

## Non-Invasive Near-Infrared Fluorescence Imaging of Neutrophil Infiltration in a Mouse Model of Acute Lung Inflammation

Catherine Robin-Jagerschmidt,<sup>1</sup> Emilie Berrocal,<sup>1</sup> Marielle Auberval,<sup>1</sup> Anna Yudina,<sup>2</sup> Jens Waldeck<sup>3</sup>

Author Information: 1 - Galapagos SASU 102, avenue Gaston Roussel 93230 Romainville France; 2 - Bruker BioSpin, 34 rue de l'Industrie, 67166 Wissembourg, France; 3 - Bruker BioSpin MRI GmbH, Rudolf-Plank-Str. 23, 76275 Ettlingen, Germany

The presence of bacterial endotoxin, lipopolysaccharide (LPS) in the lung can induce a major inflammatory response leading to acute lung injury. LPS is thought to immediately activate resident macrophages, leading to the production of various cytokines, including TNF- $\alpha$ . This will, in turn, prompt the recruitment of inflammatory cells, mainly neutrophils, into the lung through increases in capillary permeability and alveolar edema.<sup>1</sup> Neutrophil infiltration into the lung can be monitored by certain, activatable (smart) fluorescent probes. This study evaluates the use of an activatable optical probe that is optically silent upon inhalation and produces a fluorescent signal only after selective cleavage by the serine protease elastase produced by neutrophils.

### Material and Methods

#### *In vivo* Imaging Model

Six week-old female BALB/c mice (Janvier labs) were maintained on 12 hours light/dark cycle at 22 °C with ad libitum access to tap water and food. One week before initialing experiments, mice were maintained under low chlorophyll diet (Dietex).

The day before treatments, mice were anaesthetized using isoflurane and their chests were shaved and cream-epilated.

All compound administrations (including probe) were performed by intranasal route (i.n.) on mice anaesthetized with isoflurane.

Twenty-three hours before imaging, the mice were split into 3 groups (n = 6 per group). Mice of group 1 and 2 were given LPS (100 µg in 40 µL PBS; Sigma); group 3 mice (sham mice) were treated with PBS.

Four hours before imaging, Neutrophil Elastase 680 FASTTM probe (4 nmol; Perkin Elmer) was administered alone (group 3) or together with the chemotactic peptide N-formylmet-leu-phe (fMLP, 200 nM; Sigma) (groups 1 and 2). Fifteen minutes before probe administration, mice from group 2 were treated with Sivelestat (SE, 5 mg/kg; Sigma), a selective Neutrophil Elastase inhibitor (see also Table 1).

Imaging was performed using the Bruker In-Vivo Xtreme imaging system. The mice were anaesthetized by intraperitoneal injection of xylazine (5 mg/kg) and ketamine (75 mg/kg). All images were captured by a deeply cooled 4 MP CCD camera with the following parameters: f-stop 1.1, binning 2 x 2, 5 sec acquisition times, Excitation filter: 630 nm, Emission filter: 700 nm. For anatomical co-registration, an X-ray image (1.2 sec acquisition time, f-stop 2.8, binning 1 x 1, X-ray Filter: 0.2 mm, X-ray energy: 45 kVp) was performed. For precise co-registration of several mice, all images were taken with a 190 x 190 mm field of view (FOV).

### Image Analysis and Presentation

Image analysis was performed using Molecular Imaging Software version 7.1 (Bruker BioSpin, Billerica, MA, USA). A region of interest (ROI) was drawn around lung of one representative mouse using the free form option in the manual ROI tool in MI software. This ROI was then saved as template and applied to the other images using "Apply Template" function in the Manual ROI panel of the MI Navigation tool. The signal dynamic range was set from 1500 to 5000 counts for all acquired images. Data were exported and figures were compiled in Microsoft Excel®. The fluorescent signal was then overlaid on the corresponding X-ray image and exported as JPEG and/or TIFF file, respectively.

	Group 1	Group 2	Group 3 (sham)
<b>1 week b.i.</b>	Low chlorophyll diet	Low chlorophyll diet	Low chlorophyll diet
<b>1 day b.i.</b>	Shaving & epilation	Shaving & epilation	Shaving & epilation
<b>23 h b.i.</b>	LPS	LPS	PBS
<b>4 h 15 min b.i.</b>	-	Sivelestat	-
<b>4 h b.i.</b>	Elastase680 + fMLP	Elastase680 + fMLP	Elastase680
			Imaging

Table 1: Experimental workflow. LPS: 100 µg LPS in 40 µL PBS; Elastase680: 4 nmol Neutrophil Elastase 680 FASTTM probe; Sivelestat: 5 mg/kg; fMLP: 200 nM chemotactic peptide N-formylmet-leu-phe; b.i. before imaging.

