

Quantification of MMP Activity and Inhibition in a 3D Tumor Invasion Model

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Introduction

Metastasis, the spread of cancer cells from the primary tumor to secondary locations within the body, is linked to approximately 90% of cancer deaths. Penetration through the basement membrane is a critical step during the metastatic process, and has been linked to the formation of membrane superstructures called invadopodia. While being made of multiple substances, a notable component are matrix metalloproteinases (MMPs), and specifically MMP-14. Mounting preclinical evidence linking MMPs to cancer progression, combined with the issue of overlapping substrate specificity of MMP family members, has made the development of targeted MMP inhibitors an attractive approach to cancer therapy. Therefore, methods to selectively measure MMP-14 activity, specifically within invadopodia, in a sensitive yet easy to perform process, are necessary.

Similarly, appropriate in vitro cell models have been unable to accurately assess the ability of novel therapies to inhibit tumor invasion, including invadopodia formation. Incorporating a three-dimensional (3D) spheroid-type cellular structure that includes co-cultured cell types forming a tumoroid, provides a more predictive model than the use of individual cancer cells cultured on the bottom of a well in traditional two-dimensional (2D) format.

Here we demonstrate the ability to image and quantify MMP-14 activity, in addition to tumor invasion, using a 3D tumoroid cell model. Enzium's protease activity detection technology was incorporated into the invasion assay procedure to enable simultaneous phenotypic and mechanism of action quantification. The tumoroids comprised primary human dermal fibroblasts and MDA-MB-231 breast adenocarcinoma cells, known to be invasive and metastasize to lung from primary mammary fat pad tumors. The cells were aggregated into 3D structures using Corning Spheroid Microplates containing an Ultra Low Attachment surface. A novel cell imaging multimode reader and cellular analysis algorithms from BioTek Instruments, Inc. were incorporated to provide automated, image-based detection and quantification of invasion and enzyme activity. The combination presents an accurate, easy-to-use method to assess target-based and phenotypic effects of new anti-metastatic drugs.

BioTek Instrumentation

Cytation™5 Cell Imaging Multi-Mode Reader. Cytation 5 is a modular multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation 5 includes filter- and monochromator-based microplate reading; the microscopy module provides high resolution microscopy in fluorescence, brightfield, color brightfield and phase contrast. With special emphasis on live-cell kinetic assays, Cytation 5 features temperature control to 65 °C (37 ± 0.2 °C), CO₂/O₂ gas control and dual injectors for kinetic assays. Shaking and Gen5™ software are also standard. The instrument was used to image the tumoroids as well as the specific signal from the EnSens red fluorescent dye using the brightfield and Cy5 imaging channels, respectively.

Gen5™ Data Analysis Software. Gen5 software controls the operation of the Cytation 5 for both automated digital microscopy and PMT-based microplate reading. Image acquisition is completely automated from sample translation, focusing and exposure control. Cellular analysis allows examination of the tumoroid as a single object to enable accurate calculations of changes in tumoroid size and signal.

