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

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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 sites 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 Olac (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 (Kroepelin, 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 1×1 pixel binning and an aperture f-stop of 2.51. The exposure time for ProSense 750 and
MMPSense 750 FAST was 129 seconds with $1\times1$ 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, $1\times1$ 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 Winstar™ 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 matrix metalloproteinases (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 [28,38], 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.

**Key message**

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.

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**References**


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) on days 5, 19, 27, 33, and 40-post immunization. Images were acquired 8 hours following the administration of a Neutrophil Elastase 680 FAST (C; diamond) and ProSense 750 FAST cocktail (D; triangle) or 6 hours following the administration of a CatK 680 FAST (E; circle) and MMPSense 750 FAST (F; square) cocktail on the Kodak in vivo imaging system FX Pro. 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 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. Images were acquired 8 hours following the administration of the Neutrophil Elastase 680 FAST and ProSense 750 FAST cocktail on the Kodak in vivo imaging system FxPro. Graph (D) shows fluorescence from the Neutrophil Elastase probe acquired using a 630 nm excitation filter and a 700 nm emission filter (black columns, Vehicle;
white columns, Dex; grey columns, no probe). Graph (E) shows fluorescence from the ProSense probe acquired using a 730 nm excitation filter and a 790 nm emission filter (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. Graph (G) shows the correlation of the Neutrophil Elastase 680 FAST FI with the clinical score on day 28 and 35 post immunisation. Graph (H) shows the correlation of the ProSense 750 FAST FI with the clinical score on day 28 and 35 post immunisation. Graph (I) shows the correlation of the Neutrophil Elastase 680 FAST FI with paw thickness on day 28 and 35 post immunisation. Graph (J) shows the correlation of the ProSense 750 FAST FI with 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
Figure 2
Figure 3

A

B

D

C

E

Longitudinal molecular optical imaging in CIA [R24]
**Assessment of murine collagen-induced arthritis by longitudinal non-invasive duplexed molecular optical imaging**

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**Supplementary Figure I.** Fluorescence images showing arthritic mice 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. Fluorescent signal from the neutrophil elastase sensitive and the cathepsin sensitive probe 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 $4.89 \pm 1.03$ to $9.11 \pm 0.73$. 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.