In-vitro and In-vivo Imaging of Mmp Activity in Cartilage and Joint Injury.

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Introduction: Osteoarthritis (OA) is a degenerative disease of the articular joints characterized by both mechanical and enzymatic cartilage degradation. Matrix metalloproteinases (MMPs) are zinc-dependent degradative enzymes with principal roles in cartilage degradation and OA. Functional imaging of MMP activity in-vivo, before structural changes to cartilage are apparent, could be a powerful tool to study OA progression and the efficacy of intervention strategies. Here we characterize the utility of a functional imaging probe (MMPSense750) for OA research, using chondrocyte cultures, a cartilage explant injury model, and an in-vivo mouse model of joint injury. We demonstrate that injurious compression of cartilage explants and in-vivo joint injury cause a rapid and sustained increase in MMPSense signal, providing a promising functional imaging reagent for in-vivo and in-vitro studies of OA.

Methods:
Concentration and specificity of MMPSense
To determine the sensitivity of MMPSense750 to different MMPs, we added MMPSense750, at final concentration of 0.2, 0.7 or 2.0µM, into media containing dilutions of recombinant active MMP-3, -9 and -13 proteinases. The resulting fluorescent signal was measured at multiple time points until 72 h.

Cartilage explant injury model
Cylindrical cartilage explants with 6mm diameter and 2mm height were isolated from the femoral articular surface of bovine stifle knee, and randomly assigned to three groups as following; 1) IL-1b group: cultured in media with 10ng/ml IL-1b, 2) Mechanical injury group: loaded to 30% strain at 100%/s immediately before culture, 3) Control group: cultured without IL-1b or loading. Imaging was performed on culture media collected on day 3 and 6.

Animal model of joint injury
The right knees of adult male BALB/cByJ mice (n=8) were injured with a single mechanical compression as previously described in a paper about our post traumatic OA (PTOA) model (Ref.), which causes a transient anterior subluxation of the tibia and the anterior cruciate ligament injuries and leads to PTOA within 8 weeks. The contralateral uninjured knees served as controls. Imaging was performed 48 h after the injury (24 h after MMPSense injection) and total RNA was extracted from both knees dissected immediately after the imaging for real-time RT-PCR. All animals were maintained and used in accordance with National Institutes of Health guidelines on the care and use of laboratory animals and IACUC approved.

Imaging
An IVIS Spectrum imaging system (Perkin Elmer) was utilized to monitor fluorescent signal of MMPSense.

Results:
Determination of the optimal MMPSense750 concentration for in-vitro experiment
To examine how well the 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, with an R^2 value above 0.8 considered a strong correlation (Fig1). For all MMPs, the best correlation between MMP activity and fluorescent signal occurred at the higher concentrations of MMPSense probe, 2.0 or 0.7µM. 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 m, while MMP-3 started to become significant after 60 m, 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.

With respect to the detection limit, we examined the fluorescent signal over time for each enzyme and the higher MMPSense probe concentrations (Fig2). While higher concentrations of MMPSense yielded greater absolute fluorescence signals, the statistical analysis showed no benefit of the 2µM compared to 0.7µM MMPSense probe. Taken together, these results indicate that 0.7µM of MMPSense750 at 24 h yields the best assay to measure the activities of the three OA-related MMPs over the greatest range of concentrations.

**MMP activity in cartilage explants**

IL-1b was used to induce a catabolic response in bovine cartilage explants. The MMPSense signal in culture media of the IL-1b group at day 3 was significantly greater than that of the control group both at 60 m and 24 h after adding MMPSense750. At day 6, fluorescence intensity in the IL-1b group was significantly greater than in the control group at 24 h (but not 60 m) after adding MMPSense750. Rapid mechanical compression was used to mimic injury of the cartilage explants. In the mechanical injury group, MMPSense signal at day 3 was significantly greater than that of the control group at both 60 m and 24 h after adding MMPSense750. At day 6, the trends are similar to the IL-1b treatment, with greater fluorescence at the 24 h, although this did not reach statistical significance.

**MMP activity in-vivo after knee injury**

In vivo, 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 (Fig3). We further measured the mRNA expression of MMP-3, -9, and -13 immediately after the imaging was completed. The 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.

**Discussion:** MMPs are secreted as inactive pro-enzymes, and mRNA expression does not necessarily correlate with the enzymatic activity that causes cartilage degradation in OA. Quantification of enzyme activity can offer insight into the degradative processes occurring within the joint before structural changes are evident radiographically. We found that MMPSense750 is a convenient tool to non-invasively detect MMP activities for in-vitro study with cartilage, as well as in-vivo studies of knee injury. The best concentration of MMPSense750 for in vitro study was 0.7µM, and the best time to measure fluorescence intensity was highly dependent on the type of MMP enzyme (MMP-13: early, MMP-3: intermediate, MMP-3: late). Mechanical impact or cytokine treatment on cartilage explant increased the fluorescent signal in culture media. Injured knees of mice showed significantly higher fluorescent signal than uninjured.
Significance: There is no method that can non-invasively detect real time MMP activity which has strong relationship to symptom or severity of OA in clinical setting. This study can contribute to develop such methods.

![Graphs showing normalized average radiant efficiency](image)

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

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<tr>
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<th>MMP-3</th>
<th>MMP-9</th>
<th>MMP-13</th>
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<tr>
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![Graphs showing R (Pearson Correlation Coefficient)](image)

A. ![Image A](image)

B. ![Image B](image)
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