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# *In-vitro* and *in-vivo* imaging of MMP activity in cartilage and joint injury

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ABSTRACT

Non-destructive detection of cartilage-degrading activities represents an advance in osteoarthritis (OA) research, with implications in studies of OA pathogenesis, progression, and intervention strategies. Matrix metalloproteinases (MMPs) are principal cartilage degrading enzymes that contribute to OA pathogenesis. MMPSense750 is an in-vivo fluorimetric imaging probe with the potential to continuously and non-invasively trace real-time MMP activities, but its use in OA-related research has not been reported. Our objective is to detect and characterize the early degradation activities shortly after cartilage or joint injury with MMPSense750. We determined the appropriate concentration, assay time, and linear range using various concentrations of recombinant MMPs as standards. We then quantified MMP activity from cartilage explants subjected to either mechanical injury or inflammatory cytokine treatment in-vitro. Finally, we performed in-vivo MMP imaging of a mouse model of post-traumatic OA. Our in-vitro results showed that the optimal assay time was highly dependent on the MMP enzyme. In cartilage explant culture media, mechanical impact or cytokine treatment increased MMP activity. Injured knees of mice showed significantly higher fluorescent signal than uninjured knees. We conclude that MMPSense750 detects human MMP activities and can be used for in-vitro study with cartilage, as well as in-vivo studies of knee injury, and can offering real-time insight into the degradative processes that occurring within the joint before structural changes become evident radiographically.

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## 1. Introduction

Osteoarthritis (OA) is a degenerative disease of the whole joint organ characterized by cartilage degradation [1], and the number of OA patients continues to increase, estimated at nearly 27 million in the United States [2] and there is no effective treatment to prevent OA or restore joints after the onset of OA. At this time, the gold standard for clinical OA diagnosis and evaluation are morphologic assessments, such as radiography [3–5], computed tomography (CT) [5,6], and magnetic resonance imaging (MRI) [3,5,6]. These imaging technologies primarily reveal the morphological changes that become evident at the later stages of OA, but do not offer insight into the process of cartilage degradation. As the field of OA research moves toward OA prevention it is becoming important to also measure the biological processes responsible for joint degradation, processes that precede the morphological and structural changes.

It is generally accepted that enzymatic activities contribute to cartilage degradation and loss in OA, and that elevated enzymatic activity precedes morphological joint space narrowing [7,8]. The ability to non-destructively image and quantify enzymatic activity would be an important tool to assess OA initiation and progression, and the efficacy of intervention strategies. While primary OA is considered idiopathic, in post-traumatic OA (PTOA) the time point of OA initiation (trauma) can be easily identified, and this is therefore an appropriate model to study the enzymatic activities during the early phases of OA.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent degradative proteinases with roles in the enzymatic cartilage degradation and OA progression. MMP-mediated degradation of type II collagen fibrils is considered one of the first irreversible steps in OA pathogenesis (reviewed in Ref. [9]), and the presence of MMPs correlates with OA symptoms, including joint effusion and pain [10,11]. Although serum level of MMP-3 is used as a biomarker for rheumatoid arthritis (RA), there is no clinically

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established MMP biomarker for OA [12]. Most studies investigating MMP activity in OA rely on assays such as ELISA [13,14] or Western Blotting [14,15], and RT-PCR [14,16] to estimate protein levels and mRNA expression, respectively. However, these assays are not suitable for in-vivo use, and can only measure the amount of MMP protein but do not directly assay MMP activity. Direct measurements of MMP activity include zymography [14,16,17] and more recently fluorimetric MMP assays [14,16], but again these assays are generally not suited for *in-vivo* imaging. The development of a method to visualize MMP activities *in-vivo* hence may offer new insight into OA initiation and treatment efficacy.

An *in-vivo* fluorimetric probe was recently developed that allows non-destructive imaging of activity from a broad spectrum of MMPs (MMPSense™ 750 FAST PerkinElmer, Inc., Boston, MA). This near-infrared fluorescent probe is a peptide substrate that enables the detection of MMP activities by exhibiting fluorescent signal when cleaved by MMPs [13,15,18]. In-vivo imaging with this reagent has the potential to continuously measure real-time MMP activities non-invasively. This probe has been successfully used to detect tumor progression [13,18] or ischemia reperfusion in brain [15] with *in-vivo* mouse model, but to our best knowledge there is no previous report investigating MMPSense750 for the assessment of MMP activity in cartilage or joint injury. Moreover, although this probe is expected to be utilized for human patients in clinical setting in future, no paper has studied the kinetics of this substrate using human MMPs. The objective of the present study is to investigate the potential of MMPSense750 for detection of human MMPs and to use in an assay with cartilage explants, as well as an *in-vivo* mouse model of knee injury leading to PTOA.

