

A new simple method of staining exogenous surfactant in experimental research

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A new simple method of staining exogenous surfactant in experimental research. M.F. Krause, M. Orlowska-Volk, E.R. Hendrik, D. Gommers, B. Lachmann. ©ERS Journals Ltd 2000.

ABSTRACT: Two commonly used techniques in experimental lung research have helped to determine which variables influence surfactant distribution within the lung: radioactive labelling of surfactant components and admixture of coloured microspheres to surfactant. However, neither technique allows the description of surfactant distribution at the alveolar level. The aim of this study was to establish a new technique using histology colourants for admixture to exogenous surfactant to make exogenous surfactant visible by light microscopy.

In a step by step approach the authors evaluated the properties of a variety of green colourants when added to a natural porcine surfactant preparation for their ability to homogeneously mix with surfactant, to bind to surfactant, to adhere to a glass slide, to not be "overstained" by standard haematoxylin-eosin and Elastica van Giesson staining, to not influence *in vitro* surface tension properties of surfactant using a Wilhelmy balance, to not influence oxygenation and ventilation in a lung-lavage rat model and to preserve their colour and adherence to exogenous surfactant on lung specimens visualized by light microscopy.

Only one of the tested green histology colourants (Green Dye®) fulfilled all requirements and showed a brilliant green colour in a distribution pattern typical of surfactant at the alveolar level.

It is concluded that the authors have established a new, simple and inexpensive method of staining exogenous surfactant for evaluation of its distribution by light microscopy at the alveolar level.

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Surfactant replacement therapy for neonatal respiratory distress syndrome has now been used as a standard therapeutic modality since before 1989 [1]. Several variables determine surfactant distribution after its instillation into the endotracheal tube, such as the surfactant preparation itself, age of the newborn at treatment, volume and concentration of surfactant, the time interval for surfactant administration if repeatedly used, alternative ways of surfactant administration (*e.g.* nebulization), and the style of mechanical ventilation [2]. Homogeneous distribution of exogenous surfactant is of paramount importance for a favourable clinical response in treated infants because atelectatic lung areas with a substantial intrapulmonary right-to-left shunt and consecutive hypoxia should be avoided. However, most data focusing on surfactant distribution come from experimental research using different species of animals allowing histological or other processing of the lungs at the end of the experiment.

Two commonly used techniques have shed some light on surfactant distribution in experimental research: radioactive labelling of dipalmitoyl phosphatidylcholine or other surfactant components, and admixture of coloured microspheres to surfactant. Determination of the distribution of radioactively labelled surfactant requires considerable effort such as collaboration with nuclear medicine

departments [3, 4] and expensive discarding of these radioactively polluted animals. Lung processing for evaluating distribution of exogenous surfactant using the coloured microsphere technique needs additional staff and an extra session to cut the lung into 50–70 pieces before washing out the colorant affixed to the microspheres [5, 6].

Therefore, the aim of this study was to establish a new method for experimental lung research which allows the description of surfactant distribution at the alveolar level when analysing histological specimens. The authors studied eight histological colourants for admixture to exogenous surfactant in a step-by-step approach to visualize exogenous surfactant when using light microscopy for evaluation of lung specimens.

Material and methods

Colourants

A variety of green colourants was tested to provide a powerful optical contrast because the standard staining techniques with haematoxylin-eosin (HE) or Elastica van Giesson (EvG) for the histological evaluation of lung

specimens stain structures predominantly a deep blue, red and yellow.

The following substance was tested for its ability to homogeneously mix with surfactant, to adhere to a glass slide, and to preserve its original green colour on a slide when mixed with surfactant and stained with either HE or EvG: Green Tissue Marking Dye® (Green Dye: WAK-Chemie Medical GmbH, Bad Soden, Germany).

Green Dye was diluted 1:10 with normal saline. The diluted colourant was then further diluted by hand stirring one part with nine parts of surfactant until total homogeneous mixing was achieved, leaving a final concentration of 1% of the original dye. After the drying process the slide was stained with HE and EvG and checked for adherence of the colourant to the slide and maintenance of its original green colour.

In vitro testing of stained surfactant

The authors used a freeze dried natural surfactant preparation isolated from porcine lungs as previously described [7]. It consists of ~90–95% phospholipids, 1% hydrophobic proteins (surfactant proteins B and C) and 1% free fatty acids, the remainder being other lipids such as cholesterol and glyceride. There is no surfactant protein A in this preparation. Surfactant powder was added to warmed normal saline and stirred to a final concentration of 40 mg·mL⁻¹.