EnSens® Protease Activity Detection Technology

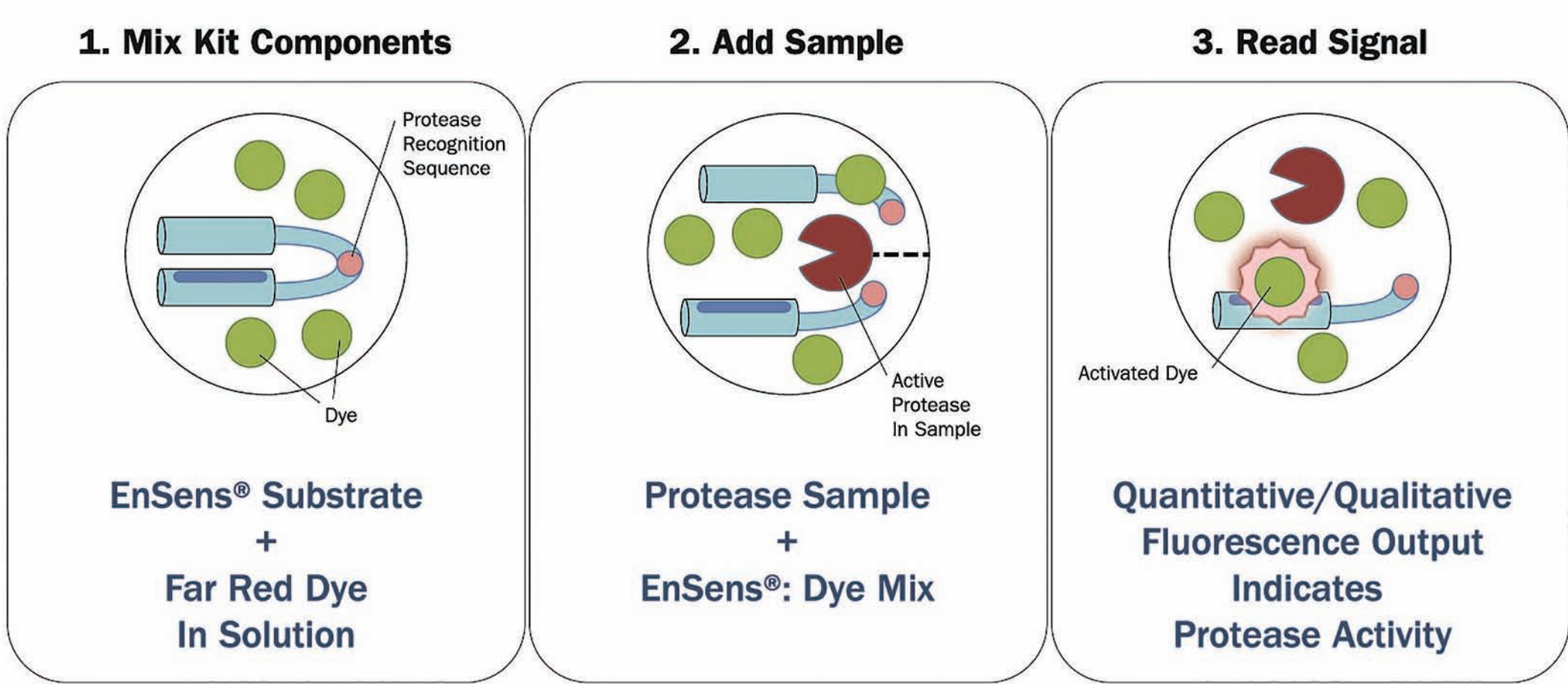


Figure 1. EnSens Protease Activity Detection Assay Procedure

The EnSens protease detection technology is based on a proprietary protein substrate that can be designed to contain a selective recognition sequence for a specific protease, in addition to a far-red shifted fluorogenic dye. The assay procedure works in the following manner: **1.** The horseshoe shaped protein substrate and dye are added to the well; **2.** In the presence of the appropriate protease, the substrate is cleaved, exposing the dye binding site; **3.** Upon binding, a far-red shifted signal is created. Therefore, little to no fluorescence is seen unless the substrate is in the presence of the correct protease.

Materials and Methods

MDA-MB-231 and fibroblast cells diluted to 5.0x10⁴ cells/mL, combined, and added to wells

- 100 µL cell suspension added to wells [2,500 MDA-MB-231 (Cell Biolabs, Inc. San Diego, CA, USA) and 2,500 fibroblast cells (Angio-Proteomie, Boston, MA, USA)]
- 96-well black, clear-bottom spheroid microplates used. (Corning® Life Sciences, Corning, NY, Catalog No. 4520)
- Plates incubated for 2 days until complete tumoroid structures form

Day 3. 70 µL media removed from wells, washed, and replaced with media + 10 ng/mL CXCL12 chemoattractant +/- inhibitor

100 µL Matrigel Matrix (1:2 dilution) + 10 ng/mL CXCL12 + 25nM EnSens substrate and 25nM far-red dye +/- inhibitor added as overlay

- Matrigel thawed on ice and plate cooled to 4 °C
- Add Matrigel to wells, centrifuge plate (300 x g for 5 min @ 4 °C)
- Incubate plate at 37 °C / 5% CO₂ for 1 hr

Cell imaging performed daily (Day 0 to Day 6) to track tumor invasion and MMP activity

Quantification of MMP-14 Activity in 3D Tumoroids and Invadopodia

An initial experiment was performed to test the ability of the assay components to work properly within the context of the 3D tumor invasion model. The MMP-14 substrate was selected for inclusion, as this particular MMP has been shown to play a critical role in conferring cells with the ability to penetrate the extracellular matrix, and is directly linked to tumorigenesis and metastasis.