## 2. Methods

### 2.1. Assessment of optimal MMPSense750 concentration

We first wanted to determine the appropriate MMPSense750 concentration for *in-vitro* studies using purified recombinant human MMPs. Human MMP-3, -9, and -13 were chosen based on their established importance in OA [11,19,20]. To achieve comparable results between the different enzymes, the amount of active enzyme in each assay was normalized using the specific activity (Units of enzyme activity per weight) provided by the manufacturer (Supplementary information 1). MMPSense750 was added to media containing the active proteases, the reactions were incubated at 37 °C, and the resulting fluorescent signal was measured at different time points as described in detail below.

Recombinant human MMP-3 (Enzo Life Sciences, Farmingdale, USA) was reconstituted to various concentrations in assay buffer consisting of 50 mM sodium acetate, 10 mM $CaCl_2$, 150 mM NaCl and 0.05% Brij-35 at pH 6.0 [9]. Recombinant human MMP-9 and MMP-13 (Enzo Life Sciences) were reconstituted in the assay buffer consisting of 50 mM HEPES, 10 mM $CaCl_2$ and 0.05% Brij-35 at pH 7.5 [21].

MMPSense750 (24 nmol per vial) was reconstituted in 1200 μl sterile phosphate-buffered saline (Invitrogen) as recommended by the manufacturer, then added into the MMP solutions at 0.2, 0.7 and 2.0 μM final concentration. Imaging was performed on an IVIS-Spectrum imaging system at multiple time points for up to 72 h after adding MMPSense750.

### 2.2. Cartilage explants

Cartilage explants were harvested from the weight-bearing area of the femoral articular surfaces of bovine stifle knee joints purchased from a local slaughterhouse (Petaluma, CA). A 6 mm dermal biopsy punch was used to isolate cartilage cylinders, which were then cut to 2 mm height from the articular surface using a custom jig. Explants were cultured for 3 days in DMEM with 10% FBS and 1% penicillin-streptomycin (all from Invitrogen, Carlsbad, CA) at 37 °C and 5% $CO_2$. Six joints were used, and 1 or 2 explants from each joint was randomly assigned to one of three treatment groups; IL-1β, mechanical injury, or control. There was no significant difference among the cartilage weights of each group. The IL-1β group was treated with 10 ng/ml IL-1β (R&D Systems, Minneapolis, MN). The explants in the mechanical injury group were mechanically compressed with an Instron 8511.20 digital servo-hydraulic mechanical testing device using displacement control. A compressive preload of ~0.5 N was applied, and then the explant was loaded to 30% strain at a strain rate of 100%/s, held at 30% strain for 100 ms, then unloaded. Following compression, all loaded explants were transferred to fresh culture medium and returned to an incubator at 37 °C and 5% $CO_2$ until the termination of the experiment. In the control group, the explants were given a preload of ~0.5 N and then returned to the culture media. The culture media were replenished at day 3. The media were collected at 3 and 6 days after IL-1β stimulation or mechanical injuries. MMPSense750 was added to a final concentration of 0.7 μM, and the fluorescence measured at 60 min and 24 h after adding MMPSense.

### 2.3. Animal model of joint injury

Eight adult male BALB/cByJ mice (9-week-old at time of injury) were obtained from Jackson Laboratory (Bar Harbor, Maine). All animals were maintained and used in accordance with National Institutes of Health guidelines on the care and use of laboratory animals. This study was approved by our Institutional Animal Care and Use Committee (IACUC). The right knees of the mice were injured with a single mechanical compression as previously described in our PTOA model [22]. Briefly, the tibial compression system consists of two custom-built loading platens; the bottom platen that holds the knee flexed, and the top platen that holds the heel. The platens were aligned vertically and positioned within an electromagnetic materials testing machine (Bose ElectroForce 3200) (Eden Prairie, MN). Mice were anesthetized using isoflurane inhalation, then the right leg of each mouse was subjected to a single dynamic axial compression (1 mm/s loading rate) to a target load of 12 N. This causes a transient anterior subluxation of the tibia, which injures the anterior cruciate ligament and leads to PTOA within 8 weeks. The contralateral uninjured knees served controls, and were used to normalize the data within each animal.

