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Research internship report

Estimation of cellular traction forces in morphogenesis of capillaries through advanced material modeling of fibrous biopolymers

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Abstract

Vaculogenesis is a process during which endothelial cells grow to form a network of interconnected vessels, to provide oxygen and nutrients to surrounding tissues. Endothelial cells microenvironment has an important impact on their growth. To understand such impact, one of the environment factors is studied in this report, i.e the traction forces that endothelial cells apply on the extracellular matrix. Those forces can modify the course of the network development. Based on a previous study, a combination of experimental and numerical approaches has been replicated for endothelial cells specifically in a three-dimension (3D) fashion. Fluorescence imaging was used to measure displacement fields with a fast iterative digital volume correlation (FIDVC). Then, forces applied on cells were determined with the technique of Traction Force Microscopy (TFM) using the Finite Element Method (FEM). The results showed that with a linear relationship between rigidity and density of the extracellular matrix, tractions, displacements, and density are independent. Furthermore, the independence between tractions, displacements and matrix density paves the way for more complex scenarios regarding the effect of matrix material remodeling.

Introduction

Regenerative therapies consist of repairing a lesion or a diseased organ by replacing the damaged parts with a new engineered cellular tissue. In that aim, those tissues need to form a functional microvasculature network to provide oxygen and nutrients to surrounding tissues as well as to assure their integration within the damaged area. A previous study [1] focused on the influence of mechanical and biochemical microenvironment during vasculogenesis, for endothelial cell growth. It has shown that environmental factors such as extracellular matrix composition, play an important role in the network morphology and although, they are not exhaustive, a computational modeling approach would allow to better understand the mechanisms behind it. In fact, cells are able to adapt to the microenvironment, especially to physical forces applied. [2]

In the continuity of the study about the influence of the mechanical and biochemical environment on endothelial cell growth, a similar controlled experimental model of formation of microvasculature on a chip was carried out to measure traction forces. However, no study has measured forces during vasculogenesis at the cellular scale and their relation to matrix stiffness heterogeneity. Nevertheless, strategies that connect mechanical deformations to matrix heterogeneity have been successful for other applications, such as structural analyses of long bones [3], in which accuracy of numerical strain in predicting experimentally measured strain was improved using reliable density-elasticity relationships.

Thus, the aim of this study is to estimate stiffness and force evolution during vasculogenesis in the fibrin hydrogel, using a controlled experimental system that allows for traction forces microscopy (TFM) experiments and further computational processing using finite element modeling (FEM).

This report firstly explains the methodology to obtain the fluorescence images and to measure matrix displacements, then the visualization of displacements in 3D with Paraview application and finally, the correlation of the localization of tractions across the sample in relation to the fibrin hydrogel stiffness.

I. Obtaining forces from confocal imaging

The aim of this study is to make an estimation of the forces applied on growing microvascular networks (vasculogenesis) in a fibrin hydrogel in vitro. The flowchart of the modeling of the forces is described as in Figure 1 and all steps will be addressed.

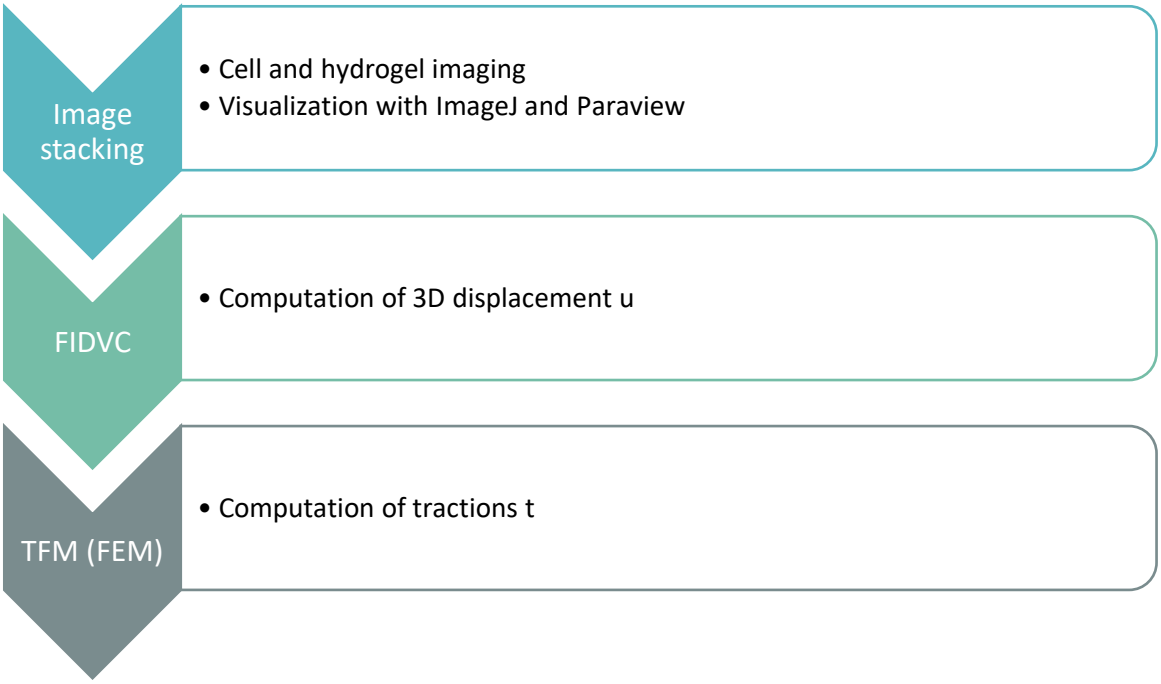

Figure 1: Flowchart of the work

A. Image stacking

Endothelial cells are seeded within a fluorescently labeled fibrin hydrogel injected [4] in a microfluidic chamber. The chamber is half a millimeter wide and a hundred of micrometers thin. The chamber length is in the centimeter range, thus allowing multiple regions of interest. Due to reduces thickness with respect to the other dimensions, the microvascular growth happens mainly on a plane, but computation of displacements is possible in 3D.

Indeed, thanks to fluorescence imaging of the fibrin hydrogel layer by layer in a 3D fashion, the data is processed to get the 3D displacement of the hydrogel as well as the 3D morphology of the cells and hydrogel local composition, and at the end, the tractions. On Figure 2 is shown the images of the acquisition of the volumetric images. On the image A is represented the 3D volumetric image where the analysis will start from. The cyan color is the hydrogel, superimposed with the cells in magenta.

From the coloration of the images, it can be said that the cells in magenta grew in majority along the horizontal plane aligned with the microfluidic chamber width and not too much in the vertical axis.

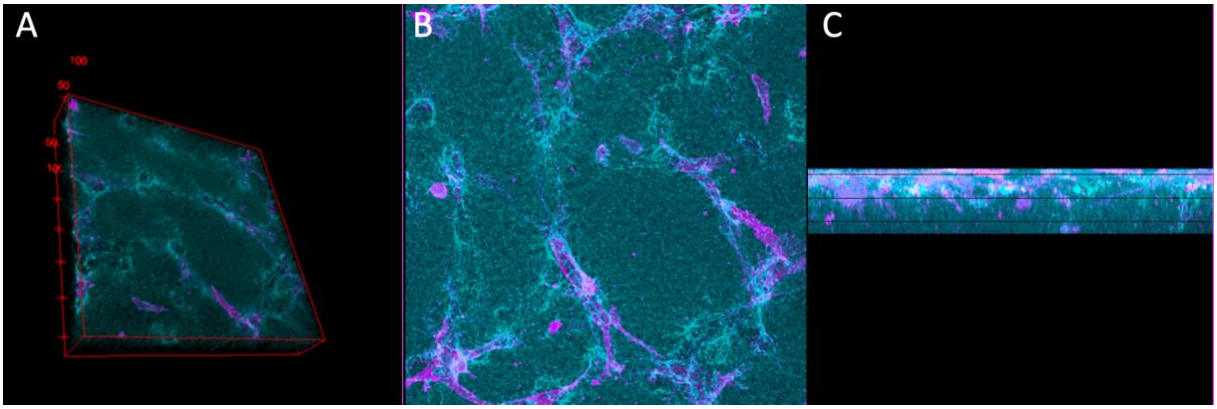

Figure 2: Imaging from cells on day 1
A - Volumetric image B – Top view C – Side view

To observe the growth of the cells, it was not possible to do the imaging in a time lapse. In fact, at different stages, the growth of cells was stopped: using a specific surfactant called Triton X, the cells were killed (decellularization) to relax all traction forces but without disrupting the hydrogel. There, the hydrogel had the time to relaxed and the difference between the undeformed and the deformed images provided the displacement of the hydrogel due to cell forces before relaxation (i.e., before Triton X decellularization). The different stages at which the cells were killed were after 1, 3 and 6 days in culture. Through this different time periods, the development of the network can be observed, and the forces can be computed at these three specific time points. The stacks of images were processed with Fiji. Fiji is an extension of ImageJ2, an application for multidimensional imaging for scientific imaging. The images were usually 640 x 640 x Z voxels, with Z the number of stack of images. Thus, the size of a voxel varies with the stacks, in order to be as accurate as possible and become a parameter for the data processing.