### Broncho Alveolar Lavage (BAL)

Immediately after imaging, the trachea was exposed through midline incision and cannulated. BAL was performed twice with 0.75 mL sterile PBS. After centrifugation (250 g, 10 min, 4°C), the cell pellet was re-suspended in PBS. Total cell count was processed with Vet ABC and the remaining volume was used to determine cell populations after Cytospin Cyto centrifuge preparation (Thermo Scientific) and Giemsa staining (Advanced Chromatic).

### Statistical Analysis

For each read-out, mean and standard error to the mean (s.e.m.) were calculated. A statistically significant difference between sham or LPS groups was evaluated with Prism® software using a one-way ANOVA followed by a Dunnett's multiple comparisons post-hoc test. \*\*: p<0.01; \*\*\*: p<0.001.

### Results and Discussion

LPS and fMLP administration induced a significant increase in fluorescence intensity upon applying the Neutrophil Elastase probe in the lungs when compared to PBS control animals. Sivelestat partially prevented the amplification of fluorescence intensity (see Figure 1). These findings are in alignment with the findings of Kossodo et al.<sup>2</sup> Lack of homogeneity of the fluorescence signal is most likely due to an uneven distribution of compounds (LPS, Sivelestat and probe) across lung surfaces after intra-nasal instillation.

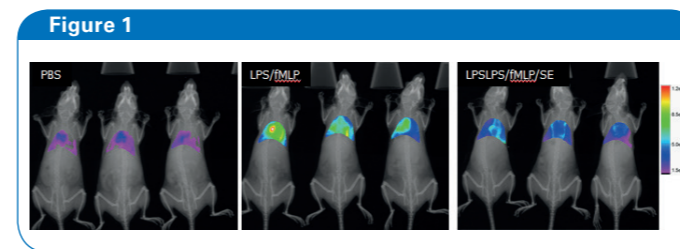


Figure 1: Multimodal *in vivo* imaging of neutrophil elastase activity in a mice model of acute lung inflammation induced by LPS inhalation. Left panel: PBS control mice; middle panel: mice treated with LPS (100 µg) and fMLP (200 nM); right panel: mice given LPS (100 µg), fMLP (200 nM) and Sivelestat (5 mg/kg). All panels: *In vivo* Fluorescent Neutrophil Elastase 680 FASTTM probe signals were overlaid onto corresponding X-ray images to demonstrate signal localization to the pulmonary space.

Image analysis showed that LPS/fMLP administration induced a highly significant, 7-fold increase in Neutrophil Elastase activity in lungs when compared to PBS control animals (p<0.001). Sivelestat partially prevented this increase in Neutrophil Elastase activity (p<0.01, see Figure 1 and 2).

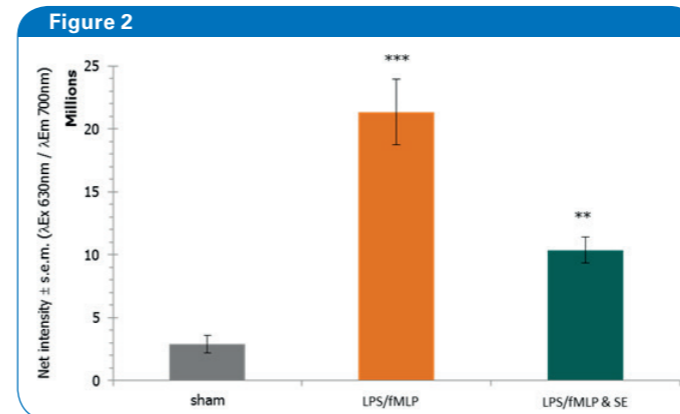


Figure 2: *In vivo* Net Fluorescence Intensities of Neutrophil Elastase activity in a mice model of acute lung inflammation. ROIs of similar areas were drawn around the fluorescent, pulmonary signals observed. Mouse treatment groups: sham: PBS control mice; LPS/fMLP: mice treated with LPS (100 µg) and fMLP (200 nM); LPS/fMLP & SE: mice treated with LPS (100 µg), fMLP (200 nM) along with Sivelestat (5 mg/kg); \*\*: p<0.01; \*\*\*: p<0.001.

Immediately after imaging, Broncho alveolar lavages (BAL) were collected for quantification of white blood cells and determination of cell distribution (see Figure 3).

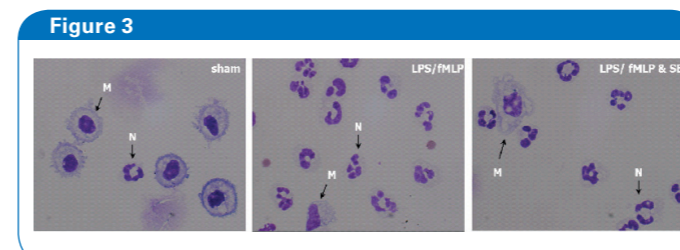


Figure 3: Cell distribution in Broncho alveolar lavage fluids from a mice model of acute lung inflammation. Cytospin Cyto centrifuge preparation and Giemsa staining were performed on Broncho alveolar lavages. Left panel (sham): PBS control mice; middle panel (LPS/fMLP): mice treated with LPS (100 µg) and fMLP (200 nM); right panel (LPS/fMLP & SE): mice treated with LPS (100 µg), fMLP (200 nM) and Sivelestat (5 mg/kg). M: macrophages; N: Neutrophils.

