

# Mechanical Properties of an Expanding Glioblastoma Tumor

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## Abstract

Glioblastoma multiforme is a highly malignant and infiltrative form of brain cancer whose patterns make total excision of the tumor nearly impossible, as invasive cells may be found in areas far from the core of the tumor. In this study, we are attempting to investigate the mechanical properties of an expanding glioblastoma tumor, through the 3D *in vitro* model of a human cell line U87MGmEGFR multicellular tumor spheroid inserted into a collagen I environment. The results of this study indicate that as the spheroid expands volumetrically it exerts outward pressure to its environment thus increasing the fiber concentration close to it, while the invasive cells in order to move forward pull inward their micro-environment as indicated by increased fiber density in front of the invasive cell tip.

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## Introduction

Glioblastoma multiforme is a highly infiltrative form of brain cancer ranked as grade IV astrocytoma. One of the reasons for low survival rate of those diagnosed with glioblastoma is its rapid volumetric growth, which leads to increased intracranial pressure with neurological consequences. The tumor volume can reach a significant fraction of the brain volume within a year from diagnosis, and the only way to decrease the pressure is partial or total excision of the tumor. However, recurrence of the tumor often takes place due to remaining cells found far from the core of the tumor. These are invasive cells that source from the highly proliferating rim of the tumor, which tend to form long multi-cellular “cords” migrating to the bloodstream or to other areas of the brain.

In this study, we are focusing on the mechanical impact the expanding tumor and the invasive cells have on their microenvironment. In order to model the tumor and its extracellular matrix (ECM) environment *in vitro*, we use spheroids of the human-derived U87MGmEGFR cell line implanted into a collagen I gel. U87MGmEGFR cells express the wildtype EGFR (Epidermal Growth Factor Receptor) as well as  $\Delta$ EGFR, a mutant type that makes the cells incapable of signal attenuation and down regulation. Another characteristic of this cell line that we exploit in our research is that after the cells reach confluence in culture, they form multicellular tumor spheroids (MTS) of diameter 200-500 $\mu$ m, which are a small-scale model of the actual brain tumors. They consist of a necrotic core surrounded by a non-proliferating volume and a highly proliferating surrounding rim. The invasive patterns begin from the outside rim and can be tracked radially outward, as they expand into the collagen I fibers. Collagen I is a protein of the collagen family, a natural biopolymer that forms a network of fibers. For our system, fiber diameter is about 500nm and length 40-60 $\mu$ m. If our hypothesis that cell traction is linked to forward movement of the cell leading to ECM remodeling is correct, then we should be able to track individual fiber movement and deformation, resulting from the applied tension. Fiber rearrangement should also be evident close to the implanted spheroid, as with time its volume is increasing and therefore fibers close to it should be pushed outward and aligned with its boundary.

## Materials and Methods

A significant part of my project was dedicated to development of the assay for the study of the mechanical properties of the expanding MTS.

We culture the U87MGmEGFR cells in DMEM medium 1X (Gibco BRL, Life Technologies™, Grand Island, NY) supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin / streptomycin) and 10mM HEPES, in a humidified environment (37°C, 5% CO<sub>2</sub>). After reaching monolayer confluence, the cells start combining in multicellular spheroids which float in the medium and can be selected individually with glass Pasteur pipettes and implanted into the assay.

We managed to form a protocol for a collagen I gel that exhibits the following properties:

- (a) has isotropically distributed fibers throughout its volume
- (b) remains stable for 7 days
- (c) retains a pH of 7.4- 7.7 for as long as it is stable
- (d) allows cell life for at least three days
- (e) its composition is known and not dependent on commercially available products which do not offer exact values of their composition.

We use sterile solution of 99% purified, pepsin-solubilized bovine dermal collagen I dissolved in 0.012N HCl (Vitrogen™ Collagen, Cohesion Technologies, CA). The choice of collagen I comes as a natural solution to the problem of modelling the ECM, since collagen I is a natural component of the human ECM. Collagen's property of fibrillogenesis serves the purpose of monitoring fiber remodelling by the spheroids. For comparison reasons, we keep the collagen concentration to the same levels with other assays used for cell motility studies (1.67mg/ml). In the collagen solution, we add 10X MEM and 1X DMEM (Gibco BRL, Life Technologies™, Grand Island, NY), 10% fetal bovine serum, 1% antibiotics (Penicillin/ Streptomycin) and to buffer the pH sodium bicarbonate. The cocktail is inserted in a plexiglass cube of 1cm edge or cylinder of 1cm diameter and 0.5cm height aligned with nylon mesh, which prevents the collagen from collapsing in its effort to avoid the plexiglass surface. Thus, the fibers anchor on the mesh grid and distribute themselves isotropically. Insertion of the spheroids and incubation at 37°C and 5% CO<sub>2</sub> lead to polymerization and fibrillogenesis within 2hours.