B. FIDVC: Fast Iterative Digital Volume Correlation

To be able to measure gel deformation, a Matlab algorithm [5] was used from another study. The program returns the displacement thanks to a digital volume correlation (DVC). The digital cross correlation comes from a simple cross correlation, that is a measure of the similarity of two data according to a time shift of one of them. Here, with images, they can differ by translation, brightness, or contrast. In our case, it is used by assimilating a voxel to its neighbors determining a displacement vector. Then, at a different time t and same localization, the same vector is compared and analyzed with a cross correlation factor named cc, which shows more similarities between the vectors when it is close to 1 [6]. This analysis is run for the whole volume with a FIDVC, which can be computed in a few minutes only (between 2 to 5 minutes). The algorithm will first save the displacement field along the x, y and z axis and their magnitude as the main output. Another output is the mapped cc.
Additionally, the cell geometry is also extracted thanks to an image segmentation. The technique used by this segmentation consists of defining a threshold and because of the different intensities of the image, a median filter is applied. The cells can thus be mapped and a triangulated mesh file of the stack of images is obtained. Finally, both displacements and cells surface are visualized, like shown on Figure 3 and 4.

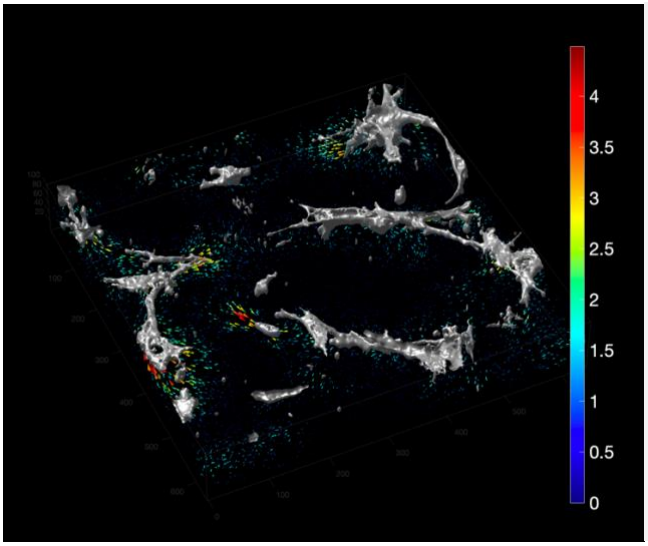

Figure 3: Displacement visualization for cells day 1

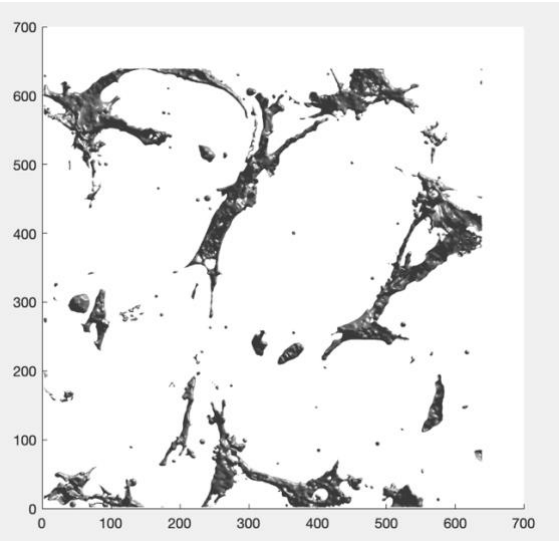

Figure 4: Cell surface visualization with Matlab for day 1

C. TFM (FEM): Traction Force Microscopy using Finite Element Model

First, the Traction Force Microscopy (TFM) is a methodology consisting in estimating traction forces exerted by cells onto the extracellular matrix. In 2D, the TFM technique is based on the quantitative analysis of the 2D substrate deformation in response to a mechanical force generated by the cells [7]. TFM calculates the tractions generated by cells (mechanical force) from a displacement field obtained from fluorescent entities cross-correlation or tracking, that will then, be used to get the deformations and finally tractions.

Strains and tractions from displacement field are calculated using the finite element method (FEM). FEM consists of modeling the sample by a structure cut into a finite number of subsets called elements (this is called "spatial discretization") to obtain an approximate model of the object under study. These elements, usually hexahedral or tetrahedral, are made up of a finite number of points called "nodes". All the elements are interconnected by these nodes and thus form the "mesh" of the initial structure. The fundamental equations of continuum mechanics are then solved at the level of each of the elements and provide a solution field at each node of the mesh, taking into account the boundary conditions.

A displacement or a material point from a position x_0 to x is written as

$$u(x) = x - x_0 \quad (1)$$

Deformations is calculated using the Lagrangian strain tensor, as follows:

$$E = \frac{1}{2}(F^T \cdot F - I) \quad (2)$$

With:

- F the deformation gradient tensor, defined like $F = I + \nabla u$,
- I the identity matrix,
- ∇u the displacement gradient.

Then, considering the small deformations ($\nabla u \ll 1$), the deformation state is updated and simplified by the linearized strain tensor:

$$\varepsilon = \frac{1}{2}(\nabla u + (\nabla u)^T) \quad (3)$$

Finally, for linear elastic materials, the stress is proportional to strains according to Hooke's law and in our case, for the FEM discretization of a linear problem, the underneath equation is solved:

$$\mathrm{K\,u} = \mathrm{f(t)} \tag{4}$$

Where:

- K is the stiffness matrix,
- u the displacement,
- f(t) a vector which depends linearly on traction field.

The resolution of this equation at each node of the mesh will give the nodal tractions. However, the easiest assumption to be made to get an idea of the tractions across the study sample was to consider a homogeneous fibrin hydrogel. Nonetheless, depending on the heterogenous stiffness, the magnitude of tractions will vary. The steps for the project are then:

1. Work with a homogeneous hydrogel, with a fixed stiffness across the whole sample,
2. Collect fluorescence density of the images,
3. Relate local fiber density to the fibrin hydrogel stiffness.

II. Materials and methods

A. Parameters for FEM & TFM algorithms

Before running the FEM and TFM functions, different parameters have been modified according to the data from the imaging. Indeed, not all the data from the different days have the same size and thus, the size of the considered voxel for the FIDVC will not be the same. The voxel sizes of the three data are:

- Day 1, [0.684 0.684 0.600] µm/pixel, with 101 stacks
- Day 3, [0.621 0.621 0.600] µm/pixel, with 131 stacks
- Day 6, [0.662 0.662 0.600] µm/pixel, with 105 stacks

On top of that, an interrogation voxel size is fixed to [124 124 64] voxel to run the FEM as an optimized size for it. [6]

B. Converting .mat file to .vtk file

Because the obtained file takes a lot of space, the software Paraview was utilized. It uses a Visualization Tool Kit (VTK). However, in order to translate data from Matlab to ParaView, a text file had to be written linking the triangled file obtained with the displacement field. Indeed, after specifying the version of the VTK, the description of the file is listed with the file format, describing the type of file, then its geometry, the connectivity and finally, if there are, the data's attributes.

In our case, the geometry is a triangle and according to the library of the VTK files [8], it corresponds to the number 5. From the Matlab algorithm to get the displacement fields, a file containing the nodes of the triangles and their vertices was used for writing the text file. An example of what the final text file looks like is presented in Figure 5.

```
# vtk DataFile Version 3.0
VTK from Matlab
ASCII
DATASET UNSTRUCTURED_GRID
POINTS 5 double
4.251830e+01 5.447060e+02 2.271296e+00
4.229596e+01 5.450796e+02 2.256319e+00
4.233991e+01 5.450591e+02 2.342394e+00
4.222003e+01 5.454443e+02 2.339020e+00
4.223078e+01 5.456545e+02 2.494035e+00
CELLS 5 20
3 0 1 2
3 1 3 2
3 2 3 4
3 2 4 5
3 3 6 4
CELL_TYPES 5
5
5
5
5
5
```

Figure 5: vtk file for a subset of 5 elements

C. Rigidity nature of fibrin hydrogel

1. Homogeneous fibrin hydrogel

We started with the assumption of having a hydrogel with the same rigidity matrix, i.e the same Young modulus E. As such, each voxel has been then associated with the same E.

