Video Article

# Measuring Global Cellular Matrix Metalloproteinase and Metabolic Activity in 3D Hydrogels

Abdulaziz S. Fakhouri[1,2,3], Jennifer L. Leight[1,2]

[1]Department of Biomedical Engineering, The Ohio State University

[2]The Ohio State University Comprehensive Cancer Center, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute

[3]Biomedical Technology Department, King Saud University

Correspondence to: Jennifer L. Leight at leight.1@osu.edu

## Abstract

Three-dimensional (3D) cell culture systems often more closely recapitulate *in vivo* cellular responses and functions than traditional two-dimensional (2D) culture systems. However, measurement of cell function in 3D culture is often more challenging. Many biological assays require retrieval of cellular material which can be difficult in 3D cultures. One way to address this challenge is to develop new materials that enable measurement of cell function within the material. Here, a method is presented for measurement of cellular matrix metalloproteinase (MMP) activity in 3D hydrogels in a 96-well format. In this system, a poly(ethylene glycol) (PEG) hydrogel is functionalized with a fluorogenic MMP cleavable sensor. Cellular MMP activity is proportional to fluorescence intensity and can be measured with a standard microplate reader. Miniaturization of this assay to a 96-well format reduced the time required for experimental set up by 50% and reagent usage by 80% per condition as compared to the previous 24-well version of the assay. This assay is also compatible with other measurements of cellular function. For example, a metabolic activity assay is demonstrated here, which can be conducted simultaneously with MMP activity measurements within the same hydrogel. The assay is demonstrated with human melanoma cells encapsulated across a range of cell seeding densities to determine the appropriate encapsulation density for the working range of the assay. After 24 h of cell encapsulation, MMP and metabolic activity readouts were proportional to cell seeding density. While the assay is demonstrated here with one fluorogenic degradable substrate, the assay and methodology could be adapted for a wide variety of hydrogel systems and other fluorescent sensors. Such an assay provides a practical, efficient and easily accessible 3D culturing platform for a wide variety of applications.

## Introduction

Three-dimensional (3D) culture systems often more closely recapitulate *in vivo* cellular responses than traditional two-dimensional (2D) culture systems, see several excellent publications[1,2,3]. However, utilizing 3D culture systems to measure cell function has been challenging due to the difficulty of cellular retrieval and further sample processing. This difficulty limits the measurement of many cellular functions in 3D culture systems. To overcome this difficulty, new techniques are needed that enable easy measurement of cell function within 3D environments. One way to address this need is the development of materials that not only support 3D cell culture but also incorporate sensors to measure cell function. For example, several hydrogel systems have incorporated fluorogenic protease cleavable moieties to enable visualization of protease activity within 3D environments[4,5,6,7]. While these systems were originally utilized for microscopic imaging, these systems can also be adapted for use in a global matrix metalloproteinase (MMP) activity assay using a standard plate reader, enabling facile measurement of a cell function in a 3D environment[8].

MMPs, a superfamily of zinc proteases, play critical roles in normal tissue homeostasis and in many diseases. MMPs degrade and remodel extracellular matrix (ECM), cleave cell surface receptors and cytokines and activate other MMPs[9,10]. MMPs play critical roles in physiological processes such as wound healing and in diseases such as arthritis, atherosclerosis, Alzheimer's, and cancer (see reviews in [9,10,11]). In cancer, high MMP expression levels are strongly correlated with cancer metastasis and poor prognosis[12]. Furthermore, MMPs contribute to tumor progression by promoting cancer cell invasion and migration, cellular processes that are inherently 3D phenomena[13,14]. Therefore, there is much interest in the ability to measure MMP activity in 3D culture in many contexts, including fundamental biological studies and drug screening assays.

PEG hydrogels are widely used for 3D cell culture due to their high water content, resistance to protein adsorption, and tunable nature. PEG hydrogels have been functionalized with a number of moieties to direct cell function, such as with ECM mimetic peptides like RGD, RLD, and IKVAV to facilitate cell adhesion, or direct tethering of growth factors such as transforming growth factor-β (TGF-β)[15,16]. More recently, PEG hydrogels have been functionalized with sensor peptides that enable measurement of cell function as well[5,8]. Specifically, the use of a PEG hydrogel system functionalized with a fluorogenic MMP-degradable peptide enabled measurement of cellular MMP activity in 3D cultures with a standard plate reader and required no further processing. These systems are also compatible with other measurements of cellular function, including metabolic activity. Here, a protocol is described for the measurement of MMP activity of cells cultured in a 3D PEG hydrogel functionalized with a fluorogenic MMP-degradable peptide, and results presented demonstrating the initial optimization experiments needed for use of this assay. Human melanoma cells (A375) were encapsulated in the fluorogenic hydrogels over a range of seeding densities to determine