Stained and uncoloured surfactant preparations were tested for their minimal surface tensions using a modified Wilhelmy balance (E. Biegler, Mauerbach, Austria) by applying increasing amounts (100 µL, 200 µL and 500 µL) to the surface of a warmed saline filled trough [8]. The surface area was compressed and expanded with a cycling time of 3 min per cycle and maximum and minimum surface areas of 64 and 12.8 cm², respectively (100% and 20%). The minimal surface tension was measured after three cycles at 20% surface area, and was expressed in millinewtons per metre.

In vivo testing of stained surfactant

This part of the study was approved by the Institutional Animal Committee at the Erasmus University Rotterdam; care and handling of the animals were in accord with the Helsinki convention.

The physiological studies were performed in male Sprague-Dawley rats (n=10) with a body weight of ~300 g. After the induction of anaesthesia with nitrous oxide, oxygen and enflurane (66/33/1–2%), a polyethylene catheter (0.8 mm outer diameter) was inserted into the carotid artery for drawing arterial blood samples. Before tracheotomy, the animals received pentobarbital sodium, 30 mg·kg body weight⁻¹, *i.p.* After a metal cannula had been inserted into the trachea it was secured in place by a peritracheal ligature. The animals were then mechanically ventilated with a Servo Ventilator 300 (Siemens-Elcoma, Solna, Sweden) in parallel (five animals simultaneously) in a pressure control mode with a continuous flow in the tubings. Muscle relaxation was achieved using pancuronium bromide, 0.6 mg·kg body weight⁻¹, *i.m.*

The following ventilator settings were used: frequency of 30 breaths per minute, positive end-expiratory pressure (PEEP) of 200 Pa (2 mbar), inspiratory/expiratory ratio of 1:2, inspiratory oxygen fraction (F_{i,O_2}) of 1.0 and a peak inspiratory pressure (PIP) of 1.2 kPa (12 mbar) yielding a carbon dioxide tension in arterial blood (P_{a,CO_2}) of 3.7–5.3 kPa (28–40 mmHg). Anaesthesia was maintained with hourly injections of 30 mg·kg body weight⁻¹ pentobarbital *i.p.* and 0.6 mg·kg body weight⁻¹ pancuronium *i.m.* Body temperature was kept within the normal range by means of a heating pad.

Respiratory failure was induced by repeated broncho-alveolar lavage as described by LACHMANN *et al.* [9]. Immediately before the first lavage PIP and PEEP were increased to pressures of 2.6/0.6 kPa (26/6 mbar). Each lavage involved the instillation and removal of 30 mL·kg body weight⁻¹ of warmed saline *via* the tracheal cannula carried out over a 30-s period. Airway lavage was repeated every 5 min until oxygen tension in arterial blood (P_{a,O_2}) was <10.6 kPa (80 mmHg). The animals were then randomly divided into two groups of five animals each: the first group received 1 mL of stained surfactant with Green Dye, and the second group served as a control group receiving 1 mL of uncoloured surfactant at a concentration of 40 mg·mL⁻¹.

Additional measurements of blood gases (P_{a,O_2} and P_{a,CO_2}) were made at 5, 30, 60, 90 and 120 min after surfactant instillation. All ventilator settings were held constant for this observational period of 2 h and were changed thereafter by steps of 200 Pa (2 mbar) to 2.4/0.4 kPa (24/4 mbar) (PIP/PEEP), 2.2/0.2 kPa (22/2 mbar), 2.0/0.0 kPa (20/0 mbar) and again 2.6/0.6 kPa (26/6 mbar) every 10 min to test deflation stability of the lungs.

At the end of each experiment the animals were killed with an overdose of pentobarbital, and the lungs were removed from the chest for histological processing.

Histologic processing

After tying off the trachea to prevent washout of stained surfactant the lungs were put *en bloc* with the heart and thymus into 4% formaldehyde for several days before the histological processing was carried out.

After embedding the specimens in paraffin four cuts of each lung at a width of 4 µm were taken from central right, central left, peripheral right and peripheral left locations and put on slides for fixation. All slides were stained with HE and EvG using standard methods. Photographs were taken to demonstrate the pattern of surfactant distribution within the lungs.

