Validation of fluorescent-labeled microspheres for measurement of relative blood flow in severely injured lungs

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The use of fluorescent-labeled microspheres for measurement of relative blood flow in severely injured lungs and after established lung injury. Across all animals, 175 pieces were selected randomly. The radioactivity of each piece was calculated for each piece from both the radioactivity and fluorescent-labeled-microsphere method. In seven sheep, lung injury was established by using oleic acid. Five pairs of radiolabeled-microsphere and fluorescent-labeled microspheres were injected before and after established lung injury. Across all animals, 175 pieces were selected randomly. The radioactivity of each piece was determined by using a scintillation counter. The fluorescent dye was extracted from each piece with a solvent without digestion or filtering. The fluorescence was determined with an automated fluorescent spectrophotometer. Perfusion was calculated for each piece from both the radioactivity and fluorescence and volume normalized. Correlations between flow determined by the two methods were in the range from 0.987 ± 0.007 (SD) to 0.991 ± 0.002 (SD) after 9 days of soaking. Thus the fluorescent microscope technique is a valuable tool for investigating regional perfusion in severely injured lungs and can replace radioactivity.

METHODS

The study was approved by the University of Washington Animal Care Committee.

Animal preparation. The experiments were performed on seven adult sheep of both genders, ranging in weight from 22.6 to 27.3 kg. The animals were premedicated with 0.6 mg/kg xylazine (Xyla-Ject, Phoenix Pharmaceutical, St. Joseph, MO), anesthetized with thiopental sodium (20 mg/kg), and maintained by a continuous infusion of thiopental sodium titrated to suppress hemodynamic responses to noxious stimuli. Once anesthetic depth was adequate, the animals were paralyzed using a continuous infusion of pancuronium (0.1 mg/kg). Anesthesia was maintained by using a continuous infusion of thiopental sodium titrated to suppress hemodynamic responses to noxious stimuli. Once anesthetic depth was adequate, the animals were paralyzed with pancuronium (0.1 mg/kg). A tracheotomy was performed, and a tube was inserted. Catheters were placed in a femoral and a pulmonary artery (Swan-Ganz thermodilution catheter) to monitor arterial, pulmonary arterial, and wedge pressures and temperature and to sample blood. A femoral venous catheter was inserted for infusion of anesthetic drugs and maintenance fluids. A central venous catheter was inserted into the left subclavian vein via the left jugular external vein and used as the injection port for the dye and microspheres. Airway pressures, arterial and pulmonary pressures and temperature were measured continuously with Validyne amplifiers (Northridge, CA) and recorded on a Western Graphitec Mark12 data-management system DMS 1000 (Irvine, CA). The end-tidal CO₂ was measured with a mass spectrometer (Perkin-Elmer medical gas analyzer MGA-1100). Cardiac outputs and blood temperatures were measured with a cardiac output computer (Baxter Edwards Sat-2). Arterial and venous pH, PO₂, and PCO₂ were measured with a blood-gas analyzer (Radiometer ABL 330, ABL Acid Base Laboratory, Copenhagen, Denmark) and corrected for temperature. Throughout the experiments all animals were relatively expensive. There are few reports about the use of nonradiolabeled microspheres in injured lungs (11). Because severely injured lung tissue is more like solid organs than normal lung tissue, digestion and filtration of lung pieces may be necessary.

The primary purpose of this study was to validate the use of fluorescent-labeled microspheres for estimating organ perfusion in severely injured lungs. Fluorescent dye was extracted from dried lung pieces, thus avoiding the time-consuming tissue digestion and filtration procedures.

Hübker, Matthias, J. Souders, E. Shade, M. Hlastala, N. Polissar, and R. W. Glenny. Validation of fluorescent-labeled microspheres for measurement of relative blood flow in severely injured lungs. J. Appl. Physiol. 87(6): 2381–2385, 1999.—The aim of the study was to validate a nonradioactive method for relative blood flow measurements in severely injured lungs that avoids labor-intensive tissue processing. The use of fluorescent-labeled microspheres was compared with the standard radiolabeled-microsphere method. In seven sheep, lung injury was established by using oleic acid. Five pairs of radiolabeled microspheres were injected before and after established lung injury. Across all animals, 175 pieces were selected randomly. The radioactivity of each piece was calculated for each piece from both the radioactivity and fluorescent-labeled microspheres. Correlations between flow determined by the two methods were in the range from 0.987 ± 0.007 (SD) to 0.991 ± 0.002 (SD) after 9 days of soaking. Thus the fluorescent microscope technique is a valuable tool for investigating regional perfusion in severely injured lungs and can replace radioactivity.