the appropriate seeding densities that are within the working range of the assay. After 24 h of encapsulation, MMP and metabolic activity were measured utilizing a standard microplate reader. Next, MMP activity was normalized to metabolic activity to determine the seeding densities within the linear range of the assay. Finally, intra-plate coefficient of variation percentages (% CV) were calculated between triplicates to reflect the reproducibility of the obtained results. This method enables simple and fast 3D cell culture, and easy measurement of protease activity with minimal sample processing.

## Protocol

## 1. Hydrogel components preparation

1. Synthesize the fluorescent protease-degradable peptides as described elsewhere[8], utilizing fluorescein as the fluorescent molecule and dabcyl as the quencher. Dissolve the peptide in DMSO to a concentration of 10 mM and store in a -80 °C freezer in small (~30 μL) aliquots to avoid repeated freeze-thaw cycles.
NOTE: These peptides can also be purchased commercially. This protocol requires a C-terminal cysteine in the peptide sequence to enable covalent incorporation into the hydrogel polymer network.
2. Prepare 8 arm 40 kDa poly(ethylene glycol) amine (PEG)-norbornene (NB) as described[17]. Verify end group functionalization of greater than 90% using ^{1}H NMR. Dissolve PEG-NB in sterile phosphate buffer saline (PBS) at 25% w/v and store in -80 °C freezer in (~300 μL) aliquots.
NOTE: PEG functionalized with norbornene can also be purchased commercially.
3. Synthesize the photo-initiator lithium phenyl 2,4,6 trimethylbenzoylphosphinate (LAP) as described elsewhere[18]. Dissolve LAP in sterile water to a concentration of 68 mM and store in a -80 °C freezer in (~300 μL) aliquots.
NOTE: As an alternative, Irgacure (2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) can be used as the photo-initiator. LAP and Irgacure can be purchased commercially.
4. Dissolve the MMP-degradable peptide crosslinker (KCGPQG↓IWGQCK) and the cell adhesion peptide (CRGDS) in sterile water to a concentration of 200 mM and 100 mM respectively, and store them in a -80 °C freezer in (~300 μL and ~30 μL) aliquots, respectively.

## 2. Assay media preparation

1. Prepare the heat-inactivated, charcoal stripped fetal bovine serum (FBS) for the MMP assay:
NOTE: Proteases in FBS can produce a high background signal with the MMP assay; therefore, it is recommended to heat-inactivate and charcoal strip the FBS for the assay media.
    1. Inactivate 100 mL of FBS by heating for 30 min at 55 °C.
    NOTE: 100 mL of FBS was utilized here for aliquoting and storage at -20 °C for future use. Smaller volumes can be used as needed.
    2. Add 0.025% of activated charcoal and 0.0026% of dextran to a small amount of FBS (approximately 5 mL) and stir until a slurry is formed. Then, add the rest of the FBS and stir for 30 min at 55 °C.
    3. Centrifuge at 1962 x $g$ for 20 min at 4 °C. Then transfer the supernatant to another vessel.
    4. Repeat step 2.1.2 but at 37 °C followed by step 2.1.3. Sterilize the supernatant using a 0.45 μm filter.
2. Prepare assay media using media supplemented with 1% charcoal stripped FBS, 2 mM L-glutamine, 10 U/mL penicillin, 10 μg/mL streptomycin.
NOTE: Media without phenol red is recommended because it has less fluorescence interference. Other additions to the assay media such as insulin, growth factors, etc. may be added as long as the absorbance and fluorescence spectrum peaks do not overlap with the sensor (494 nm/521 nm).
3. Dilute bacterial collagenase enzyme type I at 10 and 1000 μg/mL in the assay media as a positive control.