Chemistry of Green Dye and binding to surfactant

According to information from the manufacturer (WAK-Chemie Medical GmbH) Green Dye consists of green colourants fixed to latex microparticles which are inert to physiological fluids.

To assess binding of Green Dye to surfactant *in vitro* the following centrifugation protocol was used: three tubes were prepared containing: 1) Green Dye diluted 1:10 with normal saline and mixed with an equal amount of 50% dextrose in sterile water; 2) surfactant without admixture of

Green Dye with 50% dextrose; and 3) surfactant with admixture of Green Dye and 50% dextrose. The three tubes were then placed in an ultracentrifuge (Model L7-55; Beckman Instruments, Palo Alto, CA, USA) at $40,000 \times g$ and 4°C for 20 min under vacuum conditions.

Statistical methods

All data are expressed as mean \pm SD. A Wilcoxon rank sum test was used to calculate differences between the two groups. Repeated measures analysis of variance was used to assess differences in blood gases in change over time. An unpaired t-test [10] was used to determine whether there were differences between intervention and control group at individual observational times. All analyses were performed using the software program GraphPad Prism 2.0 (GraphPad Software, San Diego, CA, USA).

Statistical significance was accepted at $p < 0.05$.

Results

Colourants

By light microscopy Green Dye showed a stable fixation to the glass slide and a brilliant green colour when mixed with surfactant and "overstained" with HE and EvG.

Other colourants were also tested, but tests were abandoned at different stages because the requirements were not fulfilled: acrylic green® (Schmincke, Erkrath, Germany); methyl green®; light green SF®; Janus green B®; malachite green oxalate®; azo rubin S® (all colourants: Chroma, Köngen, Germany); resolin brillant blue BGLN 200%®; resolin brillant yellow 10 GN 400%® (DyStar, Leverkusen, Germany).

In vitro testing of stained surfactant with a Wilhelmy balance

Two sets of measurements were performed (table 1): one set with increasing amounts of surfactant immediately after preparation, and another set 3 h after preparation reflecting the time period used for the *in vivo* testing of stained surfactant in rats yielding no differences between

Table 1. – Minimal/maximal surface tensions with and without stained surfactant (surf.) preparations

	Minimal/maximal surface tension $\text{mN}\cdot\text{m}^{-1}$	
	Immediately after preparation	After 3 h of incubation
Surf. + Green Dye		
100 μL	16.5/25.9	15.5/29.3
200 μL	15.6/26.2	14.3/25.3
500 μL	8.0/16.1	11.7/17.3
Control: uncoloured surf.		
100 μL	16.8/30.0	15.9/26.8
200 μL	14.0/26.5	14.5/26.5
500 μL	8.0/13.9	10.5/17.0

A surfactant concentration of $40 \text{ mg}\cdot\text{mL}^{-1}$ was used and added to the surface of a warmed saline-filled trough of a modified Wilhelmy balance. There were no statistically significant differences between groups.

when the stained and the uncoloured surfactant preparations.

pH-measurement for Green Dye yielded a value of 6.5. When mixed with surfactant (one part colour and nine parts surfactant), the values rose to 7.0.

Stained surfactant was incubated in a cuvette at room temperature for 3 days; however, no sedimentation or dissolution of the different components could be discerned. The same procedure was undertaken adding 4% formaldehyde with 1 part of formaldehyde and one part of coloured surfactant. Again, there were no signs of sedimentation or dissolution.

In vivo testing of stained surfactant in a lung-lavage rat model

There were no differences between groups with regard to body weight (Green Dye: $290 \pm 11 \text{ g}$; control: $301 \pm 17 \text{ g}$, respectively) and the number of lavages applied (Green Dye: 6.0 ± 0.7 ; control: 5.8 ± 0.8 , respectively).

Figure 1 shows all values for P_{a,O_2} and P_{a,CO_2} before and after lavage, and for the 2-hour observation period following surfactant instillation and the three-step reduction of ventilator pressures to assess deflation stability of the terminal airways, yielding no statistically significant differences between the two groups.