The use of fluorescent-labeled microspheres for measurement of regional lung perfusion is a well-established method (7). When the number of injected microspheres is sufficiently large, and when appropriately sized microspheres are used, regional blood flow is proportional to the number of trapped microspheres (2). Our laboratory (7) showed that the extraction of the fluorescent dye can be simplified with the use of an automated spectrophotometer. We further simplified the tissue processing by avoiding digestion and filtration of the lung pieces, while still yielding excellent correlations. However, retrieving fluorescent microspheres from solid organs requires many labor-intensive steps. Radiolabeled microspheres are easier to use but pose health risks, require special precautions for use and disposal, have limited shelf lives, and are

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prone. After an initial stabilization period during which the respiratory minute volume was adjusted to maintain arterial 
PO2 between 36 and 46 Torr, baseline measurements were 
recorded. Lung injury was induced by emulsifying 15 ml of 
previously extracted blood with 0.14–0.23 ml OA/kg (C18H34O2, 
Sigma Chemical, St. Louis, MO) and injecting it with continu-
ous shaking over 30–45 min. Severe lung injury was consid-
ered established when the clinical criteria of the definition of the 
American/European consensus conference on acute respir-
atory distress syndrome (ARDS) were fulfilled (ratio of 
arterial PO2 to inspiratory O2 fraction < 200, pulmonary 
capillary wedge pressure < 19 Torr) (3). The animals were 
then studied for 120 min after the injury was established.

Microsphere validation. Details of the methods are de-
scribed by Glenn et al. (7). Briefly, five fluorescent polysty-
rene microspheres (red, orange, blue-green, yellow-green, 
and crimson) of 15 µm diameter (FluoSpheres, Molecular 
Probes, Eugene, OR) and five radiolabeled styrene-divinyl 
benzene microspheres (141Ce, 113Sn, 103Ru, 95Nb, and 46Sc) of 
15.5 µm diameter (DuPont NEN Research Products, Boston, 
MA) were used. The time points of injection were baseline 
(tbase), established injury (t0), 30 min (t30), 60 min (t60), and 
120 min (t120) after established injury. Immediately before 
injection, the microspheres were vortexed, sonicated for 90 s, 
and resuspended in a single syringe. Fluorescent (1.5 × 106) 
and radiolabeled microspheres [1.63 × 106 ± 0.6 × 106 (SD)] were 
injected simultaneously over 30 s, followed by a saline flush. 
The amount of radiolabeled microspheres given was adjusted 
to ensure a minimal radioactivity of 0.02 mCi per injection. 

RESULTS

The criteria for ARDS were attained after a mean 
time of 135 ± 65 min after the injection of OA. One 
animal died 10 min before completion of the study 
period, and the last injection of microspheres (crimson) 
could not be given. The total number of coring samples 
was therefore 150 for crimson and 175 for all other 
colors.

The mean correlations between the volume-normal-
ized relative blood flows determined by the radiola-
beled and the fluorescent microsphere techniques were 
very high and increased only slightly by prolonging the 
soaking time (Table 1). Statistically significant differ-
ences were seen for orange, comparing the 7- and 9-day 
soaking times (Table 1). The mean correlations were 
compared among colors for a given soaking time and among 
soaking times for a given color by using the two-tailed paired t-test. A one-sample two-tailed t-test was used to compare correlations to a hypothesized 
mean of zero. P < 0.05 was considered statistically signifi-
cant.

The volume-normalized relative blood flow at every time 
point was calculated for each lung piece, with the color-
specific fluorescence and the radiolabel-specific counting per-
formed separately

\[ Q_{rel,i} = \frac{x_i}{(\Sigma x_i)/n} \]

where \( Q_{rel,i} \) is the volume-normalized relative blood flow of 
the piece i; \( x_i \) is the obtained fluorescent or counted radioac-
tivity, respectively, divided by the volume of the piece (cm³); 
and \( n \) is the number of pieces. The mean normalized relative 
flow was therefore 1.0.

The mean correlations between the volume-normal-
ized relative blood flows determined by the radiola-
beled and the fluorescent microsphere technique were 
very high and increased only slightly by prolonging the 
soaking time (Table 1). Statistically significant differ-
ences were seen for orange, comparing the 7- and 9-day 
soaking times, and between orange and blue-green after 7 days of soaking. However, the magnitudes of the 
differences are extremely minor. Figure 1 shows a 
representative plot of the radiolabeled-microsphere-determined flow vs. the yellow-green-microsphere
determined flow after 9 days of soaking. In one animal, 
the correlation for crimson was slightly lower than for 
other animals: 0.94 after 7 days of soaking, increased to 
0.97 after 14 days of soaking. In this animal, the range 
of relative flow at the time of injection was very small 
compared with other animals (typical range 0.6 to 1.5). 
The random measurement error was approximately 
the same as for other animals, leading to a smaller 
signal-to-noise ratio and to a slightly smaller correla-
tion.