## 3. Hydrogel preparation and cell encapsulation

1. Prepare the hydrogel precursor solution.
    1. Add the reagents to a 1.5 mL tube in the following order, vortexing after addition of each component: 20 mM 8 arm 40 kDa PEG-NB, 12.75 mM crosslinker MMP-degradable peptide, 17.8 mM NaOH, 1 mM CRGDS, 2 mM LAP, and 0.25 mM fluorogenic MMP-degradable peptide.
    NOTE: **Table 1** shows hydrogel precursor solution contents, stock concentrations, working concentrations, volume calculation formulas and the required volumes needed to make 120 μL of hydrogel precursor solution, which is sufficient to conduct an experiment with 10 hydrogels. To account for loss of the hydrogel solution due to pipetting, increase the total volume by 20%. The commercial peptides are often supplied in an acidic hydrogen chloride solution; therefore, NaOH is added to achieve a final pH of 7. pH of the final solution should be confirmed by the user.
    2. Divide the hydrogel precursor solution into multiple 1.5 mL tubes, one tube per condition being tested.
    NOTE: Several control conditions in which hydrogels are prepared without the addition of cells are suggested. For a negative control, to account for non-specific degradation of the fluorogenic sensor, one hydrogel condition can be incubated with the vehicle control or the experimental media alone if there are no treatment conditions. For a positive control and for calibration between experiments, hydrogels can be incubated with a protease known to cleave the fluorogenic sensor. For example, two concentrations of bacterial collagenase were used here.
2. Encapsulate cells in hydrogels.
    1. Prepare a single cell suspension as appropriate for the cell type being used. For example, wash a 10 cm dish of A375 melanoma cells with 10 mL of PBS. Trypsinize cells using 0.05% trypsin and incubate at 37 °C and 5% $CO_2$ for 3 min. Count cells with a hemocytometer to determine total cell number.

2. Centrifuge the cell solution at 314 x *g* for 3 min, aspirate culture media, then re-suspend cells in PBS at approximately three times the highest required seeding density for the experiment. For example, a cell suspension with a density of $21 \times 10^6$ cells was used here to achieve a final encapsulated density of $7 \times 10^6$ cells/mL.

3. Count the cells again to ensure an accurate cell concentration.

4. Add suspended cells and PBS to each tube of hydrogel precursor solution according to the required seeding density (0.25, 0.5, 1, 2, 3, 4, 5, 6, and $7 \times 10^6$ cells/mL in this example). Add PBS to conditions with no cells in lieu of suspended cells.
   NOTE: All conditions should have the same final hydrogel precursor solution volume to ensure the ratio between the hydrogel components and PBS is constant. Do not vortex tubes that have cells in them, pipette up and down vigorously without creating bubbles in order to mix the precursor solution.

5. Dispense 10 μL of the hydrogel precursor solution into a sterile black round bottom 96-well plate, ensuring that the tip is centered in the middle of the each well while dispensing.

6. Polymerize hydrogel precursor solution by exposing the plate to ultra violet (UV) light at 4 mW/cm² for 3 min.
   NOTE: The UV lamp (UVL-56 Handheld UV Lamp, UVP, Upland, CA) produces UV-A light at a long UV wavelength (365 nm), which does not affect cellular viability.

7. Add 150 μL of assay media to all wells except for the positive control conditions without encapsulated cells. To the positive controls, add 150 μL of collagenase enzyme solution.

8. Add 150 μL of PBS to the outer wells of the plate to reduce evaporation during incubation.

## 4. Data acquisition and metabolic activity measurement

1. Measure fluorescence immediately post-encapsulation to establish a baseline fluorescence measurement and ensure uniformity in hydrogel polymerization. Read the plate using a fluorescence microplate reader utilizing an opaque 96-well plate protocol with an area scan setting at 494 nm/521 nm (excitation/emission). This will be the 0 h read.

2. Incubate plate in a humidified incubator at 37 °C and 5% $CO_2$ for 18 h.

3. Add metabolic activity reagent (resazurin) at 1:10 (v/v) for each well.

4. Incubate plate in a humidified incubator at 37 °C and 5% $CO_2$ for an additional 6 h.
   NOTE: This incubation time may vary depending on cell type.

5. Measure fluorescence at 24 h post-encapsulation. Read the plate using a fluorescence microplate reader utilizing an opaque 96-well plate protocol with an area scan setting at 494 nm/521 nm (excitation/emission) for MMP activity and 560 nm/590 nm (excitation/emission) for metabolic activity.