All mice received an injection of 2 nmol of MMPSense750 via the orbital sinus at 24-h post-injury, and IVIS imaging was performed 24 h after the injection (48-h post-injury). Mice were euthanized immediately after the imaging and both knees were dissected for isolation of total RNA and analysis of mRNA expression.

### 2.4. Quantitative real-time RT-PCR

Total RNA was extracted from injured and uninjured knees using the miRNeasy Mini Kit (Qiagen Valencia, CA) and reverse transcribed by the QuantiTect Reverse Transcription Kit (Qiagen). 2 μl of cDNA was used for quantitative RT-PCR (in a final volume of 10 μl) performed in triplicate in a 7900HT RT-PCR system with gene-specific probes according to the manufacturer's conditions. Results were normalized to the 18s rRNA and calculated as fold-change in mRNA expression relative to the untreated control, using the $2^{-\Delta\Delta CT}$ method. The probes used are shown in Supplementary information 2.

### 2.5. IVIS imaging

An IVIS Spectrum imaging system (Perkin Elmer) was utilized to monitor fluorescent signal of MMPSense750. For imaging of media cultured with cartilage explants, the samples were placed in black plates. For *in-vivo* imaging of mice, the hairs from the lower trunk and both legs were removed, and then imaging was performed under general anesthesia by isoflurane inhalation. The excitation and emission wavelengths were set to 745 and 800 nm, respectively. The fluorescence intensities were analyzed by Living Image software 4.2(Perkin Elmer). Grid type and circle type of regions of interest (ROI) were set for plates and mice, respectively. Average radiant efficiency [p/s/cm$^2$/sr]/[μW/cm$^2$] in the ROI was measured as an index of intensity of fluorescent signal. For the experiment with MMP enzymes, normalized average radiant efficiency was calculated by subtracting the value of average radiant efficiency with 0(m)U of MMPs from that with each concentration of MMPs and used for the assessment. For the purpose of clarity, we use the term "fluorescence intensity" to indicate normalized average radiant efficiency in the ROI.

### 2.6. Statistical analysis

The results were statistically analyzed using a software package (GraphPad Prism; MDF Software, Inc.). Values of all measurements were expressed as the mean with error bars representing the 95% confidence interval. Correlation between MMP concentration and MMPSense signal was evaluated with Pearson correlation coefficient. Differences of MMPSense signal with MMP enzymes and *in-vivo* study were analyzed by paired t tests. Comparison of MMPSense signal between different concentrations of MMPs and of MMPSense signal with cartilage explants were analyzed by unpaired t test. Comparison of mRNA expression was analyzed by Wilcoxon signed rank test.

## 3. Results

### 3.1. Determination of the optimal MMPSense750 concentration for in-vitro experiments

To examine how well the normalized fluorescent signal correlated to the MMP activity at each time point, Pearson's correlation coefficients (R) were calculated for each combination of MMPSense and time (Fig. 1). For all MMPs, the best correlation between MMP activity and fluorescent signal occurred at the higher concentrations of MMPSense probe, 0.7 or 2.0 μM. Perhaps more surprisingly the best time to measure fluorescence intensity was highly dependent on the types of MMP enzyme. For MMP-13, high correlations were observed as early as 15 min, while MMP-3 started to become significant after 60 min, and MMP-9 not until after 24 h. At the later time points the MMP activity and fluorescent signal were highly correlated for all three MMP enzymes.

To estimate the detection limit of the assay at each concentration of MMPSense750 over time, we statistically analyzed the differences between fluorescent intensity for each concentration increase of MMP (Supplementary Fig. 1). The intermediate concentrations of MMP generated significantly different fluorescence signal in all conditions, but there were variations in the lower and upper detection limits. With respect to the upper detection limit, we examined the fluorescent signal over time for each enzyme and the higher MMPSense probe concentrations (Fig. 2). While higher concentrations of MMPSense yielded greater absolute fluorescence signals, the statistical analysis showed no benefit of the 2 μM compared to 0.7 μM MMPSense probe.