The accuracy of the fluorescent method is indepen-
dent of the sample density, as shown by the small and 
nonsignificant mean correlations between 1) the den-
sity of the coring samples and 2) a difference defined by 
the radiolabeled-microsphere relative flow minus the 
fluorescent-microsphere relative flow (Table 2). Figure 
2 shows a representative plot of the density vs. the 
radiolabeled-microsphere-determined minus the yellow-
The correlation between the two different simultaneously injected microspheres (radioactive and fluorescent) to measure regional perfusion was not perfect. This indicates that there is a measurable, but small, error. The reasons for these errors are well known (9). Several studies have quantified the sources of error for radiolabeled microspheres (1, 6, 13). Glenny et al. (7) suggested that these errors apply also to the fluorescent microsphere technique. They concluded also that the error increases if, in the processing of the lung pieces, there are too many steps involved, leading to a loss of microspheres. They found the best correlations when the samples were only soaked. Therefore, we decided to extract the fluorescent dyes only by increasing the soaking time. We expected a longer soaking time because of the higher density of the tissue compared to noninjured lungs. Noninjured lung samples are usually soaked for 2 days (7). Although the correlations seen after 7 days of soaking were already high, a minor improvement was noted for all colors, and was significant for orange, by increasing the soaking time to 9 days. Further increase in duration of the soaking time showed only a negligible improvement of the correlations.

In most studies investigating pulmonary blood flow, lung pieces with an airway content >25% are discarded, and the relative flow is weight normalized (4, 8, 12). In severely injured lungs, a greater weight range for pieces of the same volume is noted. This is usually due to cellular infiltration or hemorrhage. We volume normalized the relative blood flows to compensate for this variability. We also included pieces up to an airway content of 50% and showed that the accuracy of the

Table 1. Mean correlations between radiolabeled vs. fluorescent-microsphere-determined volume-normalized relative flow

<table>
<thead>
<tr>
<th>Color</th>
<th>7 Days</th>
<th>9 Days</th>
<th>11 Days</th>
<th>14 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>0.986 ± 0.003</td>
<td>0.989 ± 0.002</td>
<td>0.989 ± 0.002</td>
<td>0.989 ± 0.002</td>
</tr>
<tr>
<td>Orange</td>
<td>0.985 ± 0.004*†</td>
<td>0.988 ± 0.003*</td>
<td>0.988 ± 0.003*</td>
<td>0.994 ± 0.003*</td>
</tr>
<tr>
<td>Blue-green</td>
<td>0.991 ± 0.002†</td>
<td>0.991 ± 0.002</td>
<td>0.992 ± 0.001</td>
<td>0.992 ± 0.001</td>
</tr>
<tr>
<td>Yellow-green</td>
<td>0.988 ± 0.003</td>
<td>0.989 ± 0.003</td>
<td>0.989 ± 0.003</td>
<td>0.993 ± 0.003</td>
</tr>
<tr>
<td>Crimson</td>
<td>0.982 ± 0.008</td>
<td>0.987 ± 0.007</td>
<td>0.988 ± 0.006</td>
<td>0.989 ± 0.005</td>
</tr>
</tbody>
</table>

Values are means ± SE. No. of animals = 7, for crimson = 6. No. of pieces = 175, for crimson = 150. Significant differences between soaking time: *orange, 9 vs. 7 days, P = 0.03; 11 vs. 7 days, P = 0.03. Significant differences between colors: †orange vs. blue-green at 7 days of soaking, P = 0.03.

DISCUSSION

The important finding of this study is that the fluorescent-labeled microspheres technique is an accurate method to measure volume-normalized relative blood flows in severely injured lungs. The linear correlation between perfusion estimates by use of fluorescent blood flows in severely injured lungs. The linear correlation method to measure volume-normalized relative fluorescent-labeled microspheres technique is an accurate, and narrow excitation spectrum (7). The correlation between the two different simultaneously injected microspheres (radioactive and fluorescent) to measure regional perfusion was not perfect. This indicates that there is a measurable, but small, error. The reasons for these errors are well known (9). Several studies have quantified the sources of error for radiolabeled microspheres (1, 6, 13). Glenny et al. (7) suggested that these errors apply also to the fluorescent microsphere technique. They concluded also that the error increases if, in the processing of the lung pieces, there are too many steps involved, leading to a loss of microspheres. They found the best correlations when the samples were only soaked. Therefore, we decided to extract the fluorescent dyes only by increasing the soaking time. We expected a longer soaking time because of the higher density of the tissue compared to noninjured lungs. Noninjured lung samples are usually soaked for 2 days (7). Although the correlations seen after 7 days of soaking were already high, a minor improvement was noted for all colors, and was significant for orange, by increasing the soaking time to 9 days. Further increase in duration of the soaking time showed only a negligible improvement of the correlations.