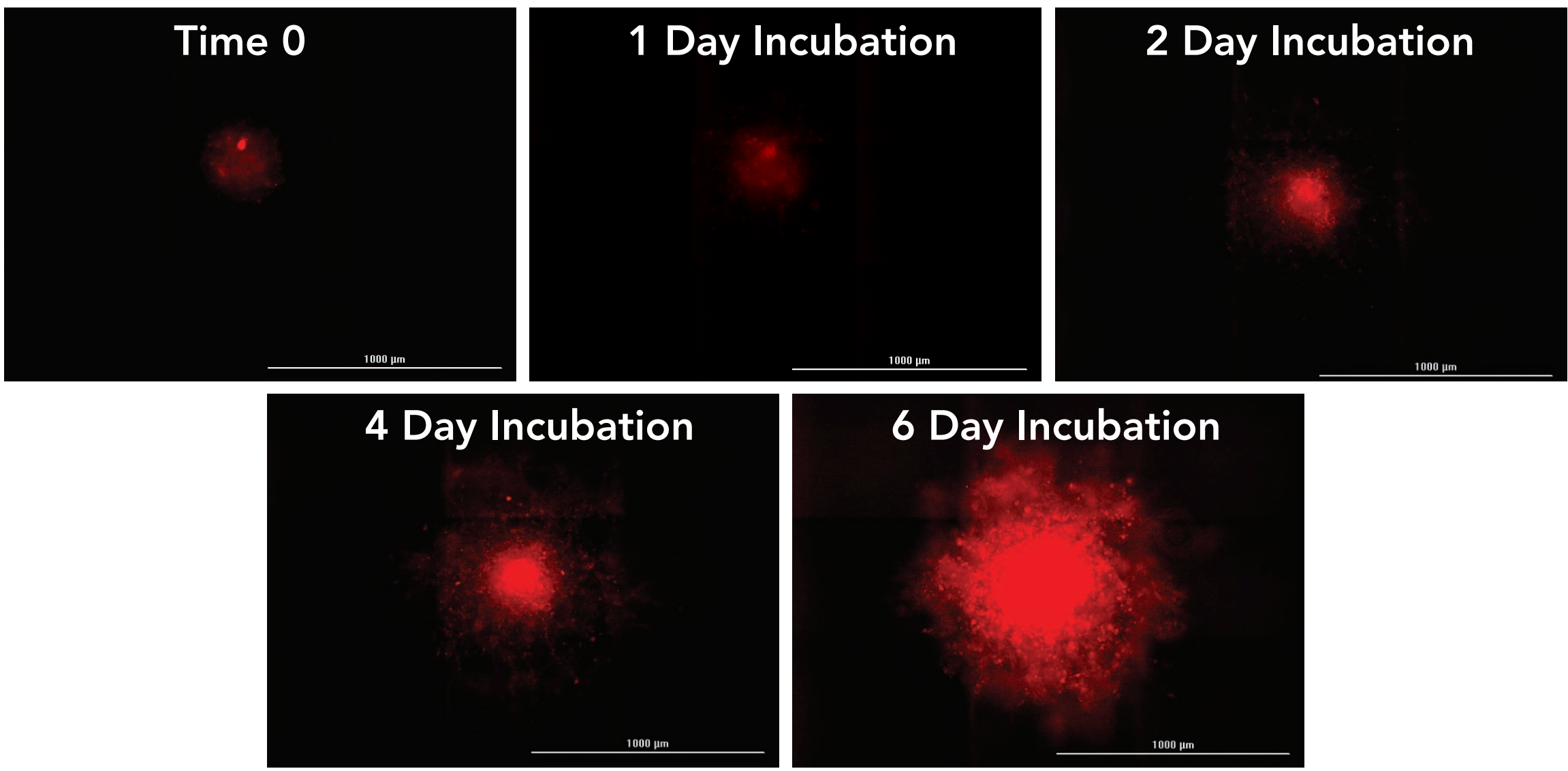


Figure 2. Image-based Monitoring of MMP-14 Activity in 3D Tumor Invasion Assay. Final stitched images from 3x3 image montages using Cy5 channel and 10x objective.