Taken together, these results indicate that 0.7 μM of MMPSense750 at a 24 h time point would yield the best assay to measure the activities of the three MMPs over the greatest range of concentrations.

### 3.2. MMP activity in IL-1β-treated cartilage explants

Three days of IL-1β treatment caused a significant increase in fluorescence intensity of culture media, indicating elevated MMP activity in cartilage explants (Fig. 3A). Specifically, the fluorescence signal of the IL-1β group at day 3 was significantly greater than that of the control group both at 60 min and 24 h after adding MMPSense750. At day 6, fluorescence intensity in the IL-1β-treated group was significantly greater than that of the control group at 24 h (but not 60 min) after adding MMPSense750.

### 3.3. MMP activity in mechanically injured cartilage explants

As observed with the IL-1β treatment, the fluorescence of the mechanical injury group at day 3 was significantly greater than that of the control group at both 60 min and 24 h after adding MMPSense750. At day 6, the trends are similar to the IL-1β treatment, with greater fluorescence at the 24 h, although this did not reach statistical significance (Fig. 3B).

### 3.4. MMP activity in-vivo after knee injury

The non-surgical joint injury caused a substantial increase in the fluorescence intensity in the injured right knee relative to the uninjured left knee of the same animal, indicating that injury increased the local MMP activity (Fig. 4A). The real-time RT-PCR results showed elevated mRNA expression of MMP-3 in the injured knee at this time point (48 h after injury), while the expression of MMP-9 and -13 were not statistically different in the injured and contralateral limb at this time point (Fig. 4B).

## 4. Discussion

Imaging technologies used in OA primarily measure structural morphology rather than the biological processes that contribute to joint degradation. The results in the present study demonstrate that the MMPSense750 is useful with human MMPs, providing insight into the parameters to consider when interpreting the data, and show a good response of the assay in *in-vitro* studies of cartilage explants and in a mouse model of joint injury. Although there are a few previous reports using MMPSense680, precedent product of MMPSense, to investigate MMP activity in OA or RA with human cartilage or mouse model [23–25], this article is the first studying OA-related assay with cartilage and joint injury using MMPSense750. This is an important contribution because MMPSense680 and MMPSense750 have different substrate specificities and different in-vivo kinetics, and MMPSense750 has the advantage that it enables a shorter time between injection and imaging (6 h versus 24 h for MMPSense680). Although human MMPs, bovine cartilage and mice were used in the current study, MMPs share a high degree of orthology among most vertebrates [26,27] and we do not expect significant species-related differences in the substrate–enzyme interactions.

With all MMPs tested, the fluorescent signal increased as the MMP activity increased. Interestingly, the reaction kinetics of the enzymes were different for the three MMPs. Namely, recombinant human MMP-13 caused a rapid increase in fluorescence within 15 min even at low enzyme concentrations, and longer incubations past 24 h decreased the assay linearity. In contrast, human MMP-9 required at least 24 h to show a dose-dependent increase in

$R^2$(R: Pearson Correlation Coefficient)

MMP-3

| Assay | MMPSense Probe | | |
|---|---|---|---|
| Time | 2.0μM | 0.7μM | 0.2μM |
| 0 min | 0.0027 | 0.0033 | 0.0841 |
| 15 min | 0.5007 | 0.3763 | 0.4709 |
| 30 min | 0.747 | 0.6724 | 0.6559 |
| 60 min | **0.9263** | **0.9191** | 0.7849 |
| 24 h | **0.9561** | **0.955** | **0.8159** |
| 48 h | **0.9345** | **0.9413** | 0.783 |
| 72 h | **0.9022** | **0.9247** | 0.734 |

MMP-9

| Assay | MMPSense Probe | | |
|---|---|---|---|
| Time | 2.0μM | 0.7μM | 0.2μM |
| 0 min | 0.1048 | 0.3791 | 0.3221 |
| 15 min | 0.04475 | 0.1754 | 0.174 |
| 30 min | 0.02352 | 0.2264 | 0.1958 |
| 60 min | 0.1129 | 0.3184 | 0.257 |
| 24 h | **0.9824** | **0.9913** | **0.9682** |
| 48 h | **0.9847** | **0.9964** | **0.9851** |
| 72 h | **0.989** | **0.9966** | **0.986** |

