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# Assessment of murine collagen-induced arthritis by longitudinal non-invasive duplexed molecular optical imaging

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**Key words:** Collagen-induced arthritis, optical imaging, ProSense 750 FAST, Neutrophil Elastase 680 FAST, MMPSense 750 FAST, CatK 680 FAST, dexamethasone.

**Abstract**

**Objectives.** In the present study we evaluated the use of four commercially available fluorescent probes to monitor disease activity in murine collagen-induced arthritis (CIA) and its suppression during glucocorticoid therapy.

**Methods.** Arthritis was induced in male DBA/1 mice by immunization with type II collagen in Complete Freund's Adjuvant followed by a boost of collagen in PBS. Four fluorescent probes from PerkinElmer in combination, ProSense 750 FAST with Neutrophil Elastase 680 FAST and MMPSense 750 FAST with CatK 680 FAST, were used to monitor disease development from day 5 through to day 40-post immunization. Fluorescence generated *in vivo* by the probes was correlated with clinical and histological score and paw measurements.

**Results.** The fluorescence intensity emitted by each probe was shown to correlate with the conventional measurements of disease. The highest degree of correlation was observed with ProSense 750 FAST in combination with Neutrophil Elastase 680 FAST, these probes were then used to successfully assess CIA suppression during dexamethasone treatment.

**Conclusion.** We have demonstrated that longitudinal non-invasive duplexed optical fluorescence imaging provides a simple assessment of arthritic disease activity within the joints of mice following the induction of CIA and may represent a powerful tool to monitor the efficacy of drug treatments in preclinical studies.

## Introduction

Non-invasive optical bioimaging techniques are of increasing interest as a means of improving both preclinical research and clinical diagnostics or monitoring of arthritic disease [1,2]. Currently there are a variety of imaging agents available that may be used to monitor disease activity. The simplest are perhaps nonspecific agents such as the Indocyanine dyes, which injected intravenously, can be used to monitor inflammation as a result of accumulation at sights of increased blood flow and vascular leakage. Indocyanine dyes have been used pre-clinically in rodent arthritis models [3,4] and clinically in rheumatoid arthritis patients where accumulation of Indocyanine Green fluorescence has been shown to correlate with both magnetic resonance imaging and ultrasound assessments of disease severity [5-7]. Imaging of this nature offers a technically simple and relatively inexpensive means to monitor disease activity. More information however can be obtained about the underlying disease mechanism by using fluorescent probes that are targeted to specific disease associated molecules or processes. For example, conjugation of fluorochromes to antibodies against F4/80, a marker for macrophages [8] or E-selectin, a marker of endothelial cell activation [9], or the labelling of small molecules such as folate, the accumulation of which has been associated with the increased metabolic activity observed at sites of inflammation [10]. Reactive probes may also be used to monitor the production of reactive oxygen species [11], or the activity of inflammation associated enzymes such as cathepsins [12-15] and matrix metalloproteases (MMPs) [16-20]. Probes such as luminol, which react with enzymes that are produced by specific cell types, may also be used to monitor the infiltration and activity of those cells into sites of inflammation [21]. More recently, cell death was assessed in collagen-induced arthritis (CIA) by using a near-infrared PSVue 794 dye [22].

In addition to monitoring inflammatory or autoimmune conditions activatable fluorescent probes have been used successfully to monitor disease and therapeutic efficacy in a variety of cancer models. MMPSense 680 fluorescence has been shown to correlate with the presence of tumours *in*

*vivo* [23], while Neutrophil Elastase 680 has been used to monitor neutrophil activity within tumours following photodynamic therapy [24]. Decreases in bone metastasis associated CatK 680 fluorescence has been shown to correlate with successful treatment in a murine model of breast cancer [25].

Despite one of the major advantages offered by non-invasive imaging being the ability to repeatedly evaluate the molecular processes occurring within arthritic joints over the entire course of the experimental model, the majority of studies to date only look at one or two time points, and in addition only evaluate a single reagent in each animal. In the present study we show the use of four commercially available fluorescent probes from PerkinElmer in combination, ProSense 750 FAST with Neutrophil Elastase 680 FAST; and MMPSense 750 FAST with CatK 680 FAST to monitor disease development in the collagen-induced arthritis (CIA) model in mice from day 5 through to day 40-post immunisation. These probes are activatable fluorescent imaging agents comprising a novel architecture termed F.A.S.T. (Fluorescent Activatable Sensor Technology) that confers an improved pharmacokinetic profile with earlier imaging time points. These probes are optically silent in their naïve state and become highly fluorescent following enzymatic-mediated activation. ProSense 750 FAST is activated by proteases such as Cathepsin B, L, S, K, V and D. Neutrophil Elastase 680 FAST produces fluorescent signal after cleavage by elastase produced by neutrophils. MMPSense 750 FAST is a matrix metalloproteinase activatable agent that produces fluorescent signal after cleavage by a broad range of MMP's including MMP 2, 3, 7, 9, 12, and 13. CatK 680 FAST produces fluorescent signal after cleavage by Cathepsin K.

The fluorescence intensity emitted by each probe was shown to correlate with the conventional measurements of disease. The highest degree of correlation was observed with ProSense 750 FAST in combination with Neutrophil Elastase 680 FAST. These probes were then used to assess the anti-inflammatory effect of dexamethasone.

## Methods

*Mice*

Male DBA/1 mice were obtained from Harlan (Bicester, UK). All mice were used at 6-7 weeks old and maintained at the Biological Services Unit, University of Glasgow in accordance with Home Office UK guidelines.

*Induction of collagen-induced arthritis*

On day 0, collagen-induced arthritis was induced under light anaesthesia in DBA/1 male mice by intradermal injection of 200 μg Bovine Type II Collagen (MD Biosciences, St Paul, MN) emulsified in Complete Freund's Adjuvant (Chondrex, Redmond, WA) into the shaved rump followed by a boost on day 21 using 200 μg of Bovine Type II collagen in PBS administered intraperitoneally [26].

*Clinical Scoring regime*

Signs of arthritis were assessed in all paws according to a 0-4 scale of ascending severity: 0= No reaction; 1= mild, but definite redness and swelling of the ankle/wrist or apparent redness and swelling limited to individual digits, regardless of the number of digits affected; 2= moderate to severe redness and swelling of the ankle/wrist; 3= Redness and swelling of the entire paw including digits; 4= maximally inflamed limb with involvement of multiple joints [26]. Paw thickness as a measure of swelling was determined in mm three times weekly using dial callipers (Kroeplin, Munich, Germany) [27], data are presented as the mean thickness of all four paws. All animals were scored and the paw measurements were taken on the day of imaging.