The spheroids sediment to the bottom of the mesh during the beginning of the gelation process and the network matrix gels *in situ* about them. The spheroids can be seen with bright field microscopy (fig.1- 521 x 521µm), while the collagen fibers are imaged with confocal reflectance laser scanning microscopy (laser wavelength 458nm). For the imaging, the Zeiss Laser Scanning Microscope, LSM510 (Zeiss Corporation, Germany) has been used and timelapse sequences of the invasive "cords" were taken for 2-5 hours in order to see the active remodeling of the fibers. Efforts were made to locate a spheroid and monitor the fiber concentration around it over the course of days, but they were not successful.

## Results and Discussion

### 1. Rheology of the collagen I gel

Since the spheroid is embedded in and tries to locally remodel a collagen I gel, we had to perform bulk rheology on the gel, before studying the deformations of the microenvironment of the spheroid. From previous studies<sup>1</sup> it has been found that spheroids of this type can overcome

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<sup>1</sup> Helmlinger, Netti, Lichtenbeld, Melder, Jain, *Solid stress inhibits the growth of multicellular tumor spheroids*, Nature Biotechnology, **15**, 78 (1997)

pressures up to 6KPa, therefore we had to have a much softer gel in order to successfully study rapid invasion. We used the ARES (Rheometric Scientific) rheometer, with the 5cm in diameter

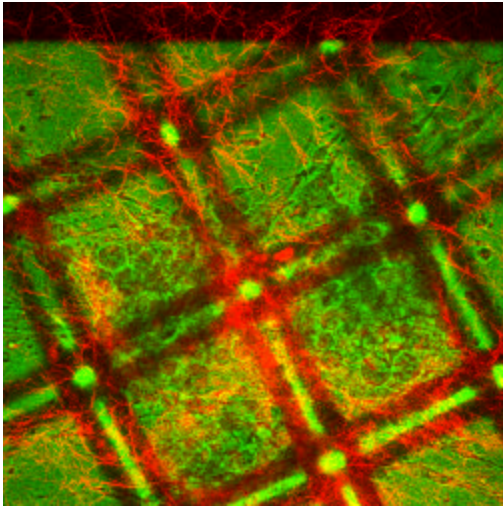


Fig.1. overlaid bright field (green channel) and confocal reflectance (red channel) image of a spheroid, 2 days after incubation, side 521µm

cone-and-plate geometry and the most sensitive transducer to measure the bulk moduli of the gel. The polymerization took place in the rheometer's gap, as temperature was controlled to 37°C. Further care was taken to prevent dehydration, preventing shrinking of the solvent, a form taken by the gel in the cubes only after 8 days which corresponds to a period after cell death. Strain and frequency test sweeps showed that for frequencies 0.1Hz – 80Hz and strain amplitudes less than 0.05, the gel is a soft, solid like gel with storage modulus  $G' \sim 100\text{Pa}$  and loss modulus  $G'' \sim 60\text{Pa}$ , according to the equation  $s(t) = \epsilon \epsilon_0 [ G'(\omega) \sin(\omega t) + G''(\omega) \cos(\omega t) ]$  used by the rheometer's program. These results are indicative of a gel permissive to invasive patterns, although an order of magnitude stiffer than the previous assay used for similar studies based on the commercially available Matrigel™ (Biocat, Becton Dickinson, NJ). To ensure the reproducibility of the results, more tests have to be performed, and even compared with the previous assay used.

## 2. Volumetric expansion of the MTS

One of the advantages of the confocal imaging technique is the accurate measurement of the xy-dimensions of the MTS for every height, as well as the control of the optical slice in the z-direction. Only the depth at which we can reach in the z-direction is limited by the objective. Thus, by using confocal reflectance microscopy to monitor the fibers over time very close to the MTS, we hoped to relate the fiber density as a function of time, and find how far the effect of the pressure reaches and how it may or may not affect the invading cells, which are located close to the MTS the first day, but soon their distance is more than 100 µm radially outward from the MTS surface.