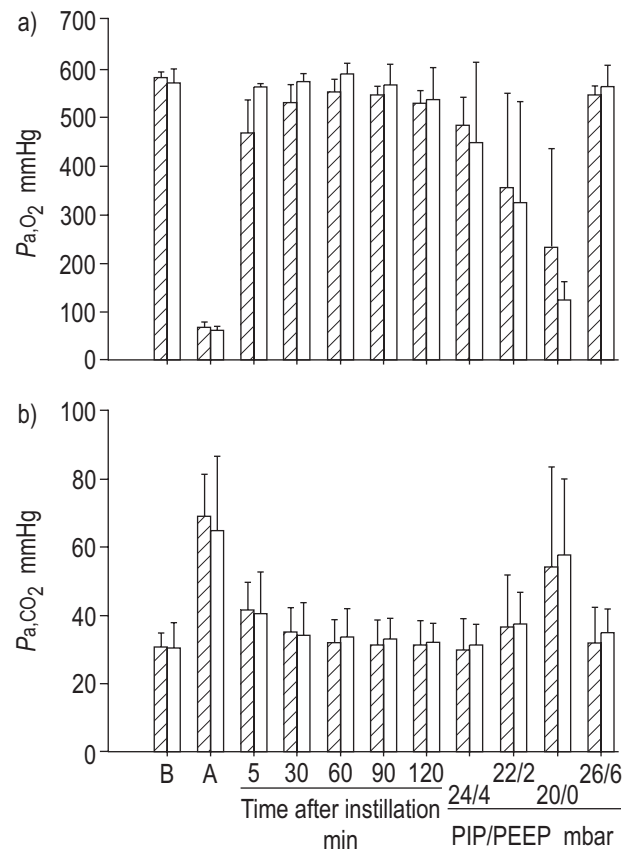


Fig. 1. – Data on oxygenation and ventilation of the two groups: a) arterial oxygen tension (P_{a,O_2}); and b) arterial carbon dioxide tension (P_{a,CO_2}). Data are presented as mean \pm SD (n=5). There were no significant differences between Green Tissue Marking Dye® (▨) and uncoloured surfactant (control; □). B: before lavage; A: after lavage. 1 mmHg=0.133 kPa.

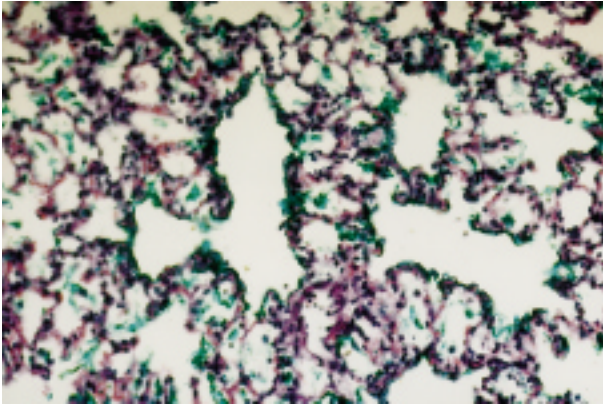


Fig. 2. – Homogeneous distribution of green coloured surfactant in the alveoli and along the alveolar septa. Haematoxylin-eosin stain; internal scale bar=25 μm .

Histological processing

Surfactant coloured with Green Dye could easily be found on all slides using both staining techniques. Figure 2 (HE staining) shows homogeneous depositions of green coloured surfactant in the alveoli and along the alveolar septa, whereas almost no green material could be found in the larger bronchi indicating good spreading properties of this coloured surfactant preparation. Figure 3 shows a layer of stained surfactant adjacent to the alveolar epithelium. Hyaline membranes could be identified but always with very little incorporation of stained surfactant (fig. 4). The same frequency of hyaline membranes was present on the control group slides. Macrophages within the alveoli showed incorporation of exogenous surfactant. As usual in this lung lavage model, the interstitial lung tissue was irregularly invaded by granulocytes also incorporating surfactant. Figure 5 shows the incorporation of coloured surfactant by alveolar epithelial cells, most likely type II pneumocytes, which are known to recycle and store endogenous and exogenous surfactant from the alveolar layer [11]. Green coloured surfactant was also preserved on slides with EvG staining (fig. 6).