*Probe administration protocol*

Four fluorescent probes were used in this study: Neutrophil Elastase 680 FAST, ProSense 750 FAST, CatK 680 FAST and MMPSense 750 FAST (all from PerkinElmer, Llantrisant, UK). Probes

were administered as cocktails: Neutrophil Elastase 680 FAST together with ProSense 750 FAST, and CatK 680 FAST with MMPSense 750 FAST. The absence of significant bleed through between probes was previously determined (Supplementary Fig. I). Probe cocktails were administered intravenously on days 5, 19, 27, 33 and 40 to naive (n=3) and CIA induced (n=6) animals. An additional group of animals were imaged without the administration of probe (n=3). Probes were administered at a dose of 4 nmol/25 g mouse according to the manufacturers instructions.

In a separate experiment, Neutrophil Elastase 680 FAST and ProSense 750 FAST were administered intravenously on days 28 and 35 to CIA induced animals treated intraperitoneally from day 21 with either the vehicle (5% ethanol in PBS) (n=6) or dexamethasone (Sigma-Aldrich, Dorset, UK) at 1 mg/kg/day (n=6). An additional group of animals were imaged without the administration of probe (n=3). Following imaging on day 35, mice were culled and the rear paws collected and fixed in neutral buffered formalin.

*In vivo Optical Imaging*

Images were acquired on a Kodak *in vivo* imaging system FX Pro (Carestream, Hertfordshire, UK). Animals were anaesthetised using isoflurane and oxygen and fur removed from the limbs using a depilatory cream (Boots Pharmacy Ltd, UK). Black electrical insulating tape was used to cover the abdomen to reduce nonspecific signal from probes degraded during excretion, and the mouse was placed in the prone position in the imaging unit. Imaging was performed eight hours following intravenous administration of Neutrophil Elastase 680 FAST and ProSense 750 FAST and six hours following the administration of CatK 680 FAST and MMPSense 750 FAST. Band pass excitation filters of 630 ± 10 nm was used for Neutrophil Elastase 680 FAST and CatK 680 FAST and 730 ± 10 nm for ProSense 750 FAST and MMPSense 750 FAST. Emission filters were 700 ± 17.5 nm and 790 ± 17.5 nm respectively. The entire acquisition time per animal was approximately 10 minutes. The exposure time was 90 seconds for Neutrophil Elastase 680 FAST and CatK 680 FAST, with 1x1 pixel binning and an aperture f-stop of 2.51. The exposure time for ProSense 750 and

MMPSense 750 FAST was 129 seconds with 1x1 pixel binning and aperture f-stop of 2.51. X-Ray images were taken using a 0.4 mm aluminium filter with an exposure time of 60 secs, 1x1 pixel binning and an aperture f-stop of 3.99. Fluorescent images are displayed as pseudo-coloured overlaid on the x-ray images. Scaling was determined manually and applied by the imaging software to the acquired grey-scale fluorescent images.

*Image Analysis*

Image analysis was performed using the Carestream molecular Imaging software (Carestream Health, Inc., Rochester, NY). A region of interest (ROI) tool was used to carefully draw around each paw forming 4 ROI per mouse. Background fluorescence signal was determined on a blank area of the image and the mean background pixel fluorescence intensity (FI) was subtracted from the ROI pixel FI. The total FI in arbitrary units for each ROI was then determined by the software. Fluorescence data are presented as the Total FI for all paws per mouse or the Total rear paw FI (for comparison to histopathology data only).

*Histological Scoring*

Rear paws were sampled on day 35, fixed in neutral buffered formalin (CellPath Ltd, Powys, UK), decalcified in EDTA, stained with haematoxylin & eosin or toluidine blue (Sigma-Aldrich) [28], and scored in a blinded fashion as described below [26]. Data is presented as the mean of the sum of the scores obtained for the three parameters obtained for each paw.

For the inflammatory cell infiltrates the scoring system was as follows: 0= no infiltrate detected, 1= modest leukocyte infiltration in synovial tissue, no fluid leukocytes, 2= moderate leukocyte infiltration in synovial tissue and in fluid phase with loss of synovial architecture, 3= gross leukocyte infiltration in synovial membrane and fluid space with significant loss of synovial and articular architecture. For synovial hyperplasia, the scoring system was as follows: 0= no abnormalities detected, 1= synovial lining layer 2-4 cell thick, 2= synovial lining layer >5 cells

thick associated with moderate expansion of the sub lining layer zone, 3= synovial lining layer >5 cells thick associated with significant expansion of the sub lining layer zone and potentially with loss of synovial architecture. For erosion of cartilage and bone 0= no abnormalities, 1= fibrillation of cartilage and/or mild erosive infiltration of periosteal and subchondral bone. Nuclei intact within lacunae, 2= moderate fibrillation and loss of cartilage and/or moderate erosive infiltration of periosteal and subchondral bone, 3= significant loss of cartilage and/or erosive infiltration of periosteal and subchondral bone. Nuclei show apoptosis within lacunae across a wide area of cartilage/bone.

*Statistical Analysis*

Where appropriate data are expressed as the group mean ± standard error of the mean (SEM). Statistical significances were determined by students t test using the Winstat™ add in for Excel™. A *P*-value of less than 0.05 was taken to be significant. Correlations between the arthritis score, paw measurements, histopathology score and FI were performed using the Spearman Rank Correlation Analysis. A *P*-value of less than 0.05 was deemed to be significant.

**Results**

*Fluorescence from commercially available activatable probes correlates with clinical disease score in collagen-induced arthritis*

As is typically observed in this model, very little disease was observed prior to the second collagen injection on day 21, following this the clinical score increased peaking on day 33 (Fig. 1A and B). The naive groups (n=3) remained disease free throughout the study, excepting a single animal in the Neutrophil Elastase 680 FAST/ProSense 750 FAST treated group which developed a transient score of 1, which was probably a minor injury and consistent with normal activity.

The increase in clinical score observed in the CIA mice was mirrored by the FI from the neutrophil elastase and cathepsin (both the multi cathepsin sensitive ProSense and the cathepsin K specific

CatK) sensitive probes (Fig. 1C, D and E). MMPSense gave the more variable fluorescence in both the naive and the arthritic animals (Fig. 1F).

Neutrophil Elastase 680 FAST and ProSense 750 FAST were the more sensitive probes, giving the highest degree of correlation between total clinical score and total FI (Fig. 1G and H). CatK 680 FAST FI, while mirroring the disease induction, was a little less sensitive showing a lower degree of correlation with the clinical score; with an increase in fluorescence above that observed in the naive animals only evident in mice with a total score of >6 (Fig. 1I). This probably relates to the degree of disease required in the CIA model before bone erosion, resulting from increased osteoclast (and therefore cathepsin K) activity, develops [29,30]. The lowest degree of correlation with clinical arthritis scores was observed with the fluorescence from MMPSense 750 probe, with an increase in fluorescence above that observed in the naive animals only observed in mice with a total score of >8 (Fig. 1J).