2. Heterogeneous fibrin hydrogel

Endothelial cells remodel the extracellular hydrogel continuously. Thus, the stiffness is usually higher close to cells. Therefore, to plot an inhomogeneous material, the hydrogel fluorescence of every pixel in the image was used to determine the hydrogel's density. Afterwards, density's values are plotted against the stiffness. This methodology was determined in a previous work [3] and improved the strain accuracy using the FEM. An assumption was made here, to observe the evolution of the stiffness with the equation:

$$\mathrm{E} = \mathrm{E}_0 \times \left(1 + \mathrm{C} \times \left(\frac{\rho - \rho_{\min}}{\rho_{\max} - \rho_{\min}}\right)\right) \quad (5)$$

With:

- E_0 is a baseline stiffness of the hydrogel,
- C is a coefficient considering the assumption that the hydrogel's stiffness is linearly related to density,
- ρ is the mean density after resizing the images.

III. Results and discussion

Most of the results shown have been firstly obtained with the image processing of cells after one day in culture. The data volume is less bulky, and the programs were running faster with it, instead of running the programs for cells after three and six days in culture. However, results for three days and six days in culture are presented in the annexes 1 and 2.

A. Superimposing fibrin hydrogel and cells in Paraview

First, converting the .mat file of cells into .vtk file is required to superimpose cells and fibrin hydrogel in Paraview. This superimposition of both files aims at observing and working on displacements and later, tractions. The first conversion was then only about converting the cell file itself, but it appeared not to superimpose. To make them coincide, the scaling parameters were firstly modified on Paraview to understand where the problem came from. It turned out to be the discretization size dimensions. Therefore, a downsizing of hydrogel dimensions has been decided. Instead of using the voxel size in microns per pixel, the mesh discretization was downsized from [640 640 101] to [81 81 14] nodes (and [80 80 13] elements) according to the displacement discretization size from the FIDVC algorithm. Finally, with a cross product, the approximate discretization size became [8 8 7] microns per pixel. However, even if it seems to correlate, a verification element by element will be discussed.

B. Rigidity visualization

The first assumption of considering a homogeneous hydrogel only served in the purpose of making easier the first part of understanding the methodology.

Then, considering a heterogeneous material means there is high and low densities. High density suggests that cells have recruited a lot of fibrin matrix whereas low density points out that cells have degraded fibrins by proteolysis or because they were in the cell's growth path. Thus, according to the fluorescence of the imaging, the density was determined to get the stiffness of the hydrogel. However, knowing that each voxel has a different fiber density, the average of the 8x8x7 element densities is computed and then assigned to the associated voxel for resizing. As the stiffness is higher in proximity of the cells, as shown in Figure 6, tractions are expected to change with respect to the heterogeneous case.

Figure 6: Rigidity of the hydrogel for 1 day in culture

C. Tractions

Here, by modeling a non-homogeneous hydrogel, the most rigid locations are highlighted. Indeed, the hydrogel will be more rigid with a higher concentration of fibers, a higher density, showing how cells are pulling on fibrin of the matrix when cells are killed.
The cells in pink and hydrogel are superimposed (Figure 7), and as calculated by the equation (5), the magnitude of the tractions goes up to 49 N.µm^2, however, tractions cannot be seen on the sample. Therefore, the maximum for the magnitude was scaled down to 15 N.µm^2 to visualize where they are (Figure 8), to help with traction distribution. It shows that the tractions do not necessarily correlate with cells.

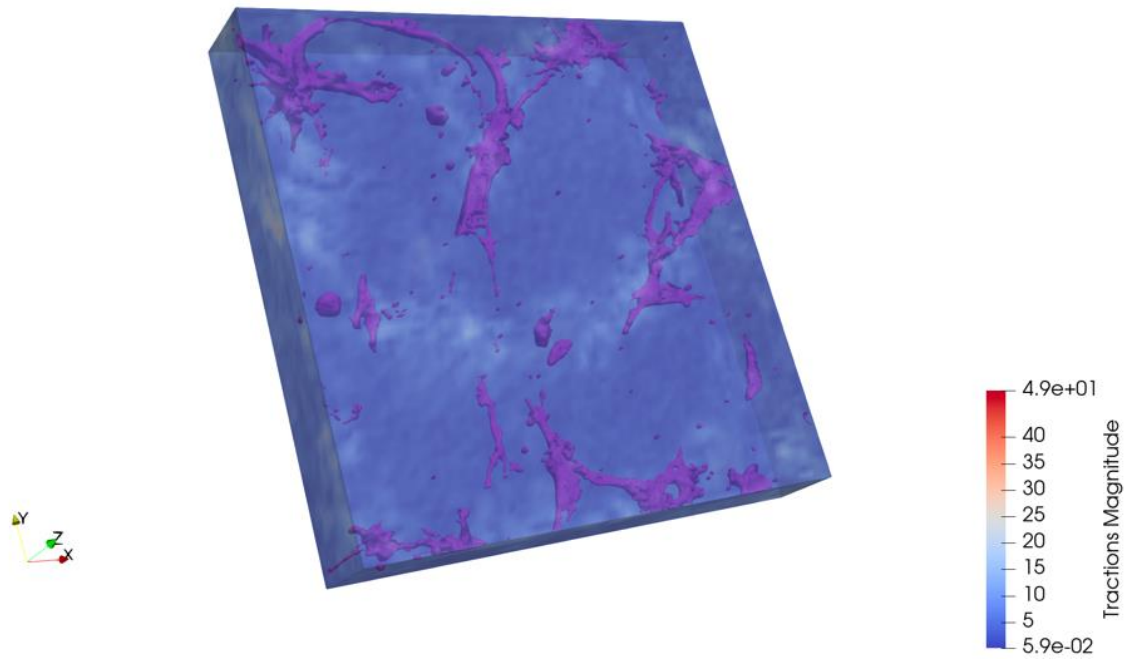

Figure 7: Tractions superimposed with cell surface

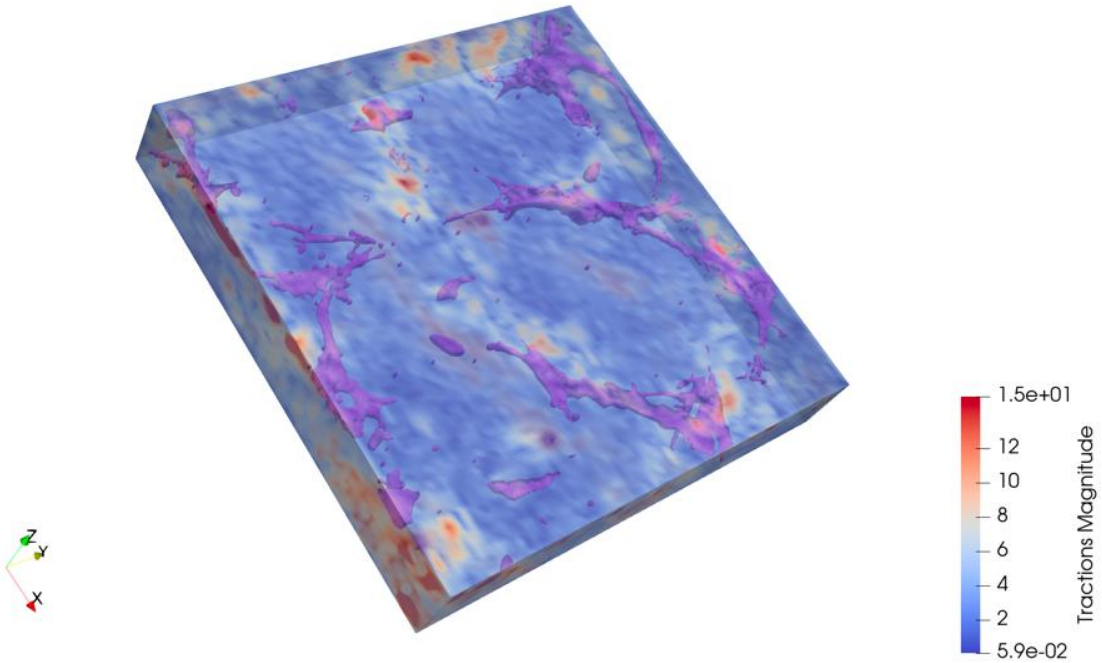

Figure 8: Traction's magnitude scaled down, superimposed with cell surface

Also, in order to test the assumption of the tractions evolving linearly with rigidity, they have been plotted against different values of the coefficient C on Figure 9 from equation (5). When C=0, the hydrogel is homogeneous and from when C=1, the hydrogel is heterogenous. Mean tractions are quite constant depending on C whereas the maximum tractions increase with C. On one's hand, considering an increase in the rigidity of the hydrogel, having the maximum tractions increasing too is expected regarding the proportionality relationship. On the other, mean tractions being constant show a distribution and compensation of traction's different magnitudes across the sample.