## Representative Results

The current assay was adapted from a previously developed and characterized 3D hydrogel culture system functionalized with a fluorogenic MMP cleavable sensor[8]. The fluorogenic MMP sensor used here consists of a peptide sequence, GPLAC(pMeOBzl)↓WARKDDK(AdOO)C (↓ indicates the cleavage site) that was previously optimized for cleavage by MMP-14 and MMP-11[19]. The peptide is labeled with a fluorescent molecule (fluorescein) and a quencher molecule (dabcyl) on either side of the cleavage site (**Figure 1**). Upon exposure of the fluorogenic sensor to the appropriate protease, the fluorophore and quencher are separated, and fluorescence increases. Here, the assay was miniaturized from a 24-well plate to a 96-well plate format, eliminating several steps in the encapsulation process and reducing the time needed to perform an experiment by 50%. Further, it reduced the volume of reagents consumed by 80%, as hydrogel volumes were reduced from 50 μL to 10 μL per well. Moreover, by using a 96-well plate, 20 conditions in triplicates could be tested instead of the 12 conditions in duplicates per 24-well plate. A schematic of the cell encapsulation process is illustrated in **Figure 2**. Label 1 and 2 correspond to steps 3.1 and 3.2 in the protocol, respectively. Labels 3 to 5 correspond to steps 3.2.5 to 3.2.7 in the protocol. Labels 6 to 9 correspond to steps 4.2 to 4.5 in the protocol.

To establish the detection limits and signal range of the assay, the hydrogels functionalized with the fluorogenic MMP-degradable peptide were incubated with a range of concentrations (0 to 2000 μg/mL) of bacterial collagenase enzyme type I. After 24 h of incubation at 37 °C, a plate reader was used to measure the fluorescence (**Figure 3**). From these measurements, the dynamic (lowest and highest detected signals) and working range (3 standard deviations above the lowest detected signal and 3 standard deviations below the highest detected signal) were determined. After 24 h of incubation, it was observed that the lowest detected signal was produced by negative controls (background noise) at 0 μg/mL collagenase, while the highest detected signal was produced by 1000 μg/mL collagenase or above, where the signal begins to plateau (**Figure 3**). From the dynamic range, the working range was calculated to be between ≈0.16 μg/mL and ≈474 μg/mL of collagenase, a wide signal range across four orders of magnitude.

For cell culture assays, the appropriate cell density that results in fluorescence readings within the working range of the assay must be determined for each cell type. Here, representative data is presented for the melanoma cell line A375, encapsulated in a range of densities from 0.25 to $7 \times 10^6$ cells/mL. Fluorescence intensity was acquired utilizing a standard microplate reader at two different time points: 1) directly after encapsulation (0 h) and 2) after 24 h of encapsulation. At 0 h, fluorescence readings were low across seeding densities (**Figure 4A**), as expected. After 24 h of culture (**Figure 4B**), MMP activity was directly proportional to the seeding density, in which more cells resulted in more cleavage of the fluorogenic MMP cleavable sensor and higher fluorescence intensity. As internal controls, hydrogels containing no cells were incubated with 0, 10, and 1,000 µg/mL of collagenase type I enzyme to indicate the low, medium and high levels of the signal respectively (as determined by the collagenase signal range characterization in **Figure 3**) and represented by dashed lines in **Figure 4B**. Further, the working range limits were calculated from the 0 and 1000 µg/mL signals and represented by dotted lines in **Figure 4B**. Seeding densities at or greater than $1 \times 10^6$ cells/mL fall within the limits of the working range. Metabolic activity measurements of the A375 cell line were also directly proportional to the cell seeding density (**Figure 4C**). Previously, MMP activity has been normalized to metabolic activity as an internal control to determine MMP activity on a per cell basis[8]. Normalizing MMP activity to metabolic activity across seeding densities resulted in no significant difference in MMP activity at seeding densities greater than $2 \times 10^6$ cells/mL (**Figure 4D**). To determine variability between triplicates in each plate (intra-plate variability), the coefficient of variation percentage (% CV) was calculated for both MMP and metabolic activity and is summarized in **Table 2**. % CV below 20% indicates that the intra-plate variability within triplicates is acceptable for the system to produce consistent results[20,21].
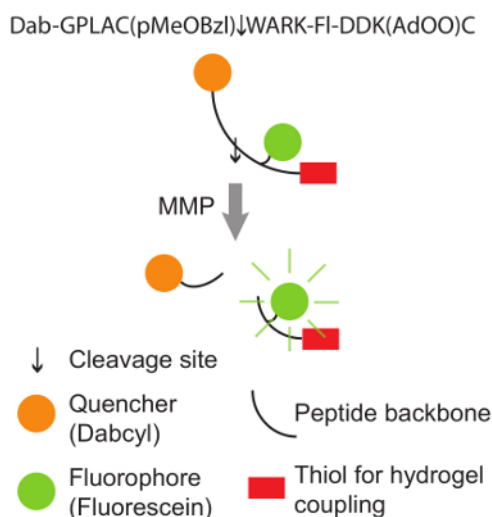