Binding of Green Dye to surfactant

When incubated with equal amounts of 50% dextrose and rotated at 40,000 $\times g$, Green Dye alone sunk to the

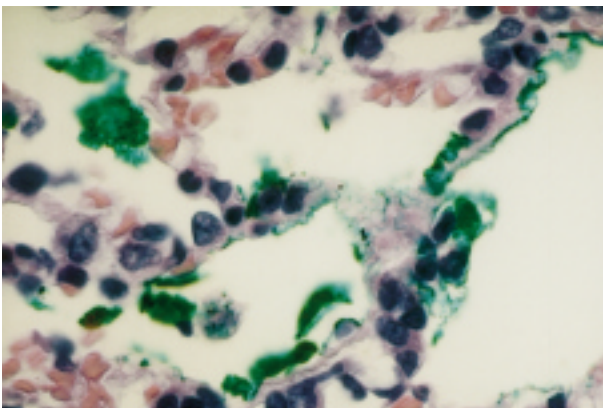


Fig. 3. – Layer of coloured surfactant adjacent to the alveolar epithelium. Haematoxylin-eosin, oil; internal scale bar=10 μm .

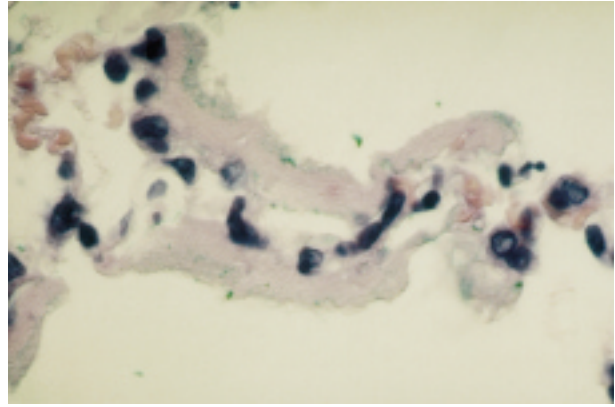


Fig. 4. – Hyaline membranes, with almost no incorporation of stained surfactant. Haematoxylin-eosin, oil; internal scale bar=10 μm .

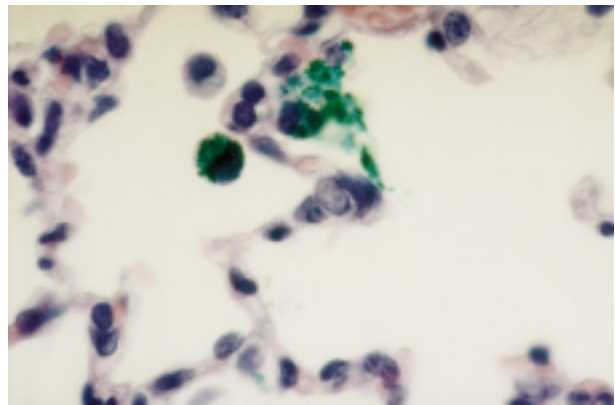


Fig. 5. – Ingestion of coloured surfactant by type II pneumocytes and macrophages. Haematoxylin-eosin, oil; internal scale bar=10 μm .

bottom of the tube. In contrast uncoloured surfactant appeared as a supernatant floating on the liquid surface as did stained surfactant leaving small amounts of unbound Green Dye on the bottom. It is concluded that Green Dye is indeed chemically bound to the bulk of surfactant without influencing the surface tension properties of exogenous surfactant.

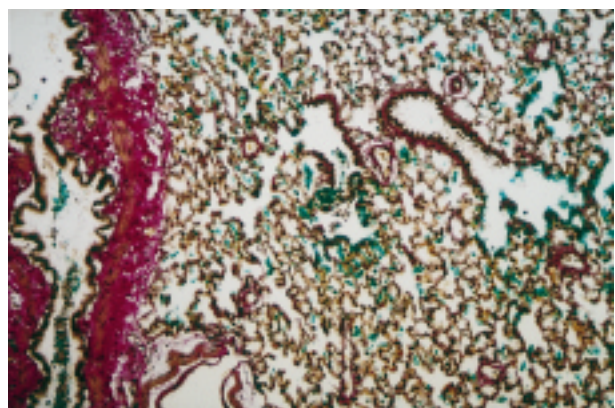


Fig. 6. – Distribution of coloured surfactant. Elastica van Giesson; internal scale bar=40 μm .