*The probes Neutrophil Elastase 680 FAST and ProSense 750 FAST can be used to monitor the efficacy of a of an anti-inflammatory drug treatment in the CIA model*

The highest degree of correlation between score and fluorescence was observed with Neutrophil Elastase 680 FAST and ProSense 750 FAST probes. These two probes were therefore carried forward to test the use of non-invasive imaging to monitor the efficacy of the anti-inflammatory drug, dexamethasone (1 mg/kg/day starting on day 21) in the CIA model.

As previously observed, only mild disease in a few animals developed prior to the boost on day 21, subsequent to the boost clinical scores increased in the vehicle treated animals and continued to do so until the experiment terminated. In the dexamethasone treated mice the mild disease that was observed prior to day 21 resolved and the mean clinical score had returned to 0 by day 27, remaining constant until termination (Fig. 2A). Paw thickness was also measured and this closely

mirrored the clinical scores, with dexamethasone significantly reducing paw thickness from day 27 until the end of the experiment compared to the vehicle treated group (Fig. 2B). Significant correlation was also observed between the clinical scores and paw thickness measurements (Fig. 2C).

The probe cocktail was administered on days 28 and 35 (prior to and at the disease peak) and the mice imaged after 8 hours. The fluorescence intensities generated by Neutrophil Elastase 680 FAST and by ProSense 750 FAST in the dexamethasone treated CIA mice was significantly reduced compared to the vehicle treated control animals at both 28 and 35 days (Fig. 2D and E). Arthritic paws are clearly visible in the pseudo coloured fluorescent image and X-ray overlays from the vehicle treated mice and absent from the dexamethasone treated animals for both Neutrophil Elastase 680 FAST and for ProSense 750 FAST (Fig. 2F). In addition to the paws there are fluorescent hot spots apparent in the knee joints of some of the vehicle treated animals (#1, 2, 4, and 6). This suggests the presence of inflammation within the knee joint, which is something that is not possible to score clinically. An additional bright spot is observed in mouse 4 over the abdomen, this signal is due to the urinary excretion of the probe localized in the bladder.
The fluorescence intensities in the paws for both probes were found to correlate significantly with both the clinical score (Fig. 2G and H) and with paw thicknesses (Fig. 2I and J).

Dexamethasone reduced the clinical score by almost 10 points at 35 days and the treated mice displayed almost no signs of arthritis. This is not always the case when new drugs are tested in the CIA model. We have therefore analysed if the fluorescent signal from the Neutrophil Elastase FAST 680 and ProSense FAST 750 probes have the potential to detect even small differences between treatment groups. To address this issue data were pooled from the control CIA mice taken from two experiments at two different time points, 4 weeks and 5 weeks, following primary type II collagen immunization (difference in clinical score of 4.22). Analysis of the data demonstrated that,

although less sensitive than the clinical score, these probes have the potential to detect small differences between groups (Supplementary Fig. II).

*Correlation with histopathological scores*

As a gold standard measure of disease activity in preclinical arthritis studies, the histological scoring data obtained from rear paws sampled at day 35 was also compared with the fluorescence imaging data. The histopathology score in dexamethasone treated animals was found to be significantly lower than that observed in the vehicle control treated animals (Fig. 3A). The rear paw histopathology scores were then compared with the rear paw thickness, rear paw clinical scores and with the rear paw FI from both probes. The clinical scores and paw measurements correlated with the histopathology scores (Fig. 3B and C, respectively); a similar degree of correlation was also observed between the FI obtained from Neutrophil Elastase 680 FAST and ProSense 750 FAST (Fig. 3D and E, respectively).

**Discussion**

Increased activity of proteases such as the cathepsins [31] and MMPs [32] and infiltration of inflammatory cells such as neutrophils [33] are associated with a variety of autoimmune and inflammatory conditions including rheumatoid arthritis. Here we demonstrate the use of 4 commercially available probes to detect increased activity of cathepsins (ProSense 750 FAST or CatK 680 FAST), MMPs (MMPSense 750 FAST) and neutrophil elastase (Neutrophil Elastase 680 FAST) in the arthritic paws of mice following the induction of CIA, albeit with varying degrees of sensitivity.

The probes, Neutrophil Elastase 680 FAST and ProSense 750 FAST (the latter activated by multiple cathepsins), showed the greatest ability to distinguish mildly diseased paws from normal paws. These results are consistent with data showing increases in gene expression and enzyme activity [34,35] or the role of neutrophils in the development of arthritis. Our data also support

previous findings demonstrating the ability of ProSense to detect cathepsin activity *in vivo* in a K/BxN serum-transfer model of arthritis [14,15]. In our hands MMPSense 750, a probe with multiple MMP sensitivities showed a relatively high degree of variability and was only able to detect differences between naïve and CIA animals once the disease was comparatively severe. Interestingly, however, evaluation of inflammatory arthritis in CIA has been recently successfully performed using a fluorogenic MMP-3-specific polymeric probe [20], and an MMP-3 specific probe has been previously used to discriminate between CIA and naïve animals prior to clinically apparent disease [16]. MMP-12 and MMP-13 specific probes have been shown to correlate with CIA disease activity at early and late phases respectively [36]. The variability of fluorescence obtained with MMPSense 750 in our hands may reflect different sensitivity of the probe to different MMPs and the role of MMPs in tissue homeostasis and at different phases of disease. In an alternative model of arthritis, the anti-collagen antibody induced model, MMPSense 680 has been shown to correlate with disease severity by being able to detect the disease enhancing properties of circulating fibrocytes [37]. More recently, MMPSense 680 was successfully used to monitor osteoarthritis development in mice, after direct injection of the dye through the patellar ligament into the knee joint cavity [19]. Demonstrating that MMP activity can be successfully assessed by near infrared fluorescence imaging depending on the experimental model and route of administration. The cathepsin K specific probe, CatK FAST 680, also showed a lower degree of sensitivity, only showing increased fluorescence in animals with higher total clinical disease scores. This may also be reflective of cathepsin K's role in bone homeostasis and erosion [38] and it therefore being present at the highest levels only in the more severely affected animals.

Dexamethasone, a synthetic corticosteroid drug with substantial anti-inflammatory activities, has been shown previously to inhibit the development of experimental arthritis following immunisation with type II collagen [39-41]. Reflecting the profound anti-arthritic effects of dexamethasone treatment in the CIA model, the fluorescence of the probes Neutrophil Elastase FAST 680 and

ProSense FAST 750 were also reduced in treated mice. This is consistent with observations in other disease models where dexamethasone reduces pulmonary neutrophil elastase production and in CIA where another steroid drug prednisolone has been shown to reduce the numbers of neutrophils present within inflamed joints [42].