BAL from sham mice contained a small quantity of white blood cells, mainly represented by macrophages (93 %). LPS/fMLP instillation induced a 9-fold increase of total cell count and dramatically raised neutrophil proportion to almost 90% (p<0.01) (see Figure 3 and 4). These results are consistent with the higher Neutrophil Elastase activity observed *in vivo* in the LPS/fMLP-treated mice when compared to control animals (see Figure 2).

In line with lower Neutrophil Elastase activity observed after administration of Sivelestat (see Figure 2), the effect of LPS/fMLP on cell recruitment and neutrophil infiltration was also partially blunted (Figure 4). The effect of Sivelestat was mostly driven through decrease of total cell count and not by a modification of neutrophil proportion among the total cell population (not shown).

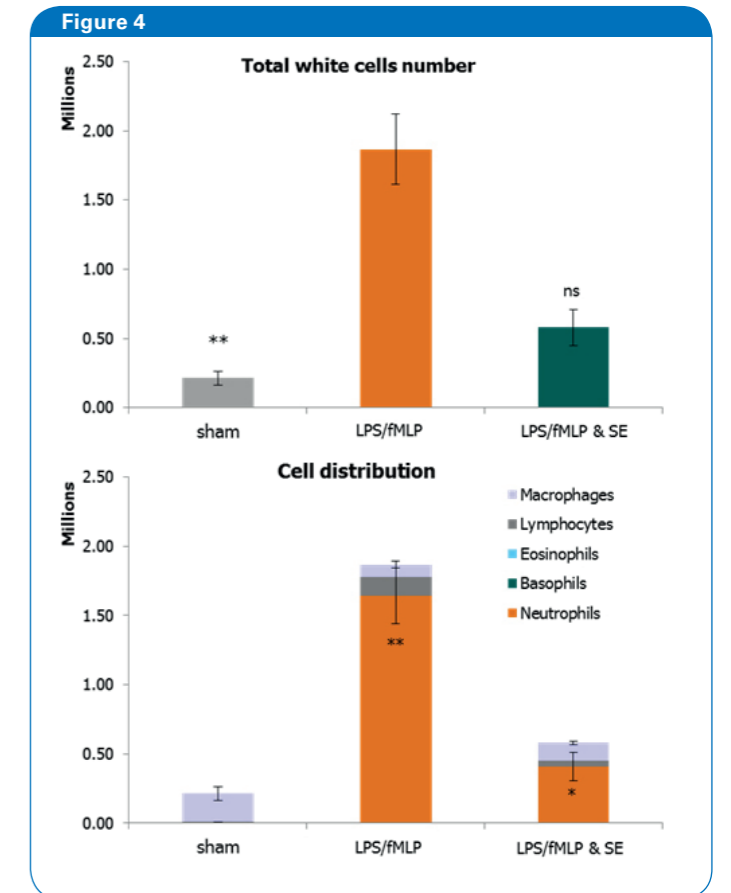


Figure 4: Total white cell number and cell distribution in Broncho alveolar lavage fluids from a mice model of acute lung inflammation. Total cell count (top panel) was processed with Vet ABC and cell population distribution (bottom panel) determined after Cytospin Cyto centrifuge preparation and Giemsa staining. For statistical analysis, see Methods.

## Conclusion

This application note describes an experimental protocol for non-invasive, *in vivo* imaging of LPS-induced neutrophil lung infiltration in a mouse model. This was achieved by monitoring the biodistribution of a neutrophil elastase activated fluorescent probe, with the Bruker In-Vivo Xtreme imaging system. As shown by the fluorescent and X-ray data presented here, the In-vivo Xtreme can provide real time, non-invasive imaging and measurement of LPS-derived lung inflammation in mouse models. It was also shown that inflammation trends observed through the In-vivo Xtreme correlated well with those seen by

traditional experimental methods. Additionally, the non-invasive approach of the In-vivo Xtreme was free of the inconveniences in traditional experimental methods: no mice had to be killed for BAL data, no extensive animal handling was involved and no preparations for neutrophil counts were needed. Finally, the In-vivo Xtreme's non-invasive approach can allow investigators to observe lung pathology evolutions over time, with one cohort of mice per treatment group. This minimizes the number of experimental mice needed, and it eliminates arbitrary data variability per treatment group over time.

## References

- [1] Grommes J, Soehnlein O. Contribution of neutrophils to acute lung injury. *Mol. Med.* 2011 Mar-Apr; 17(3-4):293-307.
- [2] Kossodo S, Zhang J, Groves K, Cuneo GJ, Handy E, Morin J, Delaney J, Yared W, Rajopadhye M, Peterson JD. Noninvasive *in vivo* quantification of neutrophil elastase activity in acute experimental mouse lung injury. *Int. J. Mol. Imaging*, 2011:581406