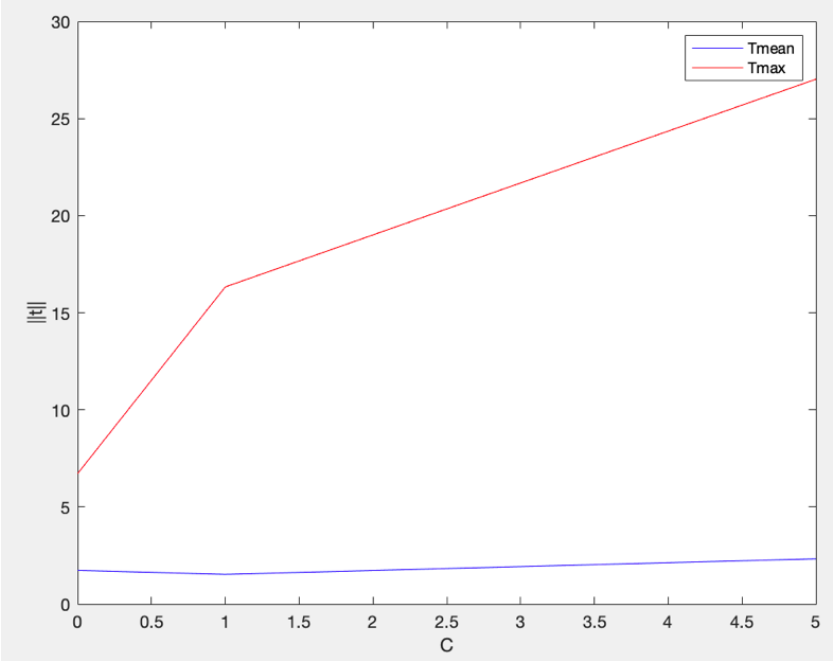

Figure 9: Mean and Maximum tractions against coefficient C

D. Verification of correlation between rigidity and tractions and rigidity and displacements

1. Correlation between rigidity and tractions

Since tractions do not seem to coincide at all points with cells and displacements, to localize them, they are plotted against rigidity (Figure 10). But a previous step was to associate tractions to elements and not nodes which was the case with the discretization before. A first function was programmed to link coordinates of stiffness to the right coordinates of tractions, then, tractions of the nodes are collected, and an average traction is calculated for the element of discretization like stiffness is. Finally, it could be plotted with element-based rigidity.

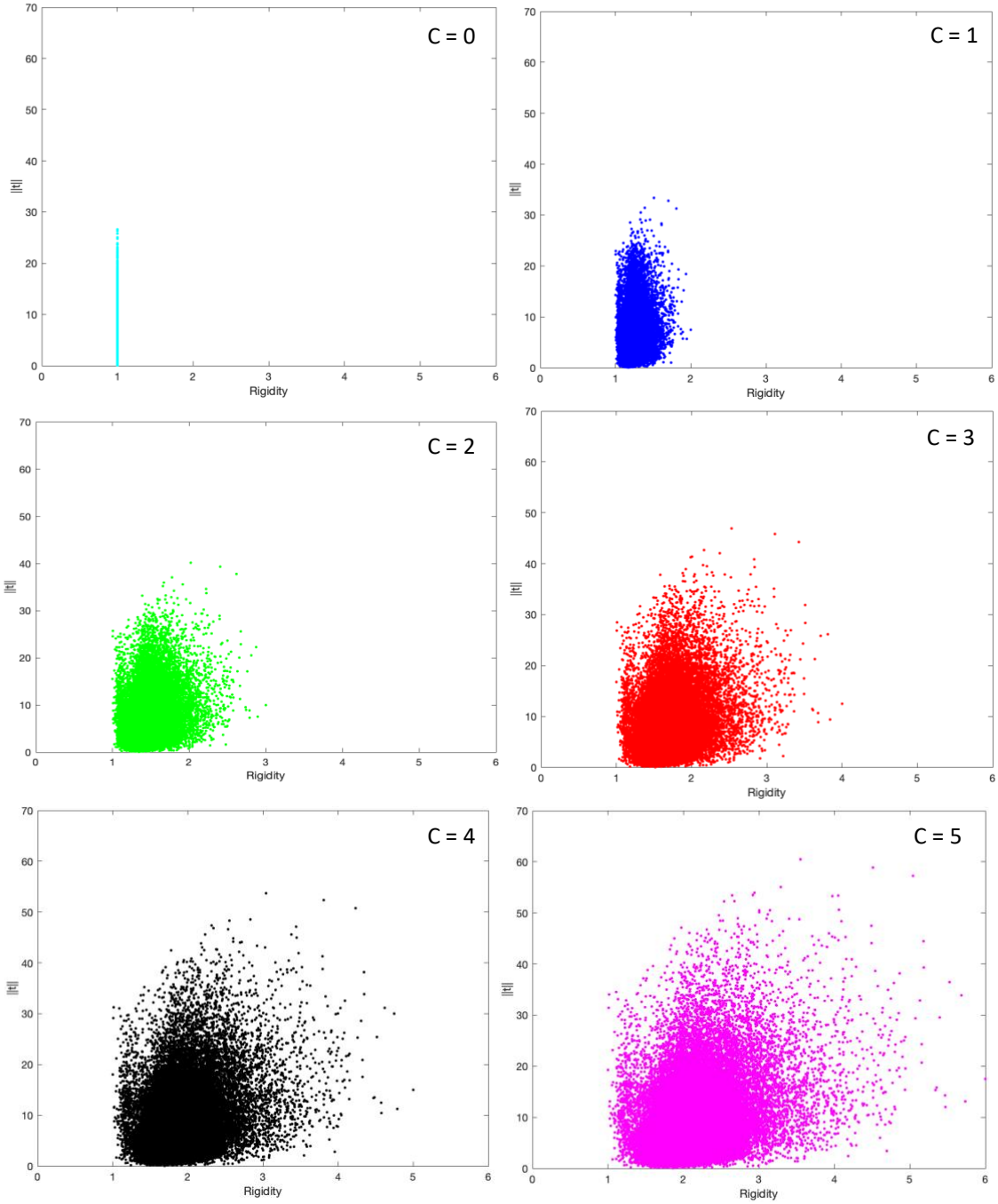

Figure 10: Tractions related to stiffness points for each element of the mesh for the different values of the coefficient C

As shown on the graphs, tractions against rigidity form a cloud of points quite scattered, pointing out that they do not correlate for C ranging between 1 and 5. On the contrary, compared to C=0, tractions and density correlates perfectly, displaying a vertical line.

2. Correlation between rigidity and displacements

This correlation, as shown on Figure 11, also presents a cloud of points, that has an L shape. The displacements, obtained with FIDVC method, are not that scattered compared to the previous correlation for C=1 (considering a heterogeneous hydrogel), but also point out like previously that there is no very poor correlation with displacements.

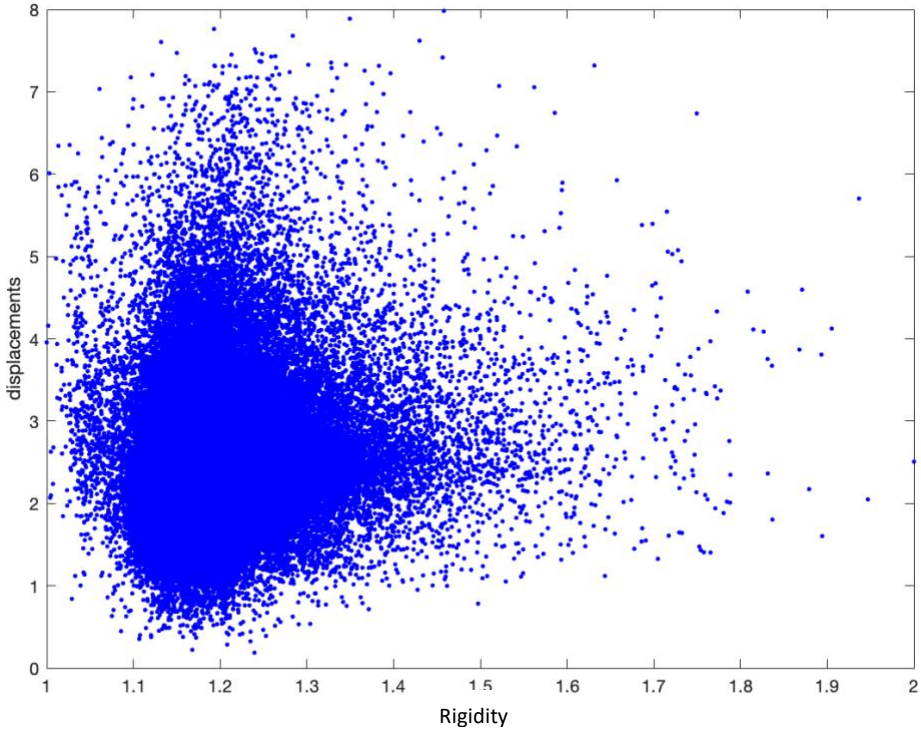

Figure 11: Displacements related to density points for each element of the mesh

To get the rigidity of the hydrogel, density of the mesh was used and a linear relationship between rigidity-density has been assumed. Considering the previous graphs, multiple rigidity-density linear relationships showed that density of the mesh is independent from tractions and displacements. But, looking at the shape of the points, that is also very pronounced for other data images in the annexes 1 and 2, in the correlation parts, two suppositions can be said:

- Low traction/displacement are obtained at high density,
- High traction/displacement are obtained at low density.

Therefore, cells might not pull stronger where a dense point is located. Indeed, it could be because of the polarization of cells along their major axis where traction forces will be higher at the extreme of that line, thus regardless of the stiffness. No conclusion can be drawn, and further analysis is necessary.

Conclusion

This report presents a modeling approach to evaluate the tractions forces across a sample of the fibrin hydrogel during vasculogenesis of endothelial cells. It involved the combination of experimental and numerical methodologies. Fluorescent images served as input for displacement and traction calculations through Matlab, and results were displayed through Paraview. The technique required the use of:

1) A fast digital volume correlation to measure displacement fields with fluorescent images,
2) Traction force microscopy using finite element model to calculate tractions,
3) Fluorescence density used to model the heterogeneous hydrogel stiffness.

The results indicate that tractions and displacements were independent from the local density of fibres in the fibrin hydrogel, and consequently from the local stiffness calculated with a linear rigidity-density relationship. To this end, a correlation between cell position and tractions could be explored in future studies. It will allow to determine whether tractions are higher where cell boundary is located or not.

However, this also opens to consider a more complex scenario than cells pulling stronger in stiffer places.

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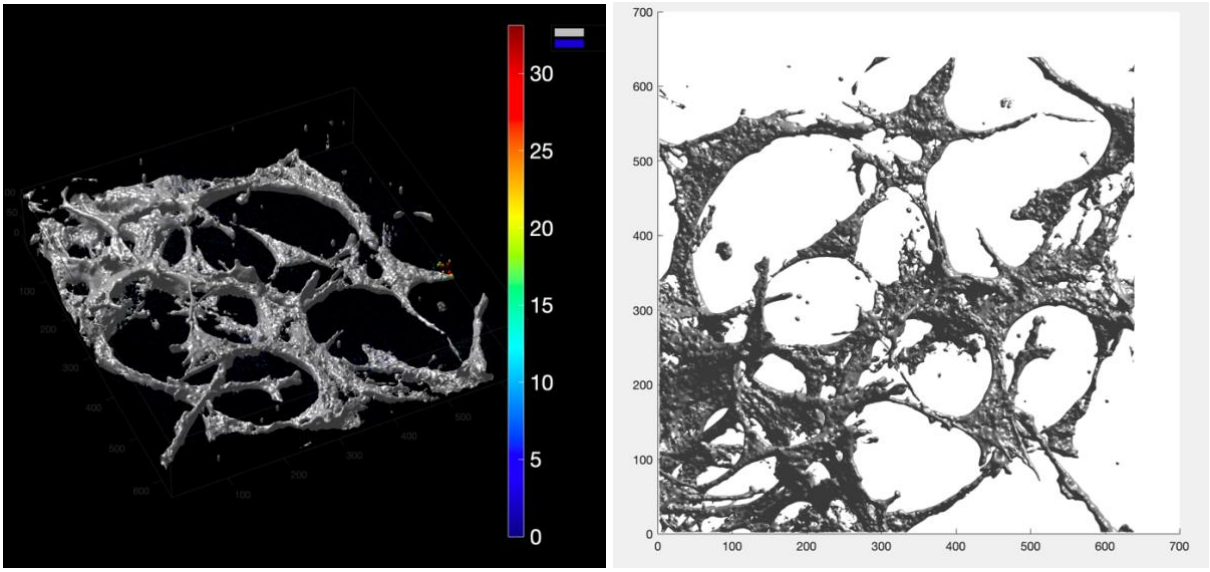
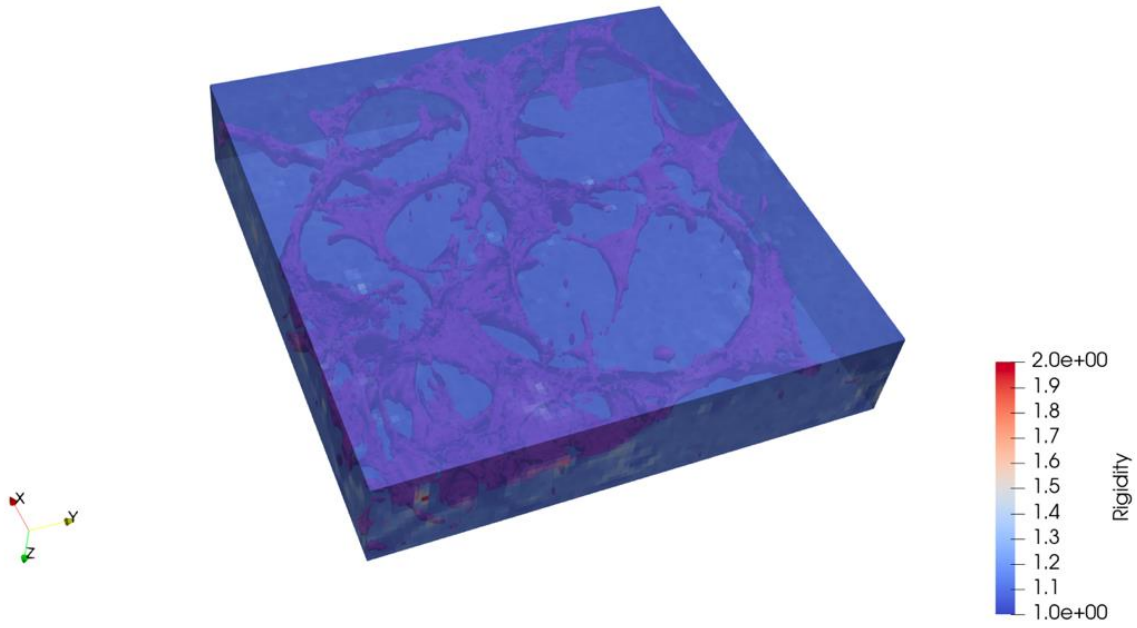
[8] The VTK user's guide – Kitware (2003)