The use of activatable probes and whole body imaging for disease monitoring has a number of advantages over the standard methods. Firstly, like clinical scoring, imaging can be used to assess the same animal over multiple time points - limited only by the half-life of the selected probes. However, unlike a simple clinical score, the activatable probes provide information about the molecular processes occurring within tissues. The standard means of assessing the molecular processes of diseases in both animal models and clinically is assessment of serum titres of biomarkers [43,44]. While repeated blood sampling is possible in animal models and in human patients, this may not fully reflect what is occurring at the site of pathology. In addition, where a mediator such as neutrophil elastase is localized on or in specific cells, this type of assessment allows the longitudinal monitoring of the infiltration and clearance of a specific cell population on an individual basis. An alternative method is to look for enzyme activity within joints [38], however this is more difficult in a clinical setting involving the biopsy or surgical removal of tissues and would usually be a terminal procedure in an animal model. Thus repeated sampling from the same individual is not usually possible. Therefore, with the rapid development of new imaging probes, optical imaging may soon offer the possibility to assess a broad range of molecular events in a non-invasive and multiplexed manner.

Most importantly, we have shown correlation between the probe FI and the histological score. Therefore, the proposed approach may decrease the number of animals required per experiment because tissue damage will not necessarily require evaluation by harvesting joints for histology. The specificity of the different probes also provides information regarding the pathological processes occurring within the tissues. In pre-clinical studies the imaging may also offer insight into

disease in joints that cannot usually be investigated by standard scoring techniques such as the knees.

In conclusion, we have demonstrated that longitudinal non-invasive optical fluorescence imaging may represent a powerful tool for the assessment of murine CIA and efficacy of drugs. Optical imaging probes are beginning to make their way into the clinic. The relative ease and non-invasive nature of fluorescent molecular imaging techniques make them an attractive addition to disease assessment both in patients and in preclinical studies.

**Key message**

Longitudinal non-invasive optical fluorescence imaging is a powerful tool for the assessment of murine CIA.

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**References**

1. Put S, Westhovens R, Lahoutte T, Matthys P. Molecular imaging of rheumatoid arthritis: emerging markers, tools, and techniques. Arthritis Res Ther 2014;16:208.

2. Mountz J M, Alavi A, Mountz J D. Emerging optical and nuclear medicine imaging methods in rheumatoid arthritis. Nat Rev Rheumatol 2012;8:719-28.

3. Meier R, Krug C, Golovko D, *et al.* Indocyanine green-enhanced imaging of antigen-induced arthritis with an integrated optical imaging/radiography system. Arthritis Rheum 2010;62:2322-7.

4. Hansch A, Frey O, Hilger I, *et al.* Diagnosis of arthritis using near-infrared fluorochrome Cy5.5. Invest Radiol 2004;39:626-32.

5. Werner S G, Langer H E, Schott P, *et al.* Indocyanine green-enhanced fluorescence optical imaging in patients with early and very early arthritis: a comparative study with magnetic resonance imaging. Arthritis Rheum 2013;65:3036-44.

6. Werner S G, Langer H E, Ohrndorf S, *et al.* Inflammation assessment in patients with arthritis using a novel in vivo fluorescence optical imaging technology. Ann Rheum Dis 2012;71:504-10.

7. Schäfer V S, Hartung W, Hoffstetter P, *et al.* Quantitative assessment of synovitis in patients with rheumatoid arthritis using fluorescence optical imaging. Arthritis Res Ther 2013;15:R124.

8. Hansch A, Frey O, Sauner D, *et al.* In vivo imaging of experimental arthritis with near-infrared fluorescence. Arthritis Rheum 2004;50:961-7.

9. Gompels L L, Madden L, Lim N H, *et al.* In vivo fluorescence imaging of E-selectin: quantitative detection of endothelial activation in a mouse model of arthritis. Arthritis Rheum 2011;63:107-17.

10. Chen W T, Mahmood U, Weissleder R, Tung C H. Arthritis imaging using a near-infrared fluorescence folate-targeted probe. Arthritis Res Ther 2005;7:R310-7.

11. Xie L, Lin A S, Kundu K, Levenston M E, Murthy N, Guldberg R E. Quantitative imaging of cartilage and bone morphology, reactive oxygen species, and vascularization in a rodent model of osteoarthritis. Arthritis Rheum 2012;64:1899-908.

12. Caglič D, Globisch A, Kindermann M, *et al.* Functional in vivo imaging of cysteine cathepsin activity in murine model of inflammation. Bioorg Med Chem 2011;19:1055-61.

13. Tung C H, Bredow S, Mahmood U, Weissleder R. Preparation of a cathepsin D sensitive near-infrared fluorescence probe for imaging. Bioconjug Chem 1999;10:892-6.

14. Stangenberg L, Burzyn D, Binstadt B A, *et al.* Denervation protects limbs from inflammatory arthritis via an impact on the microvasculature. Proc Natl Acad Sci U S A 2014;111:11419-24.

15. Stangenberg L, Ellson C, Cortez-Retamozo V, *et al.* Abrogation of antibody-induced arthritis in mice by a self-activating viridin prodrug and association with impaired neutrophil and endothelial cell function. Arthritis Rheum 2009;60:2314-24.

16. Ryu J H, Lee A, Chu J U, *et al.* Early diagnosis of arthritis in mice with collagen-induced arthritis, using a fluorogenic matrix metalloproteinase 3-specific polymeric probe. Arthritis Rheum 2011;63:3824-32.

17. Ally M M, Hodkinson B, Meyer P W, Musenge E, Tikly M, Anderson R. Serum matrix metalloproteinase-3 in comparison with acute phase proteins as a marker of disease activity and radiographic damage in early rheumatoid arthritis. Mediators Inflamm 2013;2013:183653.

18. Jones E F, Schooler J, Miller D C, *et al.* Characterization of human osteoarthritic cartilage using optical and magnetic resonance imaging. Mol Imaging Biol 2012;14:32-9.

19. Leahy A A, Esfahani S A, Foote A T, *et al.* Analysis of the trajectory of osteoarthritis development in a mouse model by serial near-infrared fluorescence imaging of matrix metalloproteinase activities. Arthritis Rheumatol. 2015;67:442-53.

20. Park J S, Kim S H, Kim K, *et al.* Inhibition of notch signalling ameliorates experimental inflammatory arthritis. Ann Rheum Dis 2015;74:267-74.

21. Gross S, Gammon S T, Moss B L, *et al.* Bioluminescence imaging of myeloperoxidase activity in vivo. Nat Med 2009;15:455-61.