**Figure 1: The fluorogenic MMP-degradable peptide design.** The fluorogenic MMP-degradable peptide consists of a backbone peptide GPLAC(pMeOBzl)↓WARKDDK(AdOO)C (↓ indicates the cleavage site) that determines the specificity of the sensor. The peptide is labeled with a quencher (dabcyl) and a fluorophore (fluorescein), which is unquenched (fluoresces) when the backbone peptide is cleaved by MMP. A thiol group is conjugated to the backbone peptide to enable covalent reaction with norbornene functional groups in the PEG molecule, coupling the sensor to the hydrogel. Please click here to view a larger version of this figure.
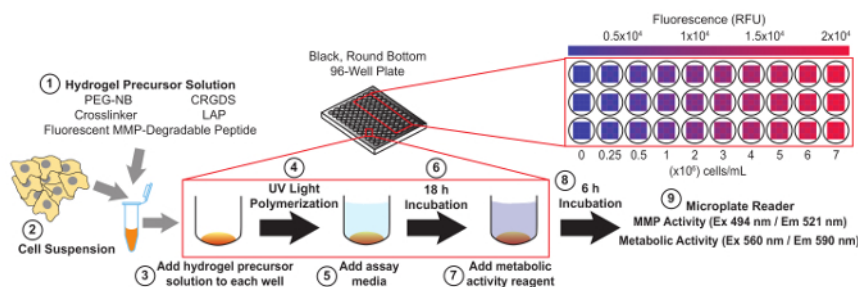


**Figure 2: Assay schematic.** The hydrogel precursor solution components are mixed with cells suspended in PBS. The precursor solution is then pipetted into black, round bottom, 96-well plates and polymerized by exposure to UV light for 3 min. Assay media is added, and plates are incubated for 18 h (37 °C, 5% $CO_2$). The metabolic activity reagent resazurin is then added, and plates are incubated for an additional 6 h. MMP and metabolic activity are measured using a fluorescent microplate reader with a well scan protocol at the indicated excitation/emission wavelengths. Please click here to view a larger version of this figure.
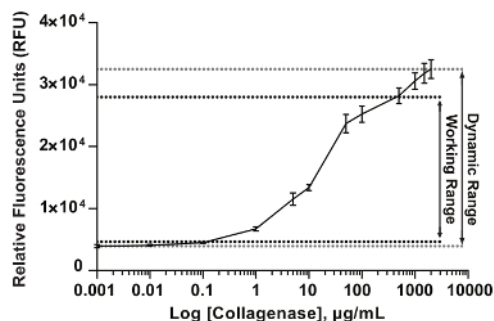
**Figure 3**: **Dynamic and working range of the assay.** Hydrogels functionalized with fluorogenic MMP-degradable peptide were incubated with a range of concentrations of collagenase enzyme type I for 24 h at 37 °C and fluorescence intensity was measured. Dotted lines represent the dynamic and working range. n = 3, mean ± standard deviation (SD). Please click here to view a larger version of this figure.
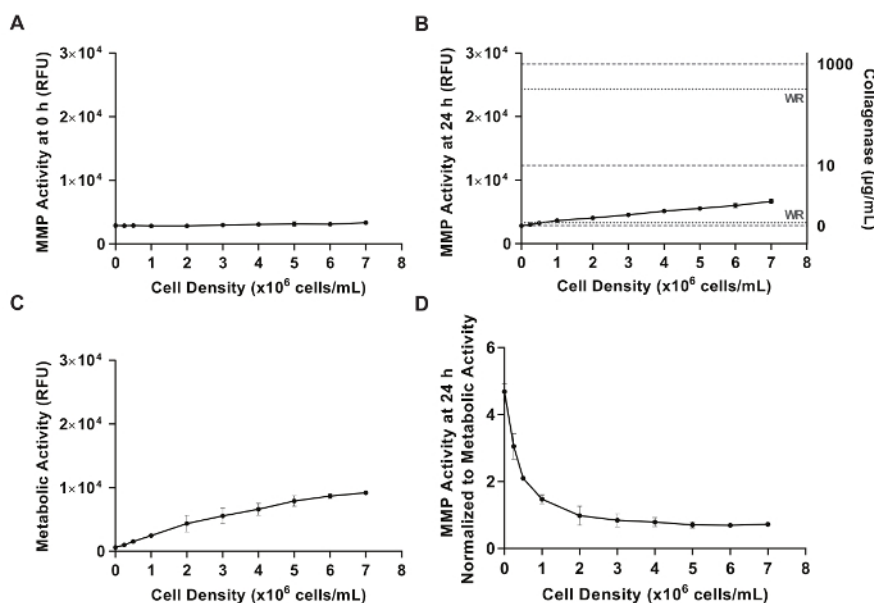