Annex 1: Results for the sample for 3 days in culture

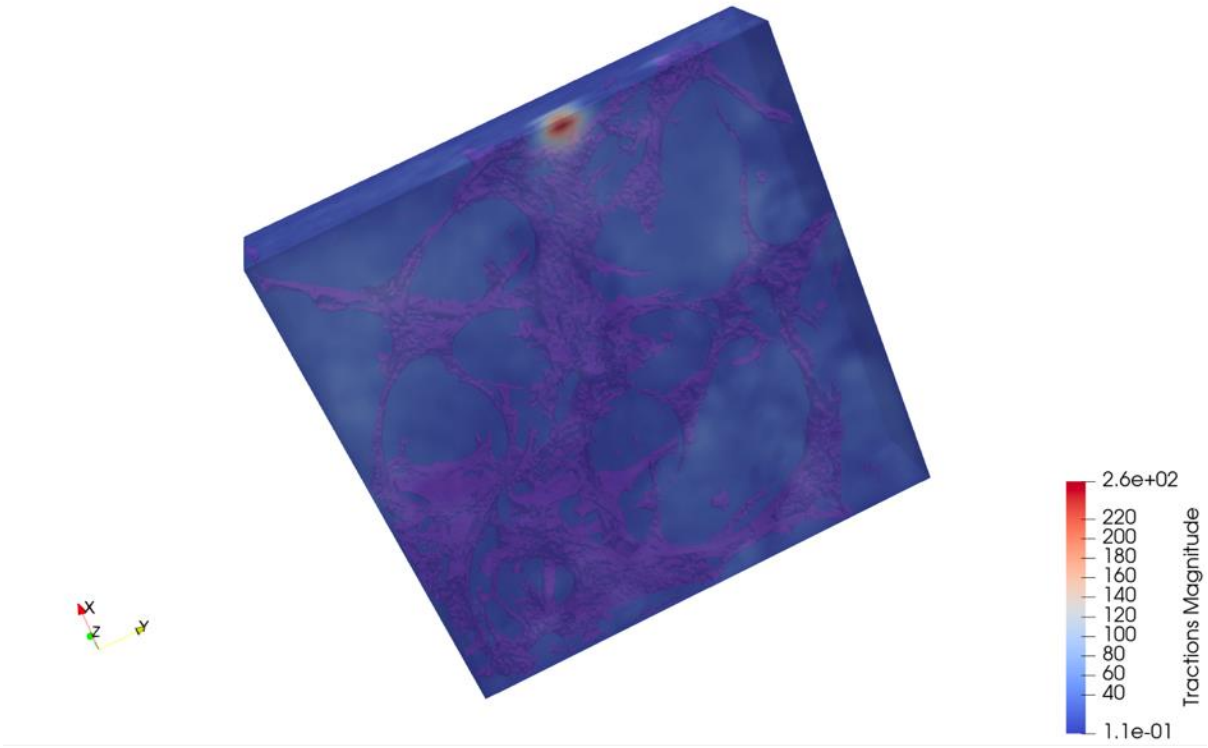
Displacement field in the hydrogel with cell surface