22. Chan M M, Gray B D, Pak K Y, Fong D. Non-invasive in vivo imaging of arthritis in a collagen-induced murine model with phosphatidylserine-binding near-infrared (NIR) dye. Arthritis Res Ther 2015;17:50.

23. Clapper M L, Hensley H H, Chang W C, Devarajan K, Nguyen M T, Cooper H S. Detection of colorectal adenomas using a bioactivatable probe specific for matrix metalloproteinase activity. Neoplasia 2011;13:685-91.

24. Mitra S, Modi K D, Foster T H. Enzyme-activatable imaging probe reveals enhanced neutrophil elastase activity in tumors following photodynamic therapy. J Biomed Opt 2013;18:101314.

25. Hoff B A, Chughtai K, Jeon Y H, *et al.* Multimodality imaging of tumor and bone response in a mouse model of bony metastasis. Transl Oncol 2012;5:415-21.

26. Ierna M, Kerr A, Scales H, Berge K, Griinari M. Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. BMC Musculoskelet Disord 2010;11:136.

27. Leung B P, McInnes I B, Esfandiari E, Wei X Q, Liew F Y. Combined Effects of IL-12 and IL-18 on the Induction of Collagen-Induced Arthritis. J Immunol 2000;164:6495-6502.

28. Maffia P, Brewer J M, Gracie J A, *et al.* Inducing experimental arthritis and breaking self-tolerance to joint-specific antigens with trackable, ovalbumin-specific T cells. J Immunol 2004;173:151-6.

29. Lubberts E, Oppers-Walgreen B, Pettit A R, *et al.* Increase in expression of receptor activator of nuclear factor kappaB at sites of bone erosion correlates with progression of inflammation in evolving collagen-induced arthritis. Arthritis Rheum 2002;46:3055-64.

30. Mori H, Kitazawa R, Mizuki S, Nose M, Maeda S, Kitazawa S. RANK ligand, RANK, and OPG expression in type II collagen-induced arthritis mouse. Histochem Cell Biol 2002;117:283-92.

31. Conus S, Simon H U. Cathepsins: key modulators of cell death and inflammatory responses. Biochem Pharmacol 2008;76:1374-82.

32. Fanjul-Fernández M, Folgueras A R, Cabrera S, López-Otín C. Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models. Biochim Biophys Acta 2010;1803:3-19.

33. Kaplan M J. Role of neutrophils in systemic autoimmune diseases. Arthritis Res Ther 2013;15:219.

34. van Meurs J B, van Lent P L, Holthuysen A E, Singer I I, Bayne E K, van den Berg W B. Kinetics of aggrecanase- and metalloproteinase-induced neoepitopes in various stages of cartilage destruction in murine arthritis. Arthritis Rheum 1999;42:1128-39.

35. Vermeij E A, Koenders M I, Blom A B, *et al.* In vivo molecular imaging of cathepsin and matrix metalloproteinase activity discriminates between arthritic and osteoarthritic processes in mice. Mol Imaging 2014;13:1-10.

36. Lim N H, Meinjohanns E, Bou-Gharios G, *et al.* In vivo imaging of matrix metalloproteinase 12 and matrix metalloproteinase 13 activities in the mouse model of collagen-induced arthritis. Arthritis Rheumatol 2014;66:589-98.

37. Galligan C L, Fish E N. Circulating fibrocytes contribute to the pathogenesis of collagen antibody-induced arthritis. Arthritis Rheum 2012;64:3583-93.

38. Hummel K M, Petrow P K, Franz J K, *et al.* Cysteine proteinase cathepsin K mRNA is expressed in synovium of patients with rheumatoid arthritis and is detected at sites of synovial bone destruction. J Rheumatol 1998;25:1887-94.

39. Kang I, Lee W W, Lee Y. Modulation of collagen-induced arthritis by IL-4 and dexamethasone: the synergistic effect of IL-4 and dexamethasone on the resolution of CIA. Immunopharmacology 2000;49:317-24.

40. Oestergaard S, Rasmussen K E, Doyle N, *et al.* Evaluation of cartilage and bone degradation in a murine collagen antibody-induced arthritis model. Scand J Immunol 2008;67:304-12.

41. Larsson E, Erlandsson Harris H, Larsson A, *et al.* Corticosteroid treatment of experimental arthritis retards cartilage destruction as determined by histology and serum COMP. Rheumatology (Oxford) 2004;43:428-34.

42. Pimentel T A, Sampaio A L F, D'Acquisto F, Perretti M, Oliani S M. An essential role for mast cells as modulators of neutrophils influx in collagen-induced arthritis in the mouse. Lab Invest 2011;91:33-42.

43. Seeuws S, Jacques P, Van Praet J, *et al.* A multiparameter approach to monitor disease activity in collagen-induced arthritis. Arthritis Res Ther 2010;12:R160.

44. Skoumal M, Haberhauer G, Kolarz G, Hawa G, Woloszczuk W, Klingler A. Serum cathepsin K levels of patients with longstanding rheumatoid arthritis: correlation with radiological destruction. Arthritis Res Ther 2005;7:R65-70.

**Figure legends**

**Figure 1.** Fluorescence from the activatable probes correlate with disease score in collagen-induced arthritis. Graph (**A**) shows the clinical arthritis score for naïve mice (n=3) (black line open shape) and CIA mice (n=6) (black line closed shape) treated with the Neutrophil Elastase 680 FAST and ProSense 750 FAST cocktail. Graph (**B**) shows the clinical arthritis score for naïve mice (n=3) and CIA mice (n=6) treated with the CatK 680 FAST and MMPSense 750 FAST cocktail. **P* <0.05. (**C-F**) Fluorescence intensity detected within the fore and rear paws of CIA mice (black line closed shape) or naïve mice (black line open shape) following the administration of a Neutrophil Elastase 680 FAST (**C**; diamond) and ProSense 750 FAST cocktail (**D**; triangle) or a CatK 680 FAST (**E**; circle) and MMPSense 750 FAST (**F**; square) cocktail. Images were also acquired at the relevant wavelengths in mice without probe treatment (n=3) (grey line open shapes). **P* <0.05. Graphs (**G-J**) show the correlation of the total fluorescence intensities and the total clinical arthritis score throughout the study. Correlation coefficient (r) and p values are shown on the graphs.