**Figure 4**: **Effect of seeding density of melanoma cell line A375 on MMP activity.** (A) Initial measurement (0 h) of MMP activity for A375 cell line encapsulated over a range of seeding densities. n = 3 mean ± SD. (B) Measurement of MMP activity at 24 h. 0, 10 and 1000 µg/mL of collagenase are represented by dashed lines. Dotted lines represent the working range (WR) calculated from collagenase controls. n = 3, mean ± SD. (C) Measurement of metabolic activity for A375 cell line encapsulated for 24 h over a range of seeding densities and incubated with resazurin for 6 h. n = 3, mean ± SD. (D) A375 MMP activity normalized to metabolic activity. N = 3, mean ± SD. Please click here to view a larger version of this figure.

| Precursor Solution Contents | Stock Conc. | Working Conc. | Volume Formula | Example volume for 10 gels (µL) |
|---|---|---|---|---|
| PEG-NB (wt%) | 25 | 10 | working conc.*total volume / stock conc. | 48.00 |
| Crosslinker MMP-degradable peptide (mM) | 200 | 8. 75 to 18.75 | Stoichiometry (thiols:enes) (0.5 to 1) | 5.25 to 7.65 |
| NaOH (M) | 1 | - | 28% of the crosslinker volume | 1.47 to 3.14 |
| RGD pendant peptide (mM) | 100 | 1 | working conc.*total volume / stock conc. | 1.20 |
| LAP (mM) | 68 | 2 | working conc.*total volume / stock conc. | 3.53 |
| Fluorescent MMP-degradable peptide (mM) | 10 | 0.25 | working conc.*total volume / stock conc. | 3.00 |
| 1X PBS (for all conditions) | - | - | total volume - sum of all precursor components | 57.56 to 49.88 |
| Total Volume = | | | number of hydrogels (10 hydrogels) * hydrogel volume (10 µL) * 20% extra | 120.00 |

**Table 1: Hydrogel precursor solution preparation.**

| %CV Mean ± Standard Deviation (%) | Seeding Density (*$10^6$ Cells/mL) | | | | | | | | | | Collagenase Control | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 0.25 | 0.5 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 1000 |
| MMP Activity | 1.81 ± 1.59 | 1.22 ± 0.48 | 1.38 ± 1.51 | 2.06 ± 0.86 | 0.90 ± 0.34 | 1.48 ± 0.20 | 2.03 ± 2.24 | 2.51 ± 0.46 | 1.33 ± 0.49 | 1.24 ± 0.33 | 4.24 ± 1.02 | 2.65 ± 0.33 |
| Metabolic Activity | 0.46 ± 0.12 | 2.85 ± 1.03 | 2.66 ± 1.64 | 1.95 ± 0.96 | 3.45 ± 1.48 | 1.96 ± 1.92 | 2.92 ± 1.46 | 3.98 ± 3.86 | 1.26 ± 0.53 | 1.05 ± 0.39 | - | - |

**Table 2: Intra-plate % CV of the assay with A375 cell line.**

## Discussion

3D in vitro cell culture recapitulates many important aspects of the in vivo environment. However, 3D culture also makes assessing cell function and signaling challenging, as many biological assays require cellular retrieval and large numbers of cells. Therefore, the development of a simple 3D culture system that enables measurement of cellular function without further sample processing would greatly increase the utility of 3D culture systems. The 3D system described here can be adapted for a variety of different applications. The sequence of the fluorogenic sensor can be changed to detect other proteases. For instance, the fluorogenic cleavable sensor sequence (GPQG↓IWGQK) which can be cleaved by MMP-1, -2, -3, -7, -8, and -9, was previously utilized to detect melanoma MMP activity in response to chemotherapeutics[22]. The use of the PEG hydrogel system also enables precise independent tuning of the cellular microenvironment, including the mechanical properties, the degradability, and the matrix adhesion moieties within the hydrogel. The adhesion molecule RGD used in this work is a fibronectin-derived sequence and enables integrin-mediated adhesion. Other adhesion molecules such as (RLD) and (IKVAV) that are derived from fibrinogen and laminin respectively could be utilized to activate other types of integrin receptors. For example, it was demonstrated that altering the adhesion molecules changed the elongation of aortic valvular interstitial cells (VIC), which may have an effect on aortic valve stenosis[15]. The hydrogel crosslinker sequence can control the degradability of the hydrogel and encapsulated cellular behavior within the hydrogel. For example, it was demonstrated that fibroblasts had increased proliferation and cell spreading when cultured in faster degrading hydrogels, which may expedite the healing process in vivo[23]. Mechanical properties of the microenvironment can also regulate cell function, and hydrogel stiffness can be modified by altering the number of arms in the PEG macromer, PEG molecular weight and the ratio between PEG and crosslinker.

