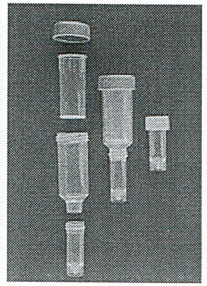
Perkin Elmer Filtration Units
Validation Experiments
September 1997

Purpose: Validate use of new centrifugation filtering devices to isolate 15 µm diameter fluorescent microspheres from solid tissue. The initial production filters tested previously had a failure rate of 2 in 10. These problems have reportedly been corrected.

Description: The devices are made of polypropylene and consist of three stages (figure). The first stage has a woven polyamid filter integrated into the bottom. Each tissue sample is placed in this first stage and digested with KOH. The digested material is filtered by suction and dried by centrifugation. The filtered sample and container are then placed within the second stage and the third stage attached to the bottom of the second stage. The organic solvent is added to the first stage and then transferred to the last stage by centrifugation. The last stage then contains the organic solvent with the extracted dyes. Perkin-Elmer also sells a separate heating block that will heat 40 tissue samples in KOH to 60° C.



The Institute for Surgical Research, Klinikum Großhadern, Munich recommends the following procedure.

Materials:

- Potassium hydroxide pellets (224.4g KOH per 1000 ml H_2O)
- 2% Tween-80™ (10 ml Tween-80 per 1000 ml potassium hydroxide)
- 100% isopropanol alcohol
- K_2HPO_4 powder (29.9 g K_2HPO_4 for 1000 ml buffer liquid)
- KH_2PO_4 powder (5.88 g KH_2PO_4 for 1000 ml buffer liquid)
- Organic solvent

Per Sample:

- 15 ml digestion solution
- 1.5 ml isopropanol
- 15 ml phosphate buffer
- organic solvent (Cellosolve™, Aldrich)

Preparation:

- Digestion solution: Dissolve 224.44 g KOH in 1000 ml H_2O by constant stirring. Add 10 ml 2% Tween-80 when the liquid has cleared (stable for 24 hours).
- Phosphate buffer: Dissolve 5.88 g KH_2PO_4 in 200 ml and 29.9 g K_2HPO_4 in 800 ml H_2O. Mix two solutions together.

Work Routine

1. Place each tissue sample in a separate first stage of the filtering device.
2. Place first stage in KOH bath or separate containers of KOH.
3. Layer 1.5 ml of isopropanol over top of each sample and seal first stage (Parafilm, tinfoil, and plastic wrap work well).

4. Heat samples to 60° C until digested (2 - 4 hr).
5. Suction digestate through first filter stage by negative suction and rinse with 15 ml of buffer.
6. Dry sample by centrifugation at 4000 rpm for 30 seconds.
7. Put first stage of filtering device into the second stage and attach third stage to bottom.
8. Add one-half volume of organic solvent to the first stage, vortex for 20 seconds and let rest for 5 min.
9. Add remaining half of organic solvent to the first stage and vortex for 20 seconds.
10. Centrifuge organic solvent to third stage of filtering device by centrifugation (4000 rpm for 30 secs.)
11. Measure fluorescence in organic solvent.

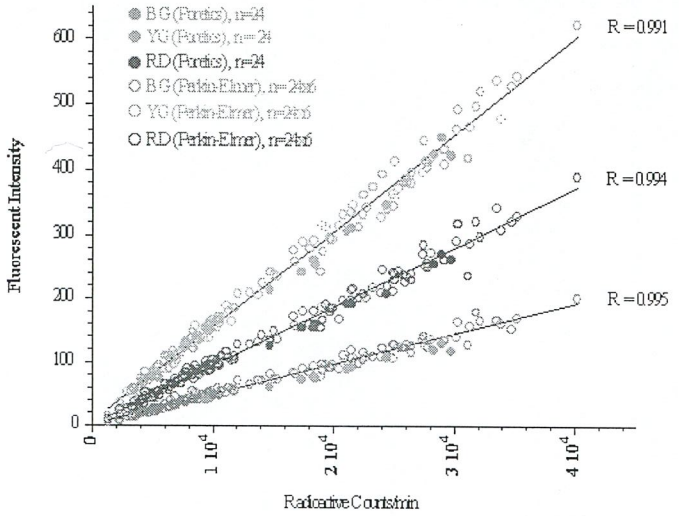
Methods: One hundred sixty-eight (168) samples of myocardium and kidney that had received both radioactive (Tin) and fluorescent (Blue-Green, Yellow-Green, and Red) microspheres via a simultaneous left ventricular injection were used for this validation study. Radioactivity was determined in each sample and corrected for background counts and decay.