**Figure 2.** Dexamethasone treatment of CIA mice reduces the fluorescent signal obtained from both the neutrophil elastase sensitive and the cathepsin sensitive probe. Graph (**A**) shows the total clinical score, graph (**B**) shows the mean paw thickness determined throughout the study, and graph (**C**) shows the correlation between the clinical score and the paw thickness at days 28 and 35 in CIA mice treated with either the vehicle control (black) or dexamethasone (white). (**D-E**) Fluorescence intensity for Neutrophil Elastase 680 FAST and ProSense 750 FAST detected within the fore and rear paws of CIA mice treated with dexamethasone (n=6) or vehicle (n=6) on days 28 and 35-post immunization (black columns, Vehicle; white columns, Dex; grey columns, no probe). Panel (**F**) shows Fluorescence-X-ray overlay images obtained on day 35 from CIA mice treated with either dexamethasone or the vehicle control. The two panels show the same animals. Graphs (**G-J**) show the correlation of the Neutrophil Elastase 680 FAST and ProSense 750 FAST FI with the clinical score and paw thickness on day 28 and 35 post immunisation. **P* <0.05, ***P* <0.01, ****P* <0.001.

**Figure 3.** Fluorescent signal from the neutrophil elastase sensitive and the cathepsin sensitive probe correlates with the histopathological score. Graph (**A**) shows the mean histopathology score obtained from rear paws on day 35 in CIA mice treated with either the vehicle control (black) or dexamethasone (white). ***P* <0.01. Graph (**B**) shows the correlation of the histological score with clinical score. Graph (**C**) shows the correlation of the histological score with paw thickness. Graph (**D**) shows the correlation of the Neutrophil Elastase 680 FAST rear paw FI with the histological score. Graph (**E**) shows the correlation of the ProSense 750 FAST rear paw FI with the histological score. Correlation coefficient (r) and p values are shown on the graphs.

Figure 1

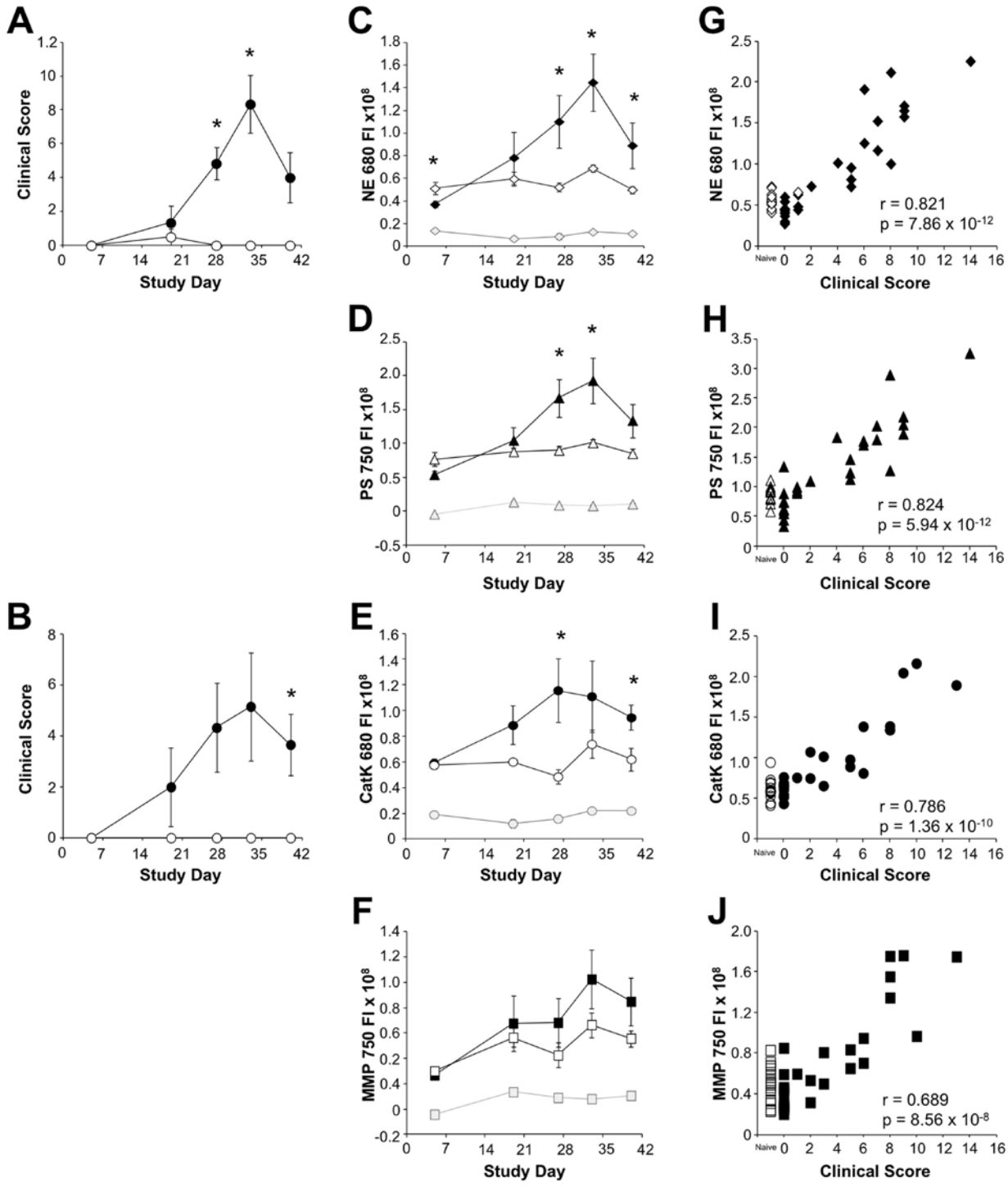

A
Clinical Score
Study Day
B
Clinical Score
Study Day
C
NE 680 FI x10⁸
Study Day
D
PS 750 FI x10⁸
Study Day
E
CatK 680 FI x10⁸
Study Day
F
MMP 750 FI x 10⁸
Study Day
G
NE 680 FI x10⁸
r = 0.821
p = 7.86 x 10-12
Naive
Clinical Score
H
PS 750 FI x10⁸
r = 0.824
p = 5.94 x 10-12
Naive
Clinical Score
I
CatK 680 FI x10⁸
r = 0.786
p = 1.36 x 10-10
Naive
Clinical Score
J
MMP 750 FI x 10⁸
r = 0.689
p = 8.56 x 10-8
Naive
Clinical Score