MMP-13

| Assay | MMPSense Probe | | |
|---|---|---|---|
| Time | 2 μM | 0.7 μM | 0.2 μM |
| 0 min | 0.5005 | 0.5053 | **0.827** |
| 15 min | **0.9487** | **0.8453** | **0.8908** |
| 30 min | **0.968** | **0.8625** | **0.9092** |
| 60 min | **0.9607** | **0.8985** | **0.9155** |
| 24 h | **0.9487** | **0.8453** | **0.8908** |
| 48 h | **0.8552** | **0.8926** | 0.6739 |
| 72 h | **0.8415** | **0.8152** | 0.6852 |

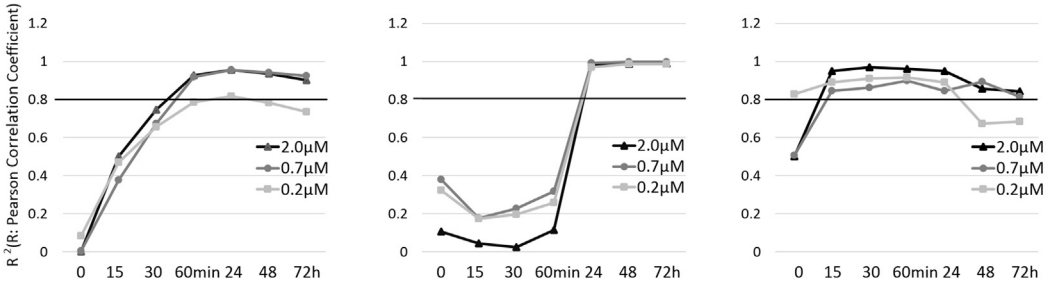


**Fig. 1.** Correlation between MMP Activity and MMPSense750 Signal. $R^2$ (R: Pearson correlation coefficient) as indices of correlation of the relationship between MMPs concentration and fluorescent signals at each time point were shown in tables and figures. $R^2$ greater than 0.8 were shown in the table with bold letters and shaded background.

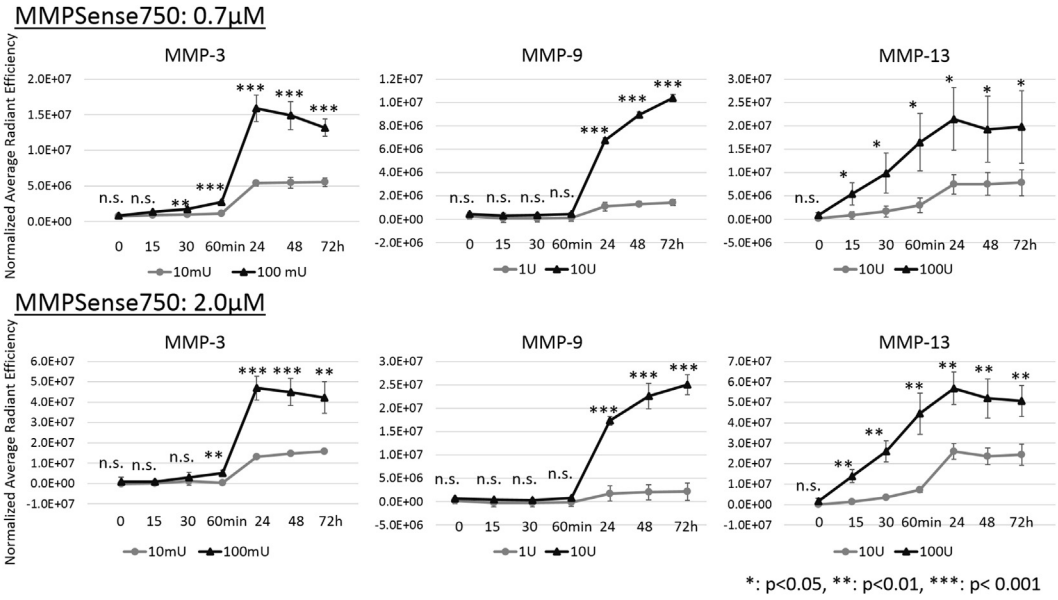


**Fig. 2.** Substrate Activation over Time. The normalized average radiant efficiency of the greatest and the second greatest concentration of each MMP with 0.7 or 2.0 μM of MMPSense750. p-values vs. the second greatest concentrations at the same time points. (n = 3).