Because MMP activity varies by cell type, for each new cell type it is important to conduct an encapsulation density optimization experiment as demonstrated here. After 24 h of encapsulation, MMP and metabolic activity were directly proportional to cell seeding density. However, at longer times the fluorescent signal can plateau, therefore the timing of the assay may have to be optimized by the user[8]. The specific protease sensor can also affect the working range and timing of the assay. The working range of the assay can be determined by conducting a signal range experiment with no cells and a proteolytic enzyme over a wide concentration. Inclusion of several no cell hydrogel conditions incubated with enzyme controls enables establishment of the low (background noise), medium and high levels of signal within each assay (0, 10 and 1000 μg/mL of collagenase here). This gives an indication of where the signals produced by cells fall within the working range of the assay. This assay is also compatible with other fluorescent sensors that do not have overlapping excitation and emission spectra, such as the metabolic activity assay used here as an internal control to calculate MMP activity on per cell basis, as previously reported[8]. MMP activity normalized to metabolic activity demonstrated the validity of this approach for seeding densities at or above 2 x $10^6$ cells/mL, in which there was no significant change across seeding densities. This normalization is crucial to identify the appropriate seeding densities that are within the working range of the MMP activity curve and within the linear portion of the normalization curve.

While the assay can be adapted for several purposes, there are several limitations and critical aspects the user should consider, specifically regarding interference with the fluorescent signal and preparation of the hydrogels. First, care must be taken with the choice of culture media and additional treatments, as these can have an overlapping absorbance spectrum or opaqueness that may interfere with the detection of fluorescence or quench the signal. It is recommended to use culture media that does not contain phenol red. Also, some drug treatments are fluorescent (e.g., doxorubicin), or have absorbance spectrum that overlaps with the excitation/emission spectrum of the fluorophore (e.g., curcuminoids). A second aspect that can affect the performance of the assay is the hydrogel preparation. Because of the high viscosity of the hydrogel precursor solution, care needs to be taken to ensure thorough mixing of the hydrogel solution and careful pipetting practices to prevent unequal fluorophore content or hydrogel solution volume in each well. Pre-wetting of the pipette tips and using low-retention tips can help reduce variability. Another important aspect of hydrogel preparation is the hydrogel shape and location in wells. The hydrogel should be centered within the well and of a uniform shape to enable accurate fluorescence measurements with less variability[15]. Here, the use of round bottom plates aids in centering the pipette tip in each well and the production of semi-spherical hydrogels consistently across all wells.

By adapting the 3D protease-degradable hydrogel system, real-time protease and metabolic activity readouts can be detected in a 3D microenvironment with minimal sample processing. In addition, the use of the synthetic PEG hydrogel reduces the batch-to-batch variability observed with naturally derived ECM hydrogels. Furthermore, utilizing PEG hydrogels enables fine-tuning of chemical and mechanical cues of hydrogels for an improved control of the microenvironment. Moreover, the PEG hydrogel polymerization described here is a photo-initiated process, making it quick and more amenable to high throughput methodologies than classic natural ECM hydrogels (i.e., collagen or Matrigel), which are slower and temperature sensitive. This quick, user-controlled polymerization allows the scaling-up of the system to be further automated using robotic liquid handlers, as demonstrated by others[15].

## Disclosures

The authors have nothing to disclose.