Figure 2

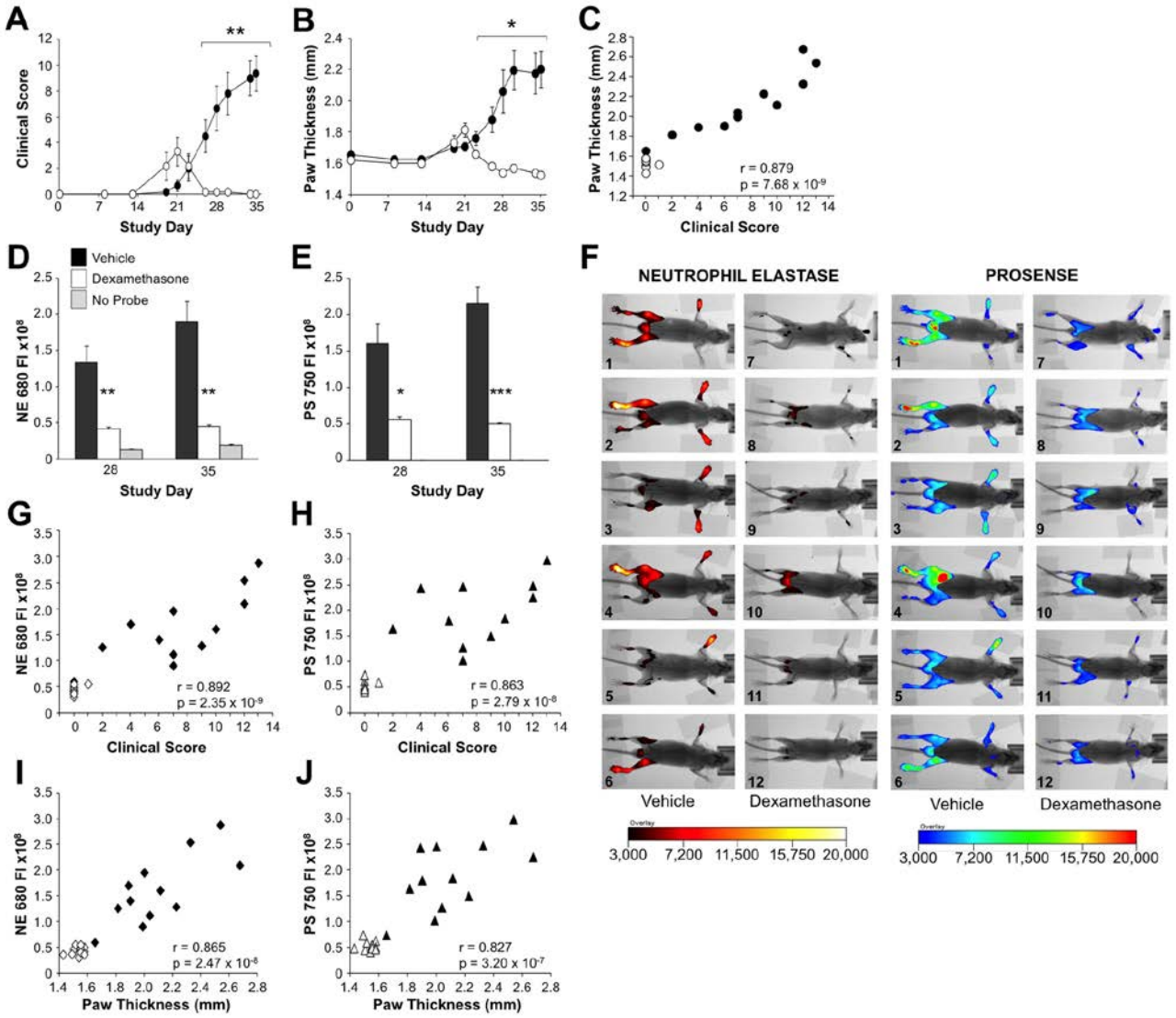
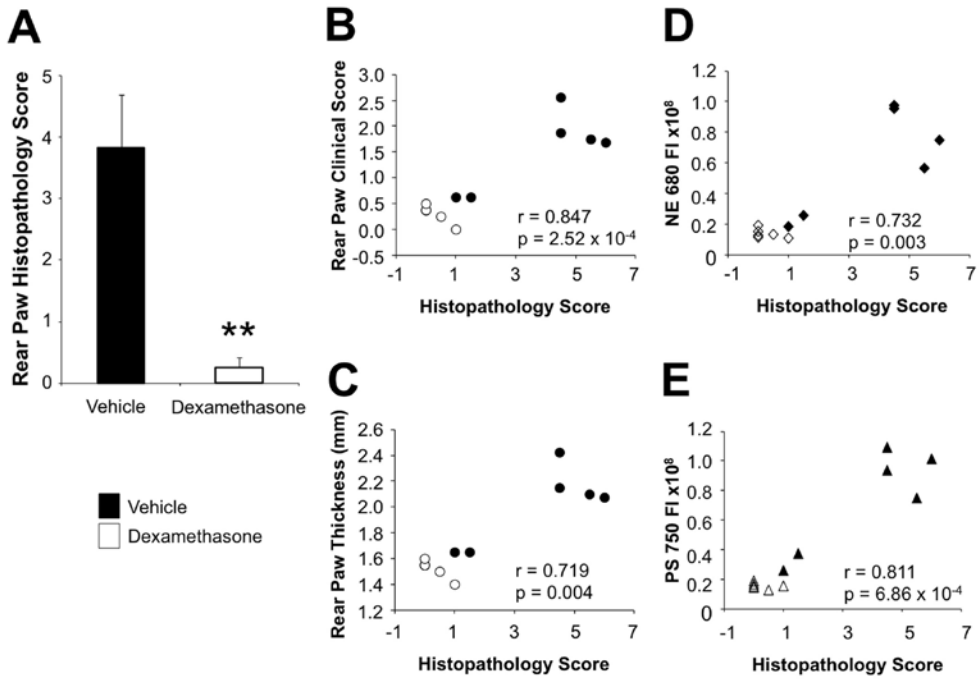
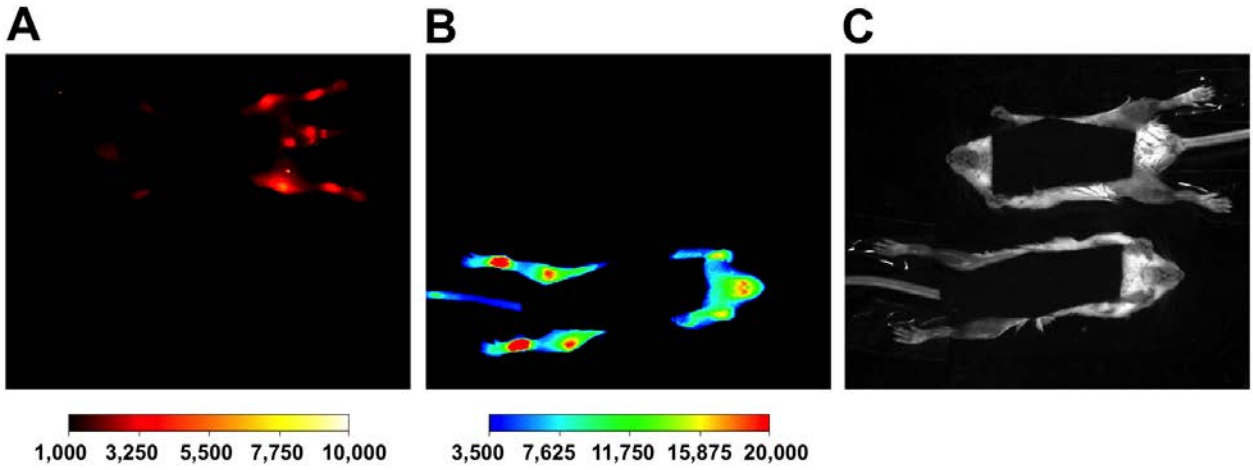