Discussion

The authors succeeded in identifying by trial and error a colourant originally designed for histology use which easily dissolved with a porcine natural surfactant preparation without disturbing the surfactant's *in vitro* or *in vivo* surface tension properties. Moreover, it could be easily detected on histology slides of rats' lungs still exerting its original brilliant green colour when staining the lung specimens with HE or EvG. On histological examination the coloured surfactant was only very slightly incorporated into hyaline membranes but was present in macrophages, granulocytes and type II pneumocytes. It did not cross alveolar membranes or pulmonary capillary endothelium (when, *postmortem*, the coloured surfactant was injected into the right ventricle and circulated through the pulmonary blood vessels). No toxic effects could be discerned during the *in vivo* test lasting ~2.5 h, a finding in line with the authors' observation that the green colour was readily bound to surfactant and did not show any signs of sedimentation or dissolution. The manufacturer has stated that no toxic chemicals are mixed to the latex microparticles. It is assumed that chemical binding of the green colorant to surfactant, as demonstrated by ultracentrifugation, is due to binding to the bulk of surfactant components (*i.e.* phospholipids) in the surfactant preparation used in the present study.

The authors are not aware of any other publication using histology colourants for staining exogenous surfactant in experimental research. However, different techniques have been used in the past to investigate the distribution of exogenous surfactant when instilled into the surfactant-deficient lungs of research animals. Most experimental studies of surfactant distribution have used radiolabelled surfactant or other easily measured substances mixed with surfactant components [11]. JOBE *et al.* [12] used a trace amount of [³H]-palmitic acid labelled sheep surfactant to assess its distribution, GILLIARD *et al.* [13] and HELDT *et al.* [14] [³H]choline labelled dipalmitoyl phosphatidylcholine (DPPC), and DAVIS *et al.* [3] [^{99m}Tc]-labelled calf lung surfactant extract. Two differently labelled surfactant preparations ([³H]-choline labelled DPPC and [¹⁴H]-palmitate labelled DPPC) were used by UEDA *et al.* [15] for differential analysis of various surfactant administration protocols. [¹²⁵I]-iodophenyldiazirine bound to albumin was used in the experiment of HENRY *et al.* [16]. Each technique has different strengths and weaknesses, giving only partial information about surfactant distribution [11]. External scanning has low resolution because of the superposition of the entire thickness of the lungs. This problem may be overcome when the lungs are divided into ~200 pieces and distribution per piece was measured using [¹⁴¹Ce]-labelled microspheres mixed with exogenous surfactant [4]. Two different radioisotopes ([¹⁴¹Ce and ¹⁰³Rn]-labelled microspheres) were used by PLÖTZ *et al.* [17] to differentiate distribution of a first and a second dose of exogenous surfactant in lung-lavaged rabbits. This technique of dividing lungs into pieces and measuring the amount of surfactant and its marker that distributes with surfactant in each piece has the advantage of being quantitative. However, the lung pieces do not provide information about alveolar distribution or the amount of surfactant not reaching the alveolar level.

Another commonly used technique involves the admixture of coloured microspheres to exogenous surfactant, and the pattern of distribution has been found to be equal to [¹⁴C]-labelled DPPC in rabbit lungs after surfactant depletion [5]. Again, the lungs were cut into 50 [6] to 70 [5] pieces after which the colourant was dissolved from the microspheres and quantified. Thus, it is a very demanding technique which requires additional technical equipment, personnel, and a second session for lung processing alone.

AMIRKHANIAN and MERRITT [18] demonstrated that the surface tension properties of natural surfactant preparations tested (including natural surfactants containing surfactant proteins B and C) are preserved within a pH range of 6.5–7.2, and that the whole range from pH 2–8 is still in accordance with almost unchanged surfactant surface tension properties. Therefore, the pH of 6.5 of Green Dye is in the ideal range of pH values for admixture to surfactant preparations.

Some questions remain that cannot be answered with the data from the present study: will the Green Dye/surfactant mixture be stable for >3 h after preparation and exert an undiminished physiological activity? Are there toxicological side effects which were not assessed (*e.g.* through monitoring of cardiovascular parameters)? How much dye is already unbound from surfactant after a study period of 3 h *in vivo* and will probably be degraded in a different manner such as unbound excess dye? Is the activity of macrophages or other cells augmented when colourants are added to surfactant? Are there any immunological reactions that were not assessed in this experiment?

In conclusion, the authors have found a stable colourant which allows staining of exogenous surfactant for experimental lung research, when the question of surfactant distribution at the alveolar level is being investigated to optimize *e.g.* the mode of surfactant administration, timing of surfactant administration or the ventilation strategy. In addition quantitative assessment of surfactant distribution may be optimized by using planimetric systems for counting green stained exogenous surfactant deposits.

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