Twenty-four (24) samples were digested using KOH and filtered with Poretic polycarbonate 10 µm filters. The fluorescent dye was extracted from each filter and the dye concentrations measured with our Perkin-Elmer LS50B. The fluorescent and radioactive signals from these samples defined the ratio of fluorescence to radioactivity expected in all other tissue samples.

Twenty-four (24) samples were digested and filtered per Institute for Surgical Research instructions. The fluorescent dye was extracted from each filter device per the protocol and the dye concentrations measured with our LS50B.

To see if the filter devices could be reused, the 24 filter devices were washed with soapy water and placed in an ultrasonicating bath for 60 minutes. Twenty-four (24) new tissue samples were digested and filtered per Institute for Surgical Research instructions, the fluorescent dye extracted from each filter device and the dye concentrations measured. This process was repeated five times so that 24 filter devices were each used a total of 6 times.

Results: Using the relationship between the Poretics filtered fluorescent signals and the radioactive counts as a standard for 100% recovery, it appears that all of the fluorescent microspheres were completely recovered by the eight Perkin Elmer filter devices (see graph below). The filter devices we tested were reusable, demonstrating 100% microsphere recovery after being used 6 times.



Conclusion: The Perkin Elmer filtering devices are an acceptable method to isolate fluorescent microspheres from solid tissue. Problems associated with the initial production run appear to have been resolved. Processing is more labor intensive than the Poretics filtration system for small numbers of samples. Significant time will be saved for larger sample numbers. The greatest advantage of the Perkin Elmer filtration system is that a tissue sample remains in a single container throughout, thereby minimizing the loss of microspheres during processing.

Although the filter devices are not meant to be reusable, we found that microsphere recovery was not compromised after being used at least 6 times. We did not continue to test them until they failed and therefore do not know how many times they can be used. We were able to determine if they failed because we had radioactive counts from the intact tissues. However, if only fluorescent microspheres are used in a study, the percent recovery will not be known and the competancy of the filters cannot be verified after multiple uses.

Ordering Information: The filter devices can be ordered through Perkin-Elmer Deutschland, Verkauf und Service, Telefon (07551)81-0, Telefax (0775)16 12. They must be purchased in sets of 160 units. The set price is 600,-- DM.

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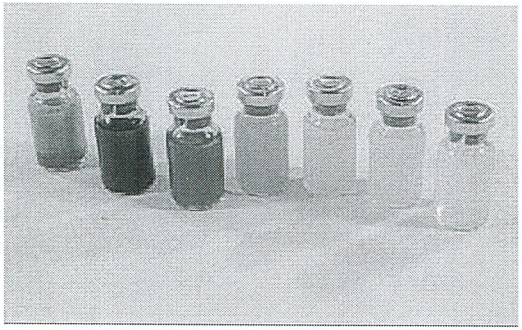
1963 - All radioactive work to present
1981 - Gottlieb et al, Fluorescent microspheres
- Identified ischemic myocardium

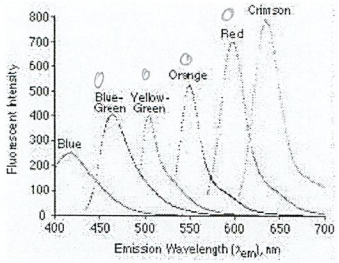
1991 - Kowalik et al, Colored microspheres
- Absorption spectroscopy

1993 - Glenny et al, Fluorescent microspheres
- Emission spectroscopy

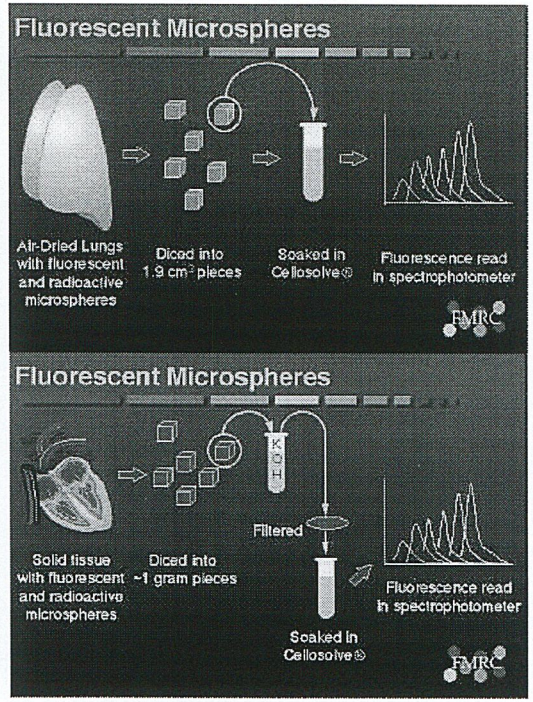
Physical properties of fluorescent microspheres
- Fluospheres™ (Molecular Probes and Triton Technologies)
  - Polystyrene
  - Diameter = 14.7 µm (scanning EM)
  - Diameter variability (CV) = 2.1% (Coulter)
  - Density = 1.02 g/ml (gradient centrifugation)
  - Fluorescence variability = 3.4 - 5.0% (FACS)
- 8 commercially available colors



