the viscosity of any compounds. A. Spurr many years ago did considerable work on the physical properties of resin with additives [17], but did not use the same additives as we did. As you know, however, once the accelerator is added, the viscosity increases logarithmically with time so that the viscosity measured before adding the catalyst is not the viscosity of the resin being injected.

A. Lametschwandter: You indicate that the size of the crystals of lead citrate and uranyl magnesium acetate ranged from 15 μm to greater than 100 μm in diameter, and you suggest they would interfere with capillary filling. Could you comment on capillary filling with Mercox with additives?
Authors: This study was entirely *in vitro*. Our next step will be to try injecting it into lungs.

S. Aharinejad: When we cast lung via the caudal vena cava or even aorta, we cast part of the bronchial circulation showing these two circulations are connected to each other. I believe these connections are at the capillary level as does Dr. Schraufnagel [15]. Since methyl methacrylate passes through capillaries, the resin carrying the tracer would then be delivered to both the pulmonary and bronchial circulation. Would not this be a problem?
Authors: Although our goal is to establish a tracer for detecting the supplies of different circulations, there will be boundary areas with a gradient of the tracer and there will, no doubt, be different conditions where one circulation will supplies a vascular bed more than the usual conditions. Developing a tracer will help answer these physiological questions.

S. Aharinejad: Have you ever tried using a low-voltage, high-resolution scanning electron microscope to observe your resin mixed with a tracer?
Authors: No.

J.L. Abraham: Other fillers such as barium, tungsten, or tantalum might be tried also. Tantalum, for example, is used clinically as a radio-opacifying additive to acrylic plastics used in surgery. Would the authors discuss the theory of solubility of methacrylate with other materials and provide references?
Authors: We agree that many different material can be tried, and the ones mentioned are common around medical environments. Methyl methacrylate is soluble in non-polar reagents and rather insoluble in polar solvents making aqueous preparations less useful. Spurr has done considerable work on this topic [17] and Roomans [13] concluded that organic or organometallic compounds make the best additives because of the lack of solubility of mineral salts and aqueous solutions. Tetraethyl lead mixes well with methacrylate but may form a sedimentation gradient although this was not studied beyond simple observation.

J.L. Abraham: Regarding working distances, why should the brightness not be different for different working distances? The solid angle of the backscatter detector should make this different at different working distances. Was some adjustment or normalizing done at the two working distance levels? How was specimen current controlled? Would not the specimen current vary from one working distance to another? In theory, there should be one-fourth the signal detected if the working distance is doubled.
Authors: The working distance should affect backscattered electron brightness which is why we tested this variable, but we compared only the difference between the control and test sample at the different working distances. We set the control baseline and kept all settings the same as we went to the test sample. We made no further adjustments for specimen current. The differences between control and test samples did not depend on working distance.

J.L. Abraham: The particular nature of the heavy metals might be important depending on the diameter of the vessels perfused. Do the authors have comments about the importance of particle size depending on different diameter vessels which they cast?
Authors: For this study our desired product was not to have particles at all because of the possibility of interfering with filling and detracting form the secondary image. However, small particles (e.g., 2-3 μm) might not interfere much with filling and could differentiate the vascular sources without need of backscatter imaging. It is clear that much more work needs to be done.

P. Motta: Did you apply your technique to an organ model? If so, were you actually able to characterize two different vascular beds supplying the same organ? In addition, could

this technique be applied to differentiate the blood supply of vascular structures such as arteriovenous anastomoses?
Authors: We have not yet applied this to an animal model, but we hope that it also will aid in understanding the vascular sources of arteriovenous anastomoses under various filling conditions.