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## References

1. Baker, B.M., Chen, C.S. Deconstructing the third dimension - how 3D culture microenvironments alter cellular cues. *Journal of Cell Science.* **125** (13), 3015-3024, (2012).
2. Duval, K. *et al. Modeling Physiological Events in 2D vs.* 3D Cell Culture. *Physiology.* **32** (4), 266-277, (2017).
3. Hoarau-Véchot, J., Rafii, A., Touboul, C., Pasquier, J. Halfway between 2D and Animal Models: Are 3D Cultures the Ideal Tool to Study Cancer-Microenvironment Interactions? *International Journal of Molecular Sciences.* **19** (1), (2018).
4. Lee, S.-H., Miller, J.S., Moon, J.J., West, J.L. Proteolytically Degradable Hydrogels with a Fluorogenic Substrate for Studies of Cellular Proteolytic Activity and Migration. *Biotechnology Progress.* **21** (6), 1736-1741, (2005).
5. DeForest, C.A., Polizzotti, B.D., Anseth, K.S. Sequential click reactions for synthesizing and patterning three-dimensional cell microenvironments. *Nature Materials.* **8** (8), 659-664, (2009).
6. Chalasani, A. *et al. Live-Cell Imaging of Protease Activity: Assays to Screen Therapeutic Approaches. Methods in Molecular Biology (Clifton, N.J.).* **1574**, 215-225, (2017).
7. Jedeszko, C., Sameni, M., Olive, M., Moin, K., Sloane, B.F. Visualizing Protease Activity in Living Cells: From Two Dimensions to Four Dimensions. *Current Protocols in Cell Biology.* **0 4**, Unit-4.20, (2008).
8. Leight, J.L., Alge, D.L., Maier, A.J., Anseth, K.S. Direct measurement of matrix metalloproteinase activity in 3D cellular microenvironments using a fluorogenic peptide substrate. *Biomaterials.* **34** (30), 7344-7352, (2013).
9. Nagase, H., Visse, R., Murphy, G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular Research.* **69** (3), 562-573, (2006).
10. Tokito, A., Jougasaki, M., Tokito, A., Jougasaki, M. Matrix Metalloproteinases in Non-Neoplastic Disorders. *International Journal of Molecular Sciences.* **17** (7), 1178, (2016).
11. Visse, R., Nagase, H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circulation Research.* **92** (8), 827-839, (2003).
12. Vihinen, P., Kähäri, V.-M. Matrix metalloproteinases in cancer: Prognostic markers and therapeutic targets. *International Journal of Cancer.* **99** (2), 157-166, (2002).
13. Yodkeeree, S., Garbisa, S., Limtrakul, P. Tetrahydrocurcumin inhibits HT1080 cell migration and invasion *via.* downregulation of MMPs and uPA[1]. *APHS Acta Pharmacologica Sinica.* **29** (7), 853-860 (2008).
14. Yodkeeree, S., Chaiwangyen, W., Garbisa, S., Limtrakul, P. Curcumin, demethoxycurcumin and bisdemethoxycurcumin differentially inhibit cancer cell invasion through the down-regulation of MMPs and uPA. *The Journal of Nutritional Biochemistry.* **20** (2), 87-95 (2009).
15. Mabry, K.M., Schroeder, M.E., Payne, S.Z., Anseth, K.S. Three-Dimensional High-Throughput Cell Encapsulation Platform to Study Changes in Cell-Matrix Interactions. *ACS Applied Materials & Interfaces.* **8** (34), 21914-21922, (2016).
16. Sridhar, B.V., Doyle, N.R., Randolph, M.A., Anseth, K.S. Covalently tethered TGF-β1 with encapsulated chondrocytes in a PEG hydrogel system enhances extracellular matrix production. *Journal of Biomedical Materials Research. Part A.* **102** (12), 4464-4472, (2014).
17. Sridhar, B.V. *et al. Development of a cellularly degradable PEG hydrogel to promote articular cartilage extracellular matrix deposition. Advanced Healthcare Materials.* **4** (5), 702-713, (2015).
18. Fairbanks, B.D., Schwartz, M.P., Bowman, C.N., Anseth, K.S. Photoinitiated polymerization of PEG-diacrylate with lithium phenyl-2,4,6-trimethylbenzoylphosphinate: polymerization rate and cytocompatibility. *Biomaterials.* **30** (35), 6702-6707, (2009).
19. Mucha, A. *et al. Membrane Type-1 Matrix Metalloprotease and Stromelysin-3 Cleave More Efficiently Synthetic Substrates Containing Unusual Amino Acids in Their P1.' Positions. Journal of Biological Chemistry.* **273** (5), 2763-2768, (1998).
20. Chai, S.C., Goktug, A.N., Chen, T. Assay Validation in High Throughput Screening - from Concept to Application. *Drug Discovery and Development.* (2015).
21. Iversen, P.W. *et al. HTS Assay Validation. Assay Guidance Manual.* at <http://www.ncbi.nlm.nih.gov/books/NBK83783/> (2004).
22. Leight, J.L., Tokuda, E.Y., Jones, C.E., Lin, A.J., Anseth, K.S. Multifunctional bioscaffolds for 3D culture of melanoma cells reveal increased MMP activity and migration with BRAF kinase inhibition. *Proceedings of the National Academy of Sciences of the United States of America.* **112** (17), 5366-5371, (2015).
23. Patterson, J., Hubbell, J.A. Enhanced proteolytic degradation of molecularly engineered PEG hydrogels in response to MMP-1 and MMP-2. *Biomaterials.* **31** (30), 7836-7845, (2010).