As the spheroid expands over the course of the few days due to the highly proliferating rim, the fiber density should increase, giving a monotonically increasing relation between the two variables. Although we have not been able to prove this, an indication that this is what might be happening comes from figure2 (magnification 63x, 1µm z-slice, xy: 180 x 180µm) where fibers seem to be aligned with the boundary of the MTS. An explanation could be that in order to reach this alignment they had to be pushed outward, the same way if one pushes a plasticine piece - a 3D material like the collagen gel - with a 2D surface - like the MTS surface- it forms wrinkles perpendicular to the direction of pressure which align with the surface boundary. It seems that the

expansion of the MTS is plastically deforming the fibers in the area, while the solution in between the fibers flows outwards.

The idea being tested is that cell traction and outward pressure exerted by the MTS are two antagonistic forces in the micro-environment of the spheroid. However, there is no evidence in the picture below that the pressure affects the environment of the distal invading cells, as fibers appear to have aligned tangentially to the surface of the MTS, but only those that are about 20 $\mu\text{m}$  far from the MTS. However, this picture we now have may as well be incomplete as it shows only a part of the remodeling over time, as we have not succeeded in observing the volumetric expansion of the MTS nor the way the fibers are aligned over the course of days.

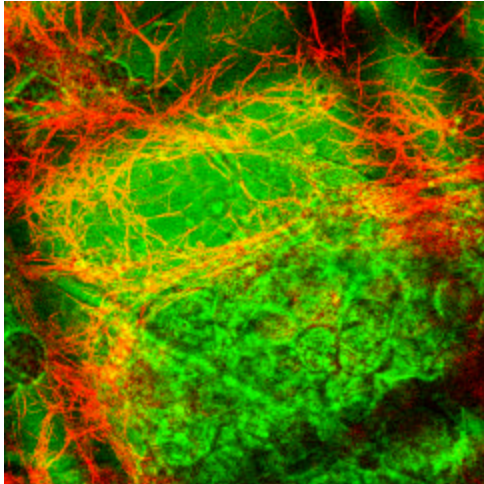


Fig.2. Fiber alignment along the MTS boundary

### 3. Invasive patterns and cell traction

One of the goals of this study was to show and relate cell invasion with cell traction. As the spheroid grows volumetrically, the pressure exerted by the outside environment on it increases. A solution leading to decrease of the overall pressure comes from increase of the total surface area. The invasion process starts with the radially outward, relative to the MTS, movement of one cell into its extracellular environment. The first cell is for haptotactic reasons followed by more cells of the highly proliferating rim, thus, from the spheroid's surface start long multi-cellular "cords" of invasive cells which move radially outwards. Whether the goal of pressure decrease is the cause of this behavior, or whether the decrease is just the outcome of this procedure, is hard to say. The real cause may be lack of nutrients close to the densely populated rim, which makes cells follow the path leading to the closest nutrient source. However, pressure seems to play role in the formation of this pattern, since cells instead of starting many separate branches follow the least resistance path; they follow one another in the path the first one has opened. This indicates that the ECM even though it serves as a mean for forward cell movement and anchoring, which produces the tension, acts as resistive medium that the cells have to overcome. Another factor enhancing the multi-cellular "cords" is the social haptotactic behavior of glial cells, which tend to secrete substances to attract other cells of the same type. In our system, multi-cellular patterns have been observed ranging from 2 to 6 cells. The cells' velocity has been measured to 11-13  $\mu\text{m}/\text{hour}$ , in agreement with what is reported in the literature for cells *in vitro*.

Indicative of cell traction is the "pulling" of fibers opposite to the direction of motion; an equal and opposite force is produced that aids the cell cytoplasm to move forward, and thus enhances the forward motion of the cell. "Pulling" is more correctly described by cell adhesion of