In most studies investigating pulmonary blood flow, lung pieces with an airway content >25% are discarded, and the relative flow is weight normalized (4, 8, 12). In severely injured lungs, a greater weight range for pieces of the same volume is noted. This is usually due to cellular infiltration or hemorrhage. We volume normalized the relative blood flows to compensate for this variability. We also included pieces up to an airway content of 50% and showed that the accuracy of the

![Figure 1](https://via.placeholder.com/150)

**Fig. 1.** Radiolabeled microsphere-determined vs. yellow-green microsphere-determined volume-normalized relative flow after 9 days of soaking. No. of animals = 7, no. of pieces = 175.

![Figure 2](https://via.placeholder.com/150)

**Fig. 2.** Typical of count data. In any case, there is a tight variability at higher values of relative flow, which is typical of count data. In any case, there is a tight distribution about the mean. Figure 3 shows a representative plot for yellow-green.

![Figure 3](https://via.placeholder.com/150)

DISCUSSION

The important finding of this study is that the fluorescent-labeled microspheres technique is an accurate method to measure volume-normalized relative blood flows in severely injured lungs. The linear correlation between perfusion estimates by use of fluorescent blood flows in severely injured lungs. The linear correlation method to measure volume-normalized relative fluorescent-labeled microspheres technique is an accurate, and narrow excitation spectrum (7).
method is independent of the density of the lung sample. The correlation with the magnitude of density was tested after a soaking time of 9 days because of the results mentioned above.

One important concern when investigating blood flow is that the accuracy of the method should be independent of the magnitude of flow for an individual piece. Whereas the mean correlation for blue-green is significantly different from zero (Table 3), the significance level is not small and could readily have arisen by chance when five hypothesis tests are carried out. In addition, the mean correlation itself is weak. Finally, even if one accepts a real relationship between the fluorescent-isotope difference and the magnitude of flow, the impact is likely to be negligible. The equation for the difference vs. flow indicates a very minor correction: blue-green = 0.033 (relative flow) (Fig. 4), based on a regression analysis pooling n = 175 pieces from seven animals. Across a typical relative flow range of only 0–2.5, the blue-green relative flow would differ from isotope relative flow by 0.03 to 0.05, an extremely minor correction. The magnitude of this correction is 1–2% of the typical flow range and could be ignored in practice. Even with the use of the equation from the animal with the greatest slope [blue-green – isotope = −0.072 + 0.072 × (relative flow)], the correction for relative flows from 0 to 2.5 would range from −0.07 to +0.11, again very minor compared with the wide range of flows encountered.

In conclusion, we have shown that the fluorescent microsphere technique is a valuable tool for investigating regional perfusion in severely injured lungs and can replace radioactive microspheres. The optimal duration of soaking seems to be 9 days, and, if only four colors are used, blue-green can be omitted.

Table 3. Mean correlation of radiolabeled minus fluorescent microsphere-determined relative flow vs. relative flow after 9 days of soaking

<table>
<thead>
<tr>
<th>Color</th>
<th>Correlation</th>
<th>P Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>0.124 ± 0.095</td>
<td>0.24</td>
<td>−0.063–0.311</td>
</tr>
<tr>
<td>Orange</td>
<td>−0.022 ± 0.061</td>
<td>0.73</td>
<td>−0.141–0.098</td>
</tr>
<tr>
<td>Blue-green</td>
<td>0.255 ± 0.090*</td>
<td>0.03</td>
<td>0.079–0.432</td>
</tr>
<tr>
<td>Yellow-green</td>
<td>0.083 ± 0.069</td>
<td>0.27</td>
<td>−0.052–0.219</td>
</tr>
<tr>
<td>Crimson</td>
<td>0.175 ± 0.086</td>
<td>0.1</td>
<td>0.006–0.344</td>
</tr>
</tbody>
</table>

Correlations are means ± SE across animals. All flows were volume normalized. No. of animals = 7, for crimson = 6. No. of pieces = 175, for crimson = 150. Significance was tested against zero with a 2-tailed t-test; *P < 0.05. See text for details.
REFERENCES