substrate activation, and longer incubations out to 3 days improved the assay linearity. Human MMP-3 was intermediate, showing linear response after 1 h out to 2 days, but decreasing at 3 days. The experiments were all performed within the reported tissue half-life of 72 h for the MMPSense750 reagent. MMPSense produces fluorescent signal upon MMP-mediated hydrolysis, and the discrepancy of the detection time between MMPs may suggest the kinetics of cleavage differs between MMPs. We examined MMP-3, -9 and -13 in the current study, but according to the manufacturer MMPSense750 can also detect the activities of MMP-2, -7, and -12. In summary, it is important to recognize that a fluorescent signal indicates activities from multiple MMPs with differing sensitivities and reaction kinetics.

The result from cartilage explants assay provides a novel non-destructive method to quantify the MMP activity. At day 6, significantly higher fluorescence was detected in the IL-1β group when compared with the control group, while there was no significant difference between the mechanical injury group and the control group at both 60 min and 24 h after adding MMPSense. A possible reason for this difference could be the continuous presence of IL-1β during the 6 days, compared to a single mechanical injury at day 0.

In the *in-vivo* mouse model, injured knees showed significantly higher signals of MMPSense750 than the contralateral uninjured knees, indicating that MMP activity was elevated 48 h after injury. This provides a novel real-time non-destructive imaging method to quantify knee injury response and the progression of cartilage

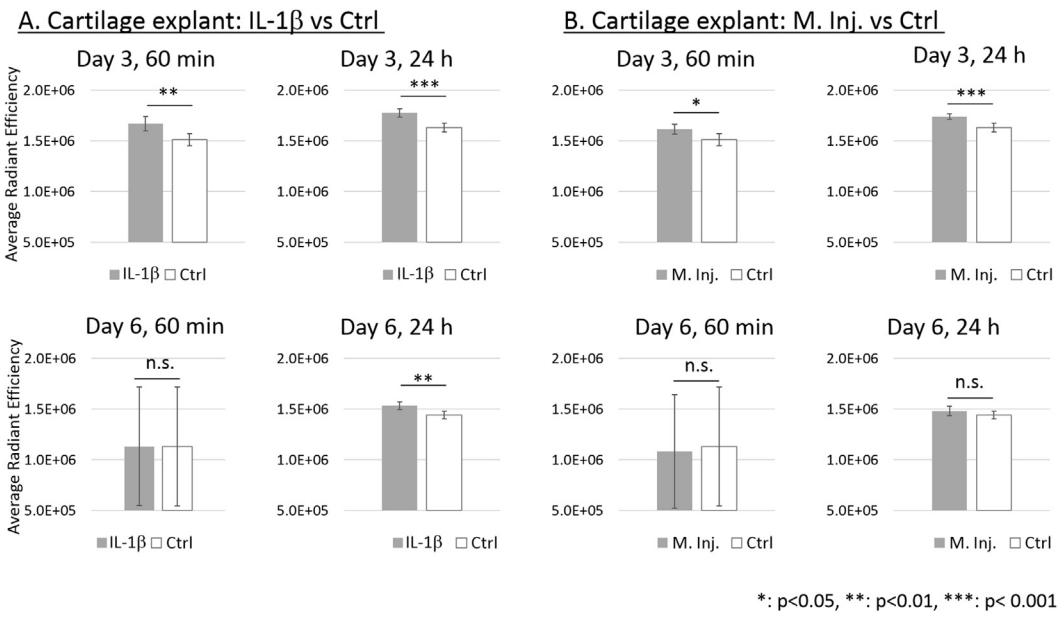


**Fig. 3.** Increased MMP Activity in Models of Cartilage Explant Injury. The average radiant efficiency in culture media at day 3 of the IL-β group (A) and the mechanical injury group (B) measured at 60 min and 24 h after adding MMPSense was significantly greater than that of the control group. As for the media collected at day 6, the average radiant efficiency of the IL-1β group measured at 24 h was significantly greater than the other, while there was no significant difference in the radiant efficiency measured at 60 min of the IL-1β group and at 60 min and 24 h of the mechanical injury group compared to the control group. M. Inj.: mechanical injury group, Ctrl: control group. (n = 10).