the outside surface of the cell via integrins to the ECM. We do not know how long it takes for the integrins to make a cycle of adhesion-detachment, which could help to explain the speed of the cells as a function of the period of attachment-detachment and local fiber density. However, cell adhesion which is correlated with cell motion produces a higher concentration of fibers close to the cell tip, indicative of active fiber remodeling. In figure 3, an invasive cell tip in the upper left corner is attached to fibers. The overall cell length is more than  $100\mu\text{m}$ , while the bulk of the cytoplasm is only  $20\mu\text{m}$  across, the remaining being the long invasive part. Compared to the fiber density in the lower right corner, the fiber density seems higher closer to the tip. Another indication of fiber traction is the orientation of the fibers, which seem to point towards the end of the tip, while the ones that happened to be found in areas further away and not in the same direction with the cell movement, seem to have been curved, with one end pointing again towards the tip. However, during the course of this project we did not proceed to quantification of fiber density, so the data we get from images like (3) are only indicative of fiber remodeling. One way to proceed is by quantifying density as a function of intensity of the corresponding channel. Another is to use 2D Fourier transforms to compare images of the isotropically distributed fibers and the oriented ones.

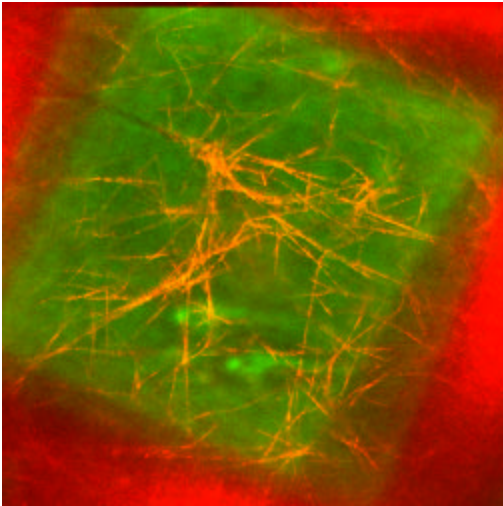


Fig.3. Cell tip with remodeled fibers ( $146 \times 146\mu\text{m}$ )

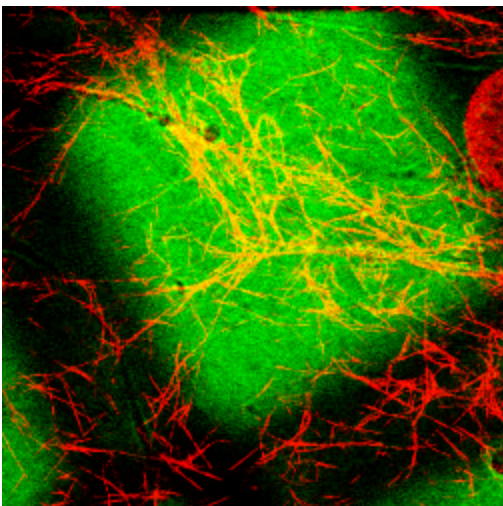


Fig.4. Cell tip remodeling the fibers ( $180 \times 180\mu\text{m}$ ) the cell comes from the right, under the red shadow, and is pulling on the fibers (in yellow). More antagonistic cells appear in the frame, but they are at slightly different heights.

The effect of fiber alignment with the direction of motion, could be mistaken for concentrated fibers in the front of the cell due to “pushing” of the cell in an effort to make its way through the viscous medium of collagen I gel. However, there is no evidence that the biopolymer collagen I resembles a stiff rod. If it were, pushing it would result in translation and rotation of the total length of the rod, but what is seen above is curving of the fibers, which precludes the “rigid rod” idea. However, if collagen I fibers are semi-flexible rods, then pushing them would result in wrinkles and compression of the individual fibers, which is not what is seen in figures 3 and 4, as the fibers retain their shape. In addition, we have evidence from a time-lapse sequence of a cell which is the head of one of the multi-cellular cords, that although its general direction of motion is forward, the cell first pulls fibers to the back, until they stretch, and then it starts its forward motion. In order to be able to observe the cell for long periods, we should develop the imaging protocol. Such efforts have included imaging the cells with CARS (Coherent Anti-Stokes Raman Spectroscopy), and finding ways to minimize the light emitted to the cells.

### **Future directions**

Fiber traction and cell invasion have been linked (movies show the active remodeling). The next step is to quantify the forces cells in the front of the multi-cellular invasive “cord” can exert, study how the motility patterns of the cells following are affected and quantify the relation of cell velocity and force exerted with the bulk moduli, or with local inhomogeneities if these affect more the cell patterns. In addition, the time course of fiber alignment and concentration near the volumetrically expanding MTS has to be studied, as well as quantified. Furthermore, experiments remain to be done with particle tracking to fully describe the network’s properties, and data to be analyzed quantitatively for a more thorough understanding of the model system.

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