Captured images over the incubation period (Figure 2) are as expected. Minimal signal from the dye is initially seen, which increases dramatically over time as invasion proceeds and the MMP-14 protease is able to cleave greater numbers of substrate molecules.

Matrix metalloproteinases, such as MMP-14, have been shown to be active in the invadopodia portions of the tumoroid structure. Therefore, a comparison of the Cy5 dye signal, to that of the complete structure as observed through the brightfield channel, was performed.

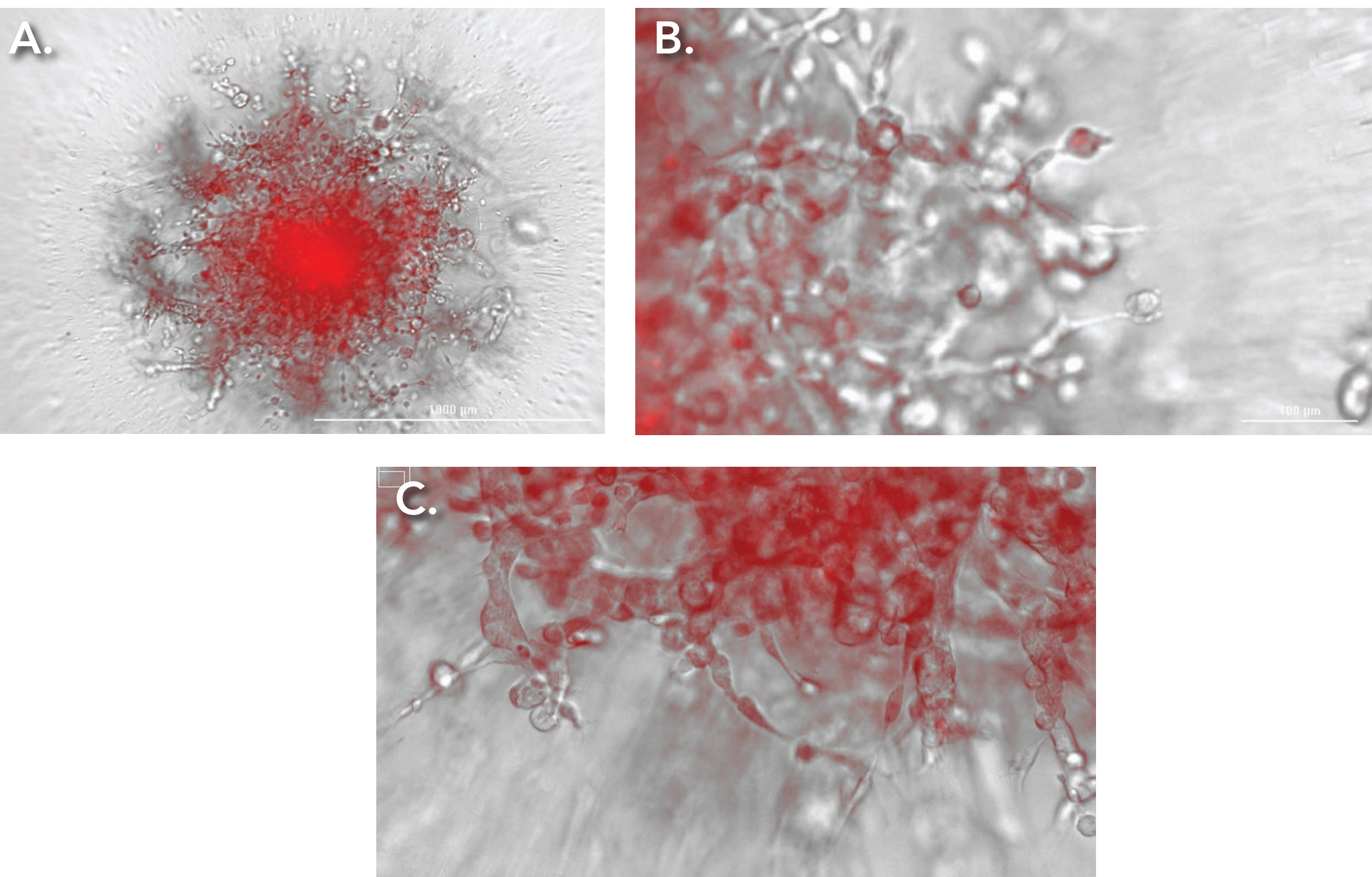


Figure 3. Visual Confirmation of MMP-14 Activity in Invadopodia. (A.) Overlay of 10x image montages from brightfield and Cy5 channels of signal emanating from entire invading structure. (B.) and (C.) Additional magnification of 10x images focusing on invadopodia tumoroid structures and their MMP-14 activity.