A
Clinical Score
Study Day
**
B
Paw Thickness (mm)
Study Day
*
C
Paw Thickness (mm)
Clinical Score
r = 0.879
p = 7.68 x 10-9
D
Vehicle
Dexamethasone
No Probe
NE 680 FI x10⁸
Study Day
E
PS 750 FI x10⁸
Study Day
F
NEUTROPHIL ELASTASE
PROSENSE
Vehicle
Dexamethasone
3,000 7,200 11,500 15,750 20,000
G
NE 680 FI x10⁸
Clinical Score
r = 0.892
p = 2.35 x 10-9
H
PS 750 FI x10⁸
Clinical Score
r = 0.863
p = 2.79 x 10-8
I
NE 680 FI x10⁸
Paw Thickness (mm)
r = 0.865
p = 2.47 x 10-8
J
PS 750 FI x10⁸
Paw Thickness (mm)
r = 0.827
p = 3.20 x 10-7

Figure 3

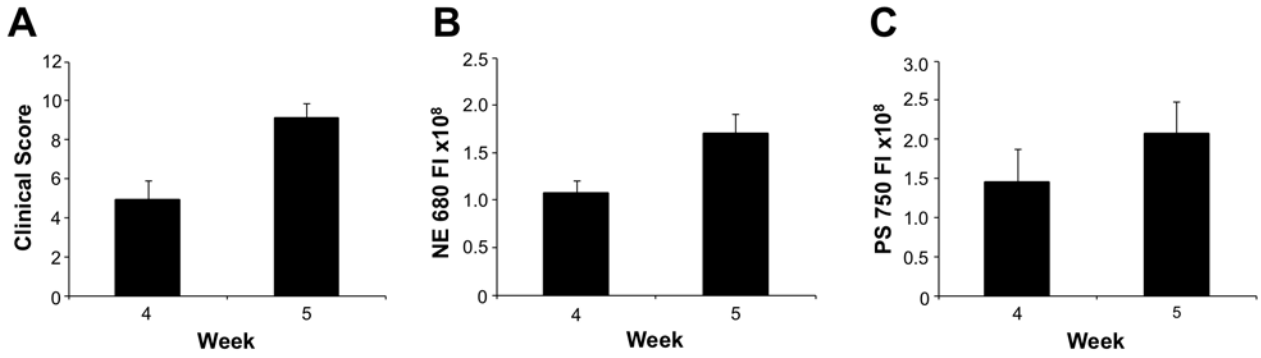

A
Rear Paw Histopathology Score
5
4
3
2
1
0
**
Vehicle
Dexamethasone
Vehicle
Dexamethasone
B
Rear Paw Clinical Score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
r = 0.847
p = 2.52 x 10-4
-1
1
3
5
7
Histopathology Score
D
NE 680 FI x10⁸
1.2
1.0
0.8
0.6
0.4
0.2
0
r = 0.732
p = 0.003
-1
1
3
5
7
Histopathology Score
C
Rear Paw Thickness (mm)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
r = 0.719
p = 0.004
-1
1
3
5
7
Histopathology Score
E
PS 750 FI x10⁸
1.2
1.0
0.8
0.6
0.4
0.2
0
r = 0.811
p = 6.86 x 10-4
-1
1
3
5
7
Histopathology Score

# Assessment of murine collagen-induced arthritis by longitudinal non-invasive duplexed molecular optical imaging

Hannah E. Scales[1,2], Michelle Ierna[2], Karen Smith[2], Kirsty Ross[3], Gordon R. Meiklejohn[1,2], Janet C. Patterson-Kane[1], Iain B. McInnes[1], James M. Brewer[1], Paul Garside[1] and Pasquale Maffia[1,4]




**Supplementary Figure I.** Fluorescence images showing arthritic mice. Images were acquired 8 hours following the administration of Neutrophil Elastase 680 FAST (animal at top of image) or ProSense 750 FAST (animal at bottom of image) on the Kodak *in vivo* imaging system FX Pro. Arthritis was induced in BALB/c mice (Charles River Laboratories, Harlow, UK) using the rapid Collagen Antibody Induced Arthritis model by the administration of 2 mg ArthritoMab (MD Biosciences, St Paul, MN ) on Day 0 and 100 μg LPS on day 3. Probes were administered on Day 5 and mice were imaged 8 hours later. Images (**A-C**) show the same two animals with images taken consecutively. Image (**A**) shows a pseudo-coloured fluorescence picture acquired using a 630 nm excitation filter and a 700 nm emission filter; fluorescence is only observed from the neutrophil elastase probe. Image (**B**) shows a pseudo-coloured fluorescence picture acquired using a 730 nm excitation filter and a 790 nm emission filter; fluorescence is only observed from the ProSense probe. Image (**C**) shows a white light image of the animals in grey scale as acquired.




**Supplementary Figure II.** Neutrophil Elastase and ProSense probes have the potential to detect small differences between treatment groups. The clinical scoring system can detect small differences between treatment groups. We have therefore analysed if this is also the case with the Neutrophil Elastase 680 and ProSense 750 probes. To address this issue data were pooled from the control CIA mice taken from two experiments at two different time points, 4 weeks and 5 weeks, following primary type II collagen immunisation (difference in clinical score of 4.22). Analysis of the data by dependent student T test showed that the clinical score (**A**) significantly ($P$ =0.0003; n=9/group) increased from 4.89 ± 1.03 to 9.11 ± 0.73. Concomitantly, the fluorescent intensity for Neutrophil Elastase 680 FAST (**B**) increased significantly ($P$ =0.0379; n=9/group). The fluorescent intensity for ProSense 750 FAST (**C**) also increased, approaching, but not reaching, significance ($P$ =0.0679; n=9/group). When data were analysed by independent student T test (the test to be applied in a real experimental scenario; i.e. for the analysis of two independent groups) the clinical score significantly ($P$ =0.004; n=9/group) increased, as did the fluorescent intensity for both Neutrophil Elastase 680 FAST and ProSense 750 FAST ($P$ =0.021 and $P$ =0.026, respectively; n=9/group). Demonstrating that, although less sensitive than the clinical score, these probes have the potential to detect small differences between groups.