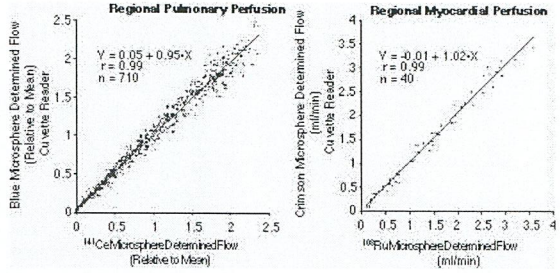
Fluorescent Spectra



Validation vs. Radiolabeled Microspheres (Glenny, Bernard, et al. 1993)



- Correlation between fluorescent and radiolabeled determined flow = 0.99



Greatest advantage of fluorescent microspheres is that they can be used in studies where radioactivity is not permitted.

- physiology studies in field
- labs that are not cleared for radioactivity
- countries that do not allow radioactivity

Sources of error

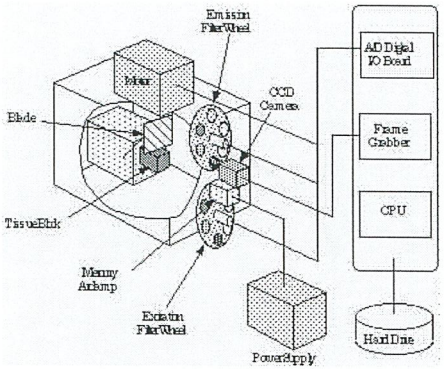
Many of the same potential errors as radiolabeled microspheres with some additional ones.

Potential Error	Solution
Signal quenching	dilute sample, less spheres
Loss of microspheres when isolating	meticulous technique, centrifugation filters, single tube processing
Low signal:noise ratio	increase number of microspheres injected or sample size
Inaccurate solvent volumes	accurate pipettes, larger volumes, robotics
Dye stability	check in solvent
Background signal	choose appropriate colors

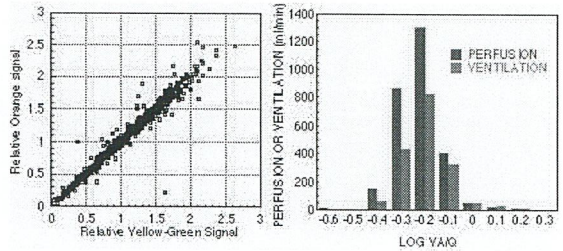
- Methodolgic noise greater than radioactive microspheres
  - solvent volumes
  - filtering methods
  - signal variability (CV = 3%)
- Time requirements greater than radioactive microspheres
  - sample handling

Future Applications of Fluorescent Microspheres

- Histology
  - Fluorescent microspheres are easily visualized, allowing perfusion to be quantified at the microscopic level.
  - Provides ability to define source of blood when more than one circulation exists.
- Cryomicrotome
  - Currently in developmental stage
  - Automatically determines the spatial location of fluorescent microspheres in small organs
    - rats and rabbit lungs
    - canine hearts
  - Produces text file with X, Y, and Z locations of each microsphere
  - Able to differentiate four different colors



Aerosolized microspheres to measure regional ventilation
- 1 µm diameter fluorescent microspheres.
- Produced dry aerosol, administered to mechanically ventilated pigs.
- Pulmonary perfusion quantified using 15 µm diameter microspheres via central vein.
- Currently in press - H. T. Robertson et. al. [tomrobt@u.washington.edu]



Chronic studies
- initial studies suggest that radiolabels leach off of radioactive microspheres but fluorescent microspheres can be measured months after injection

References

Glenny, R. W., S. Bernard, and M. Brinkley. (1993). "Validation of fluorescent-labled microspheres for measurement of regional organ perfusion." J. Appl. Physiol. 74(5): 2585-2597.

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