Overlaid Cy5 and brightfield images seen in Figure 3A demonstrate that MMP-14 activity can be observed in all portions of the tumoroid. Furthermore, when focusing solely on the areas invading furthest into the matrix (Figure 3B and C), the increased Cy5 signal emanating from the structures identified using the brightfield channel confirms that the protease is active within the invadopodia.

Cellular analysis can also be performed with the Cytation 5 using the Cy5 signal from the dye to quantify and correlate the extent of invasion and MMP-14 activity. Minimum and maximum object sizes, as well as Cy5 RFU (relative fluorescent units) threshold values are set such that a precise object mask is automatically drawn around each tumoroid in its entirety (Figure 4A and B). The same criteria are used for all images evaluated during the experiment. This allows for a quantitative comparison of the area covered and total Cy5 signal within each object mask to be completed.

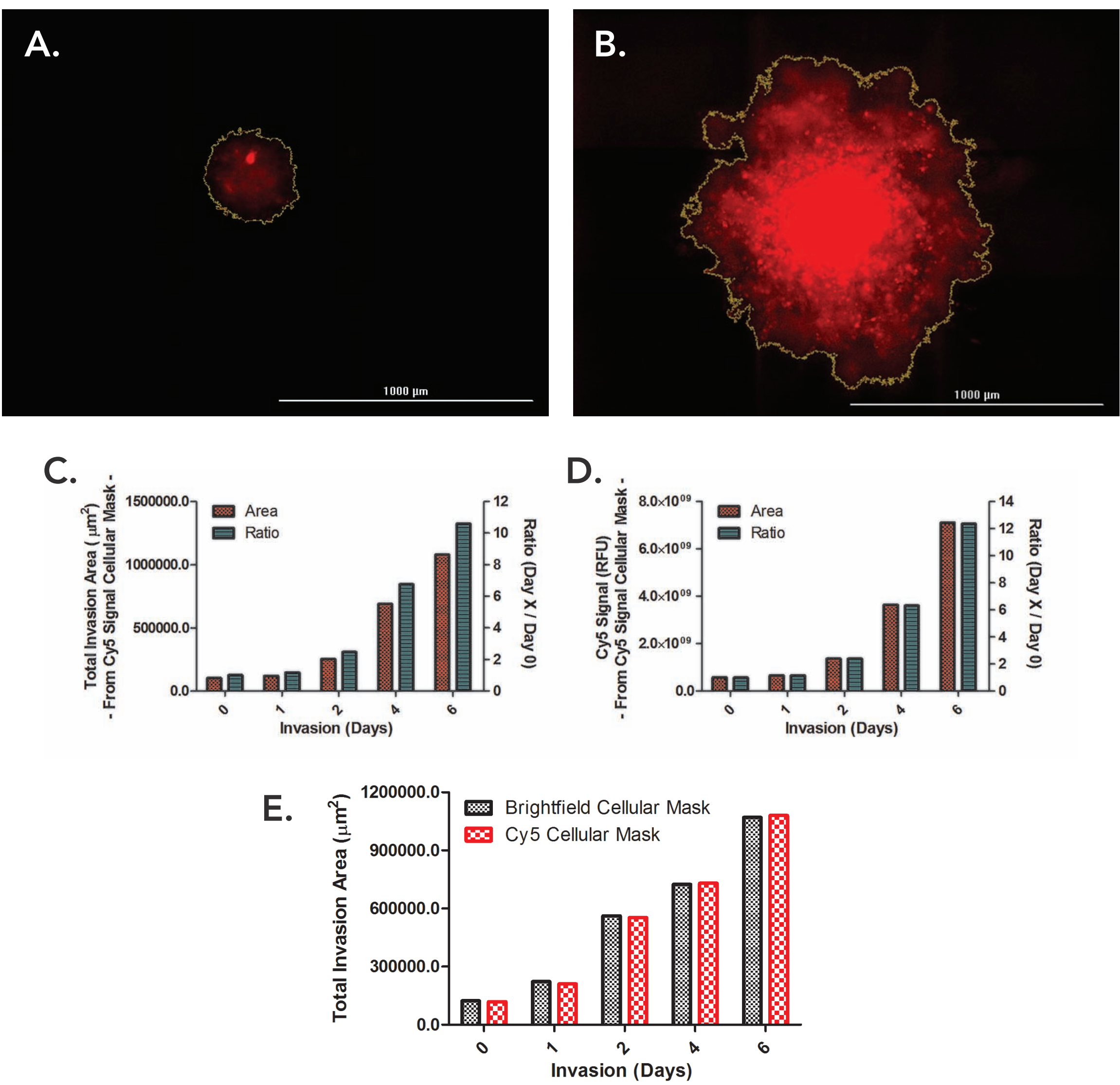


Figure 4. Quantification of MMP-14 Activity. Images of Gen5 object masks drawn around Cy5 signal meeting minimum and maximum object size and RFU threshold cellular analysis criteria after a (A.) 0 and (B.) 6 day incubation. Graphs generated using (C.) area and (D.) Cy5 signal intensity within object masks. Raw µm² and RFU values plotted on left y-axis. Ratio of values on day X compared to day 0 plotted on right y-axis. (E.) Area of invading tumoroid structure quantified using brightfield and Cy5 signals.

Figure 4C and D illustrate how the area covered by the Cy5 RFU values meeting or exceeding the set threshold increases over the total incubation period. The same trend can be seen when examining the total Cy5 signal within the object masks drawn by Gen5. When comparing the area covered within object masks drawn using the brightfield and Cy5 signal (Figure 4E), it can be observed that similar values are generated throughout the entire incubation period. This further validates that MMP-14 activity can be detected in the center mass, as well as invadopodia of the tumoroid.

Interruption of Invasion via MMP Activity Inhibition

Following confirmation that the EnSens assay components were performing correctly within the invasion matrix, and signal was observed in the expected areas of the structure, the next step was to verify that cleavage of the substrate was indeed from MMP activity and not components of the Matrigel matrix or general non-specific protease activity. GM 6001, a broad spectrum MMP inhibitor, was added to the medium and matrix surrounding the original tumoroids as previously explained, and invasion was allowed to proceed for six days.