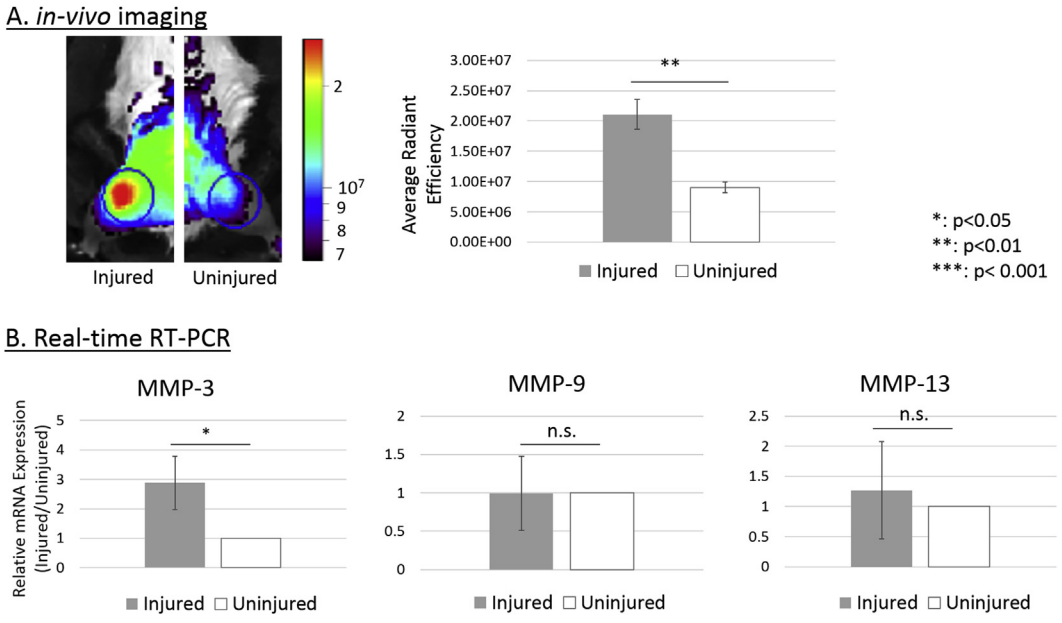


**Fig. 4.** Increased MMP Activity and mRNA in a Mouse Model of Joint Injury. A. Representative IVIS imaging of knees after MMPSense750 injection are shown. The image is a merged picture of fluorescent signal in color and a grayscale picture of the mouse. Blue circles on both knees are ROI with same shape and size as each other. The graph shows that average radiant efficiency in injured knees was significantly higher than the controls. (n = 8). B. mRNA expression of MMP-3 in injured knees were significantly greater than in uninjured knees. No significant difference in MMP-9 and MMP-13 mRNA expression was detected between the knees at this time point. (n = 8). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

degradation and OA based on MMP activity. Interestingly, when we examined MMP mRNA expression at 48 h after injury, we found that only MMP-3 was still elevated. In a separate study, we found that mRNA up-regulation of MMP expression after injury peaked at 4 h after injury and returned to baseline after 24 h using the same animal model (data not shown). MMPs are secreted as inactive pro-enzymes that are later activated in the extracellular matrix, which may explain the apparent discrepancy between the mRNA expression and the protease activity and highlight the importance of quantifying the enzymatic activity.

A limitation of the imaging technology is that we did not have sufficient resolution to determine the exact tissue source of MMP activity. It is likely that MMPs are active in multiple tissues, including cartilage, bone, and synovium. In our explant experiments we were able to detect activity of MMP secreted by cartilage, but not within the cartilage itself. Based on these observations we

speculate that the *in-vivo* source of fluorescent signal might be joint tissues other than cartilage, although this does not preclude the MMP activity in the cartilage. In future experiments we would like to localize the source of MMP activity more precisely.

In conclusion, we established experimental parameters to use the MMPSense750 imaging reagent to quantify MMP activity *in-vitro* in cartilage explants, and *in-vivo* in a mouse joint injury model. The advantages of MMPSense750 over other techniques to evaluate MMP activity include its non-destructive nature, enabling repeated measurements on the same samples. This provides an imaging opportunity to monitor the destructive enzymatic processes that contribute to OA progression, and complements traditional imaging technologies that quantify the resulting structural changes.

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The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.100.

## Conflict of interest

None.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.100.

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