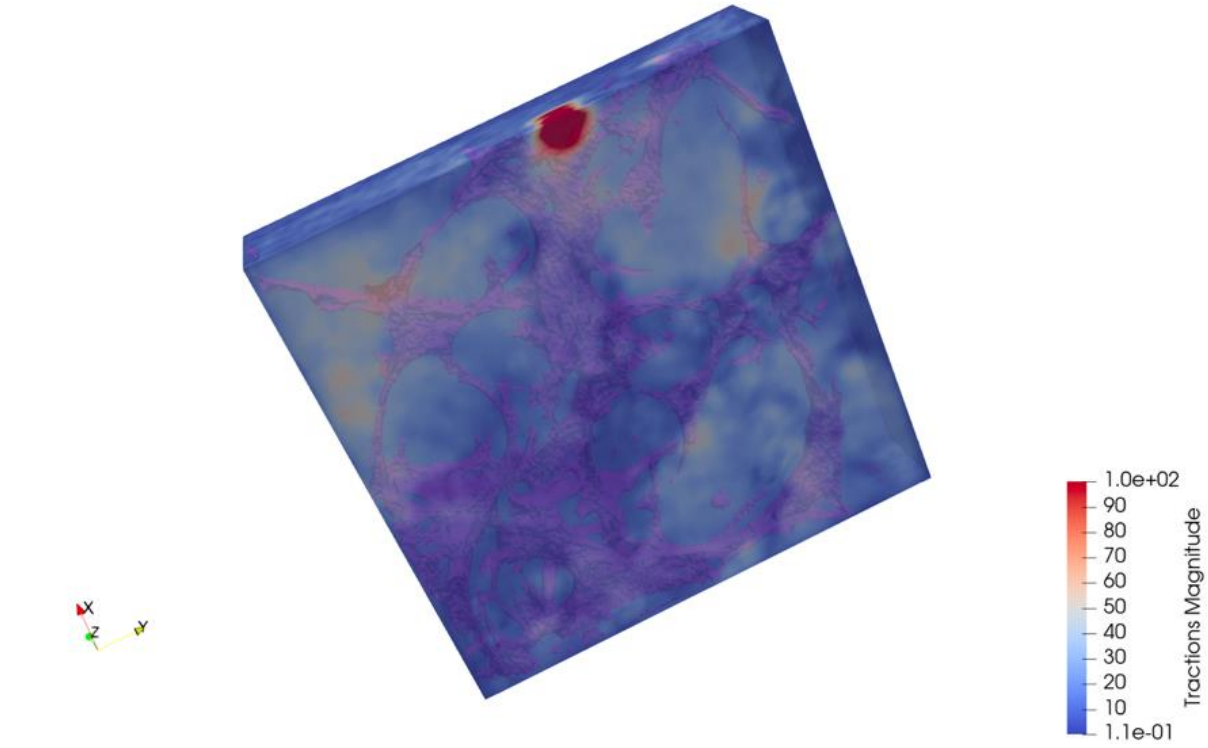


Rigidity

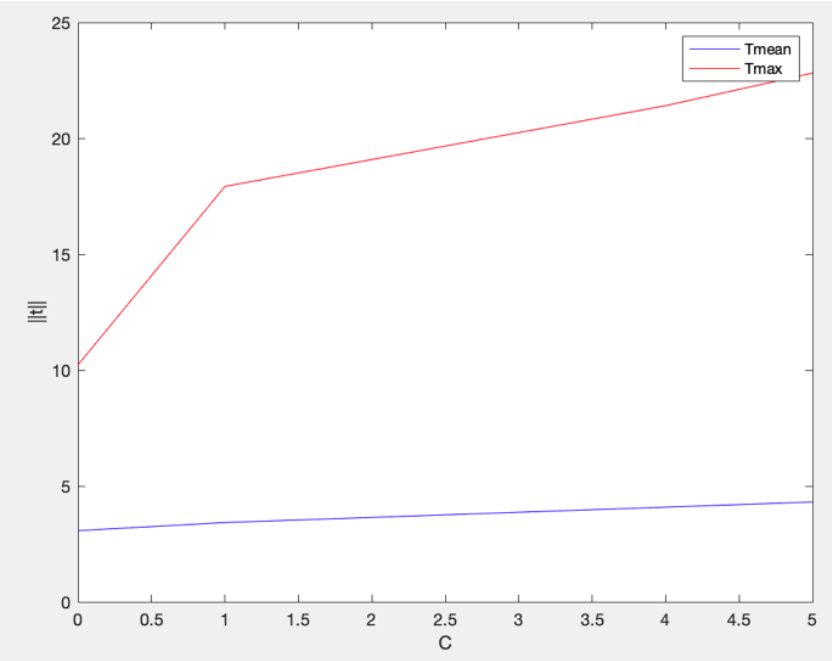


Tractions

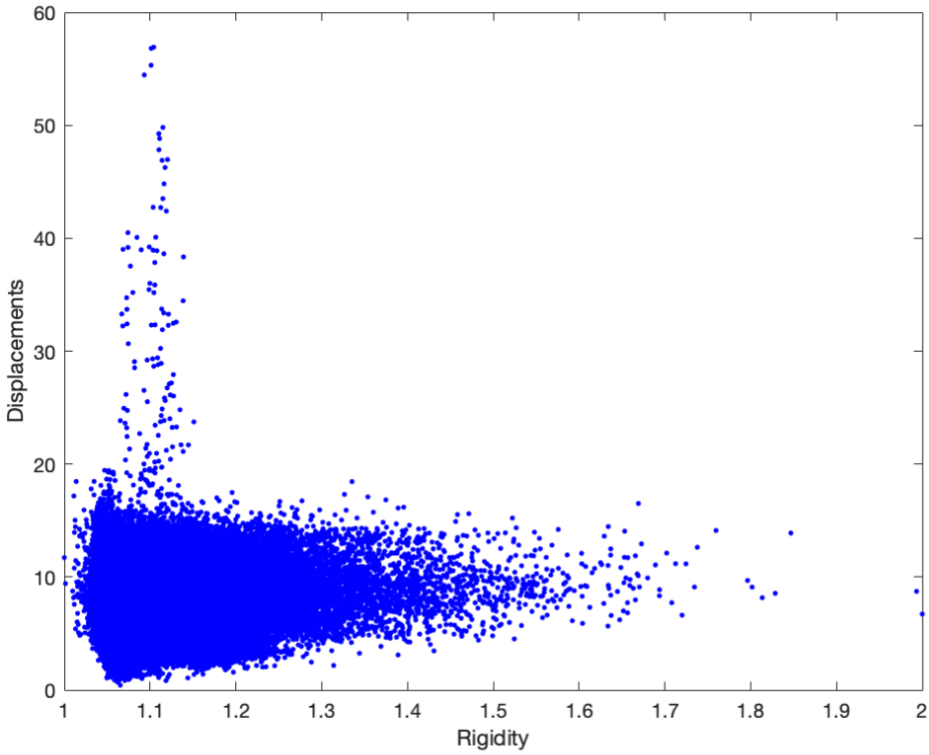

2.6e+02
220
200
180
160
140
120
100
80
60
40
1.1e-01
Tractions Magnitude


1.0e+02
90
80
70
60
50
40
30
20
10
1.1e-01
Tractions Magnitude

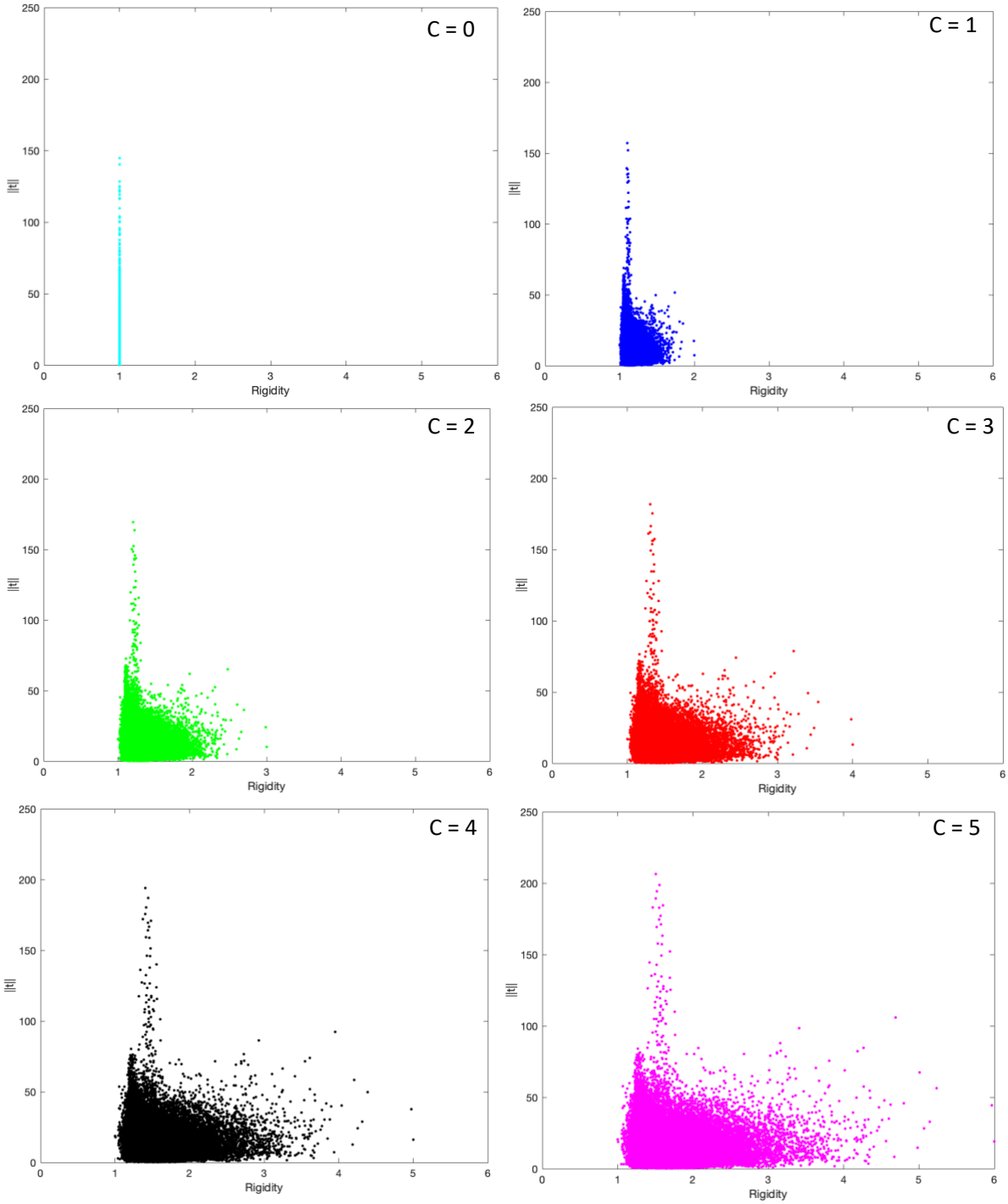
Tractions according to coefficient C from density