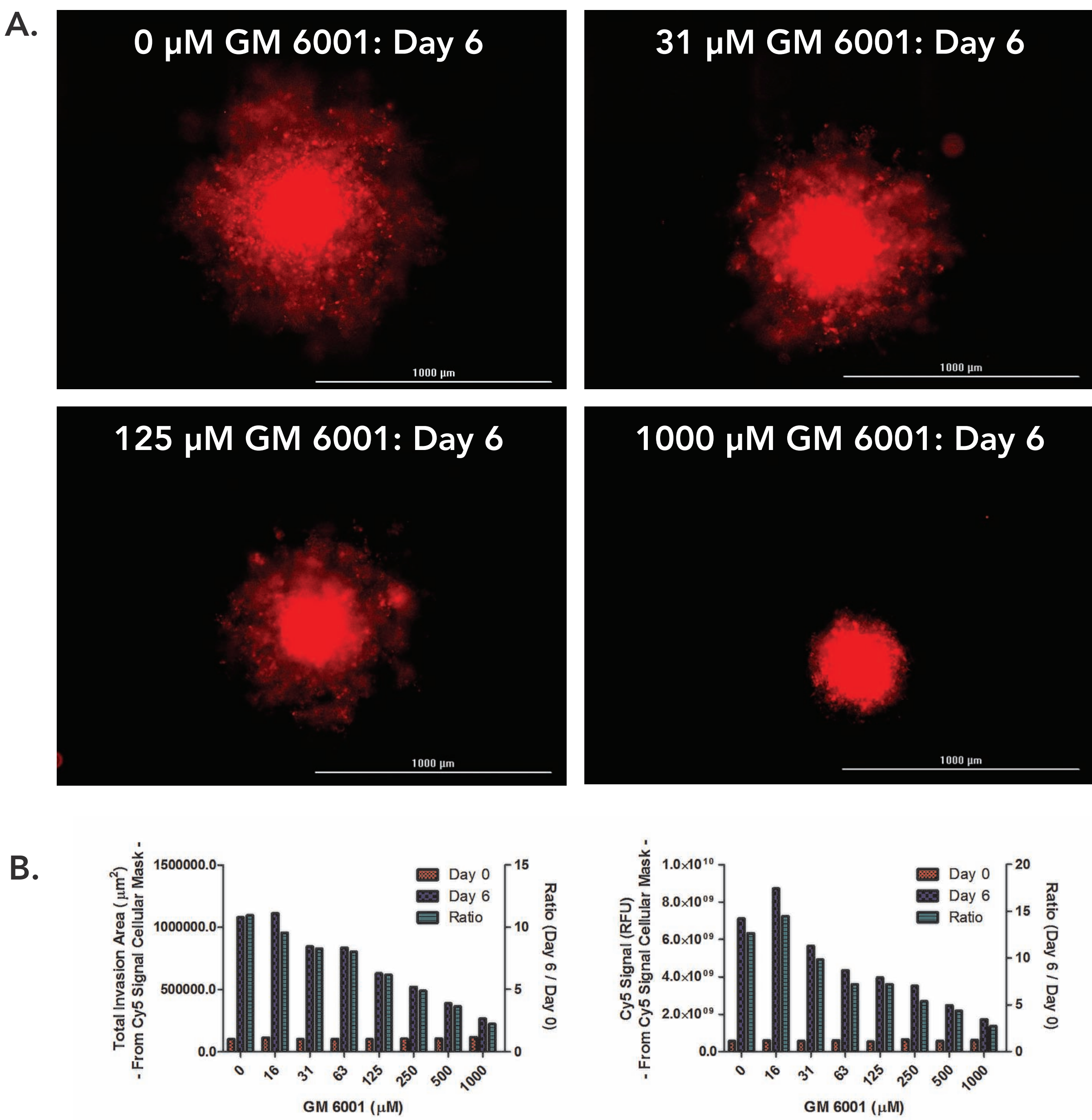


Figure 5. Demonstration of MMP Activity Detection via Inhibition. (A.) Stitched 10x images of Cy5 fluorescent signal from wells containing various concentrations of GM 6001, following a six day incubation period. Identical exposure settings used to image all wells. (B.) Quantification of area and Cy5 signal intensity within Gen5 drawn object masks.

A decrease in Cy5 signal emission is observed from the dye as the tumoroid is exposed to increasing concentrations of GM 6001 (Figure 5A), confirming that signal generation is caused by cleavage of the substrate due to MMP activity, and not from non-specific cleavage brought about by a component of the Matrigel matrix. Quantification performed (Figure 5B) also confirms that inhibiting MMP activity causes a decrease in tumor invasion into the matrix as well as MMP-14 protease activity signal generated from the included substrate and dye.

Demonstration of Substrate Selectivity

A final test was performed to confirm that cleavage of the substrate and resulting signal were specific not only to general MMP activity, but to MMP-14 protease activity. Here we incorporated selective EnSens substrates for MMP-14 and MMP-2, and NoPro substrate, which is not cleaved by any protease expressed in mammalian cells. Two types of inhibitors were tested: the broad range MMP inhibitor GM6001; and oridonin, which suppresses MMP-2, but not MMP-14, expression in MDA-MB-231 cells. Tumoroids were exposed to multiple concentrations of each inhibitor for five days, and total fluorescent signal from the EnSens dye was measured (Cy5 filter).

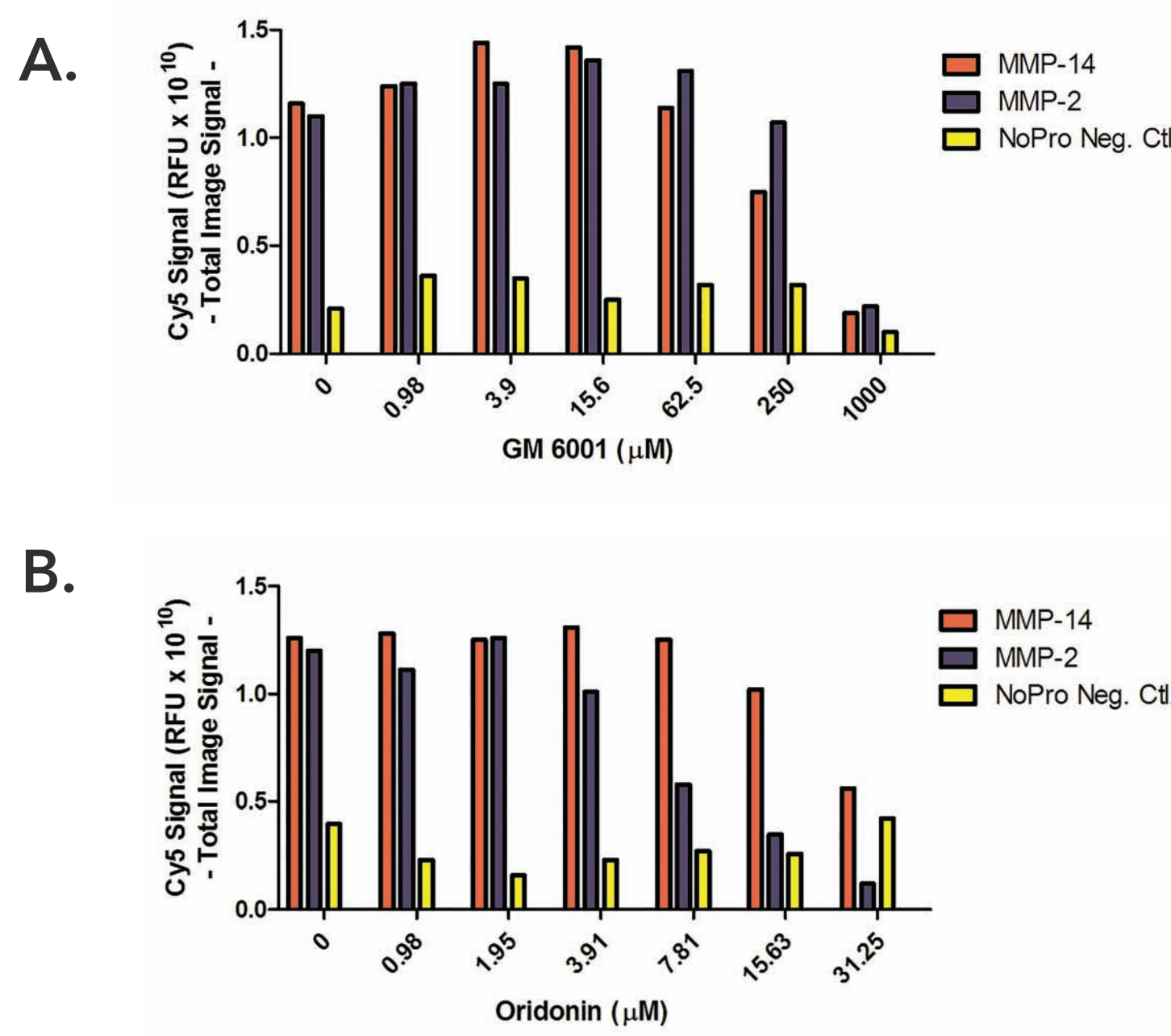


Figure 6. Total Cy5 Signal Inhibition. Total fluorescent signal from stitched 10x images following six day incubation with varying concentrations of (A.) GM 6001 or (B.) oridonin.

GM6001 inhibits substrate cleavage and signal generation in a dose dependent manner when either the MMP-2 or MMP-14 substrates are incorporated, demonstrating its pan-MMP inhibition. (Figure 6A). However, treatment with MMP-2-selective oridonin resulted in disparate outcomes for the two substrates (Figure 6B). Cy5 signal is reduced in a dose dependent manner in the presence of the MMP-2 substrate; but when the MMP-14 substrate is incorporated, no significant signal reduction is seen until the highest oridonin concentration (which results in apoptosis) is used.

Conclusions

1. 3D tumor invasion assays represent an important method to accurately quantify invasive characteristics of cancer cells and their response to test molecules
2. EnSens Activity Detection technology enables simple, definitive, and selective assessment of MMP activity and inhibition, adding value to the assay procedure by allowing live cell mechanism of action determinations
3. Monitoring the invasion process and signal generation following cleavage of the selective MMP substrate can be completed in an automated fashion via imaging using the Cytation 5
4. Gen5 Data Analysis Software allows accurate quantification of invasion, and MMP activity, in the original tumoroid and invadopodia
5. The combination of EnSens technology, Cytation 5 imaging, and Gen5 analysis presents a complete solution to assess target-based and phenotypic effects of new anti-metastatic drugs