Correlation between displacements and rigidity

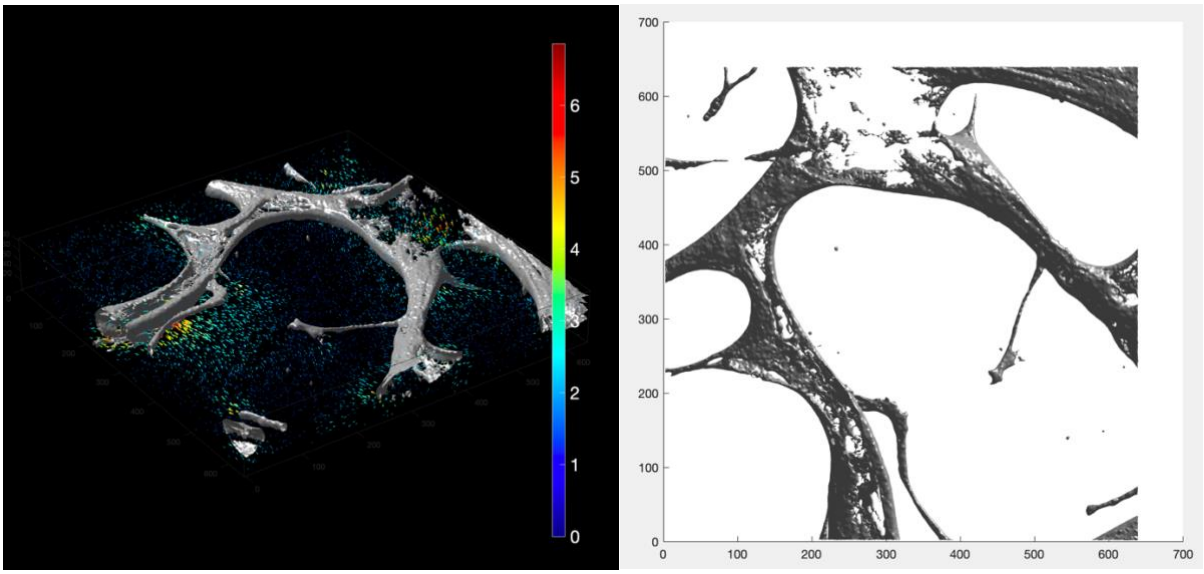


Correlation between tractions and rigidity

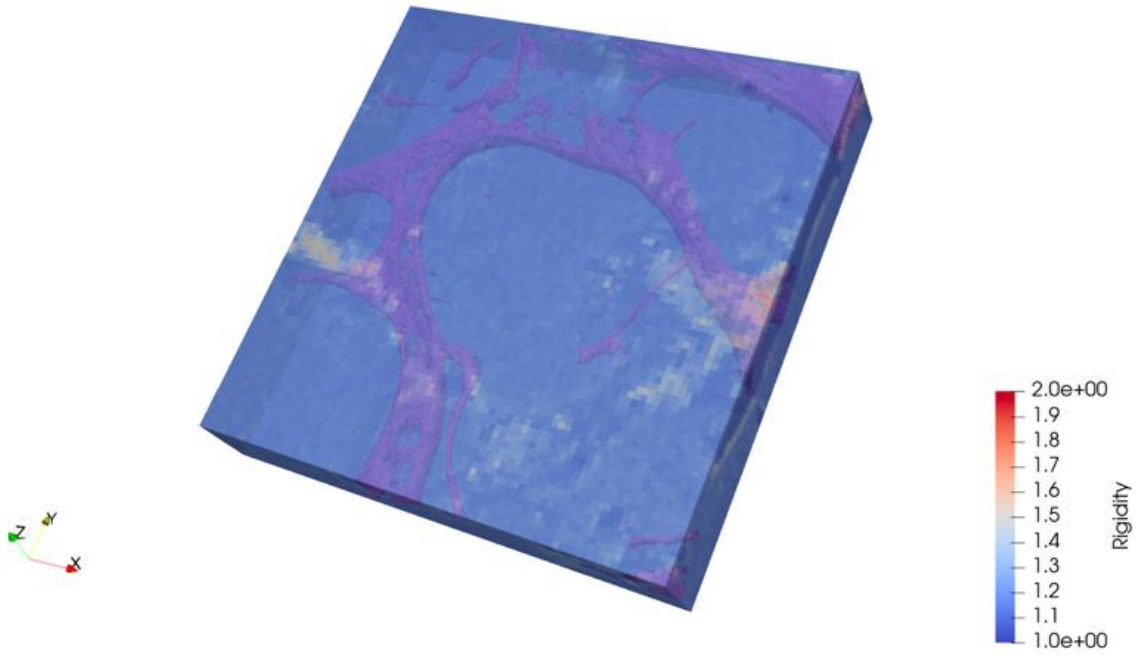


Annex 2: Results of the sample for 6 days in culture

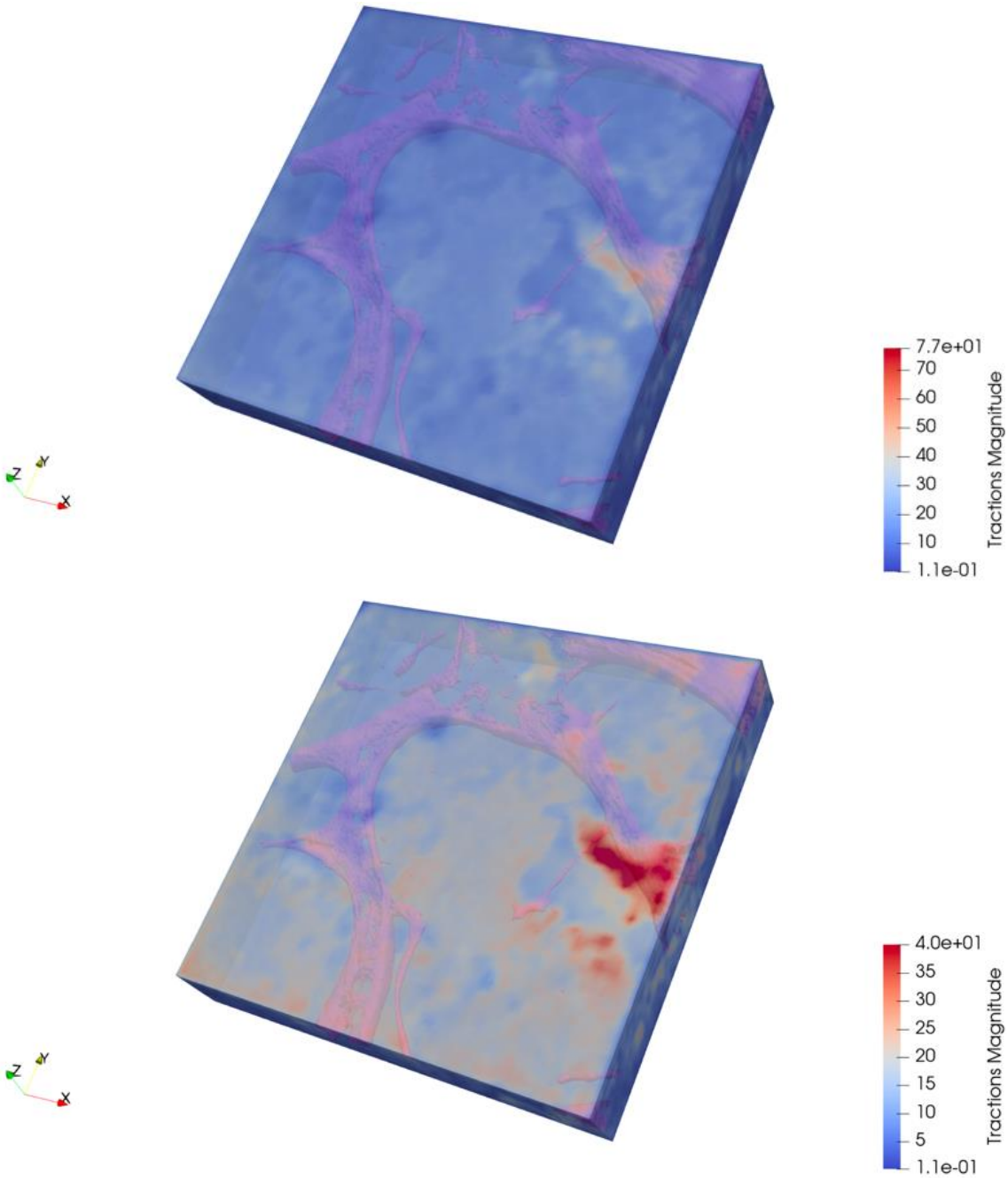
Displacement field in the hydrogel with cell surface



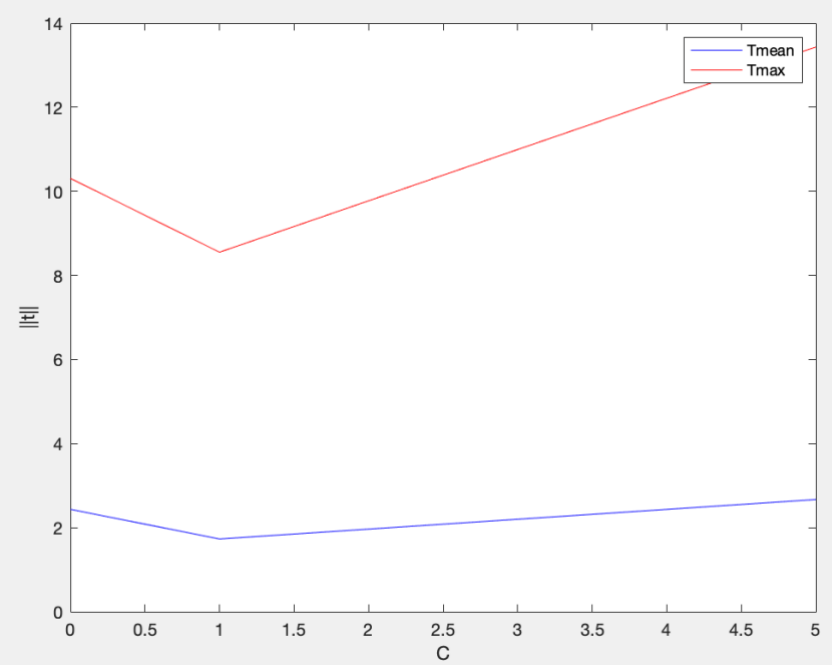
Rigidity



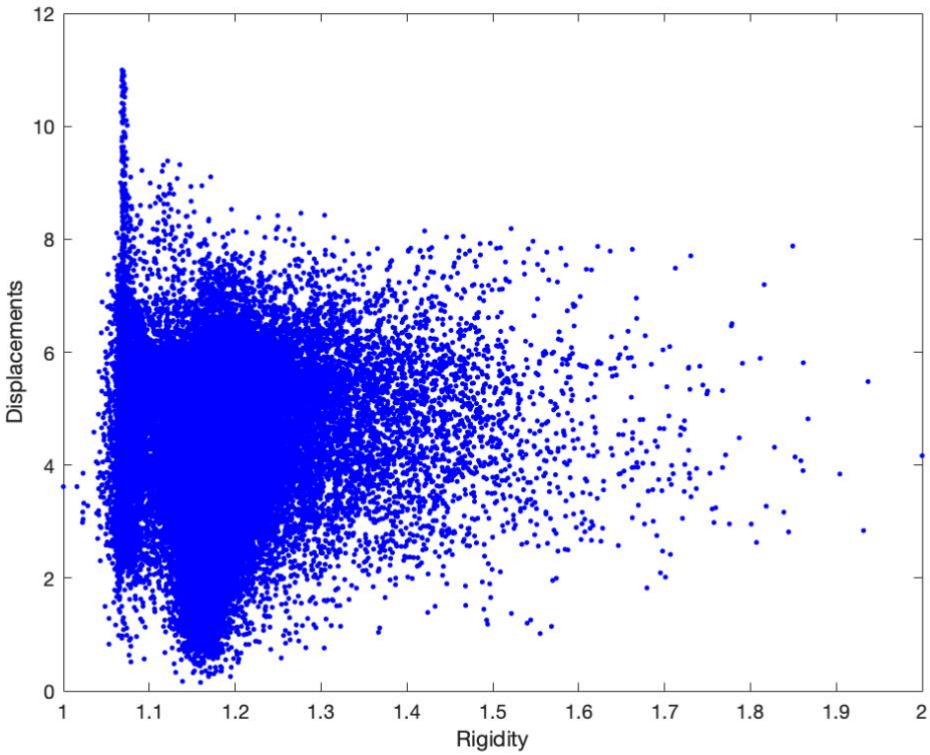
Tractions


7.7e+01
70
60
50
40
30
20
10
1.1e-01
Tractions Magnitude
Y
Z
X
4.0e+01
35
30
25
20
15
10
5
1.1e-01
Tractions Magnitude
Y
Z
X

Tractions according to coefficient C from density



Correlation between displacements and rigidity



Correlation between tractions and rigidity