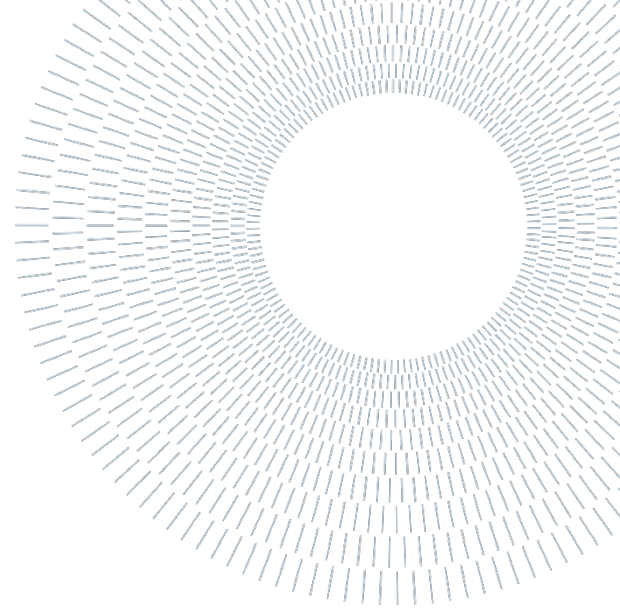




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EXECUTIVE SUMMARY OF THE THESIS

AFM based mechanical measurements on histological samples of meningioma as a novel diagnostic tool

TESI MAGISTRALE IN BIOMEDICAL ENGINEERING – INGEGNERIA BIOMEDICA

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1. Introduction

Medical statistics, as provided by the World Health Organization (WHO) and the American Cancer Society, reveal an alarming trend in the incidence of tumors [1] [2]. The incidence of meningiomas, a category of central nervous system tumors originating from arachnoid membrane cells, is steadily increasing and represents a significant portion of neurological pathologies [3] [4]. While they are often benign, they can manifest in various ways and are of great concern in neurology and oncology. Understanding their nature and properties is crucial for accurate diagnosis, appropriate treatment, and effective clinical management of patients with this condition [5] [6]. From a histological perspective, meningiomas can present in 15 different histotypes, with the most common being meningothelial and fibrous meningiomas [7].

In the first type, tumor cells form lobules surrounded by thin collagen septa. These cells resemble arachnoid cells but also exhibit morphological alterations such as oval or round

nuclei, dispersed chromatin, smooth nuclear profiles, and small, indistinct nucleoli. In the second type, cells are elongated, like normal fibroblasts, and can form extensive bundles that intertwine with each other. The amount of collagen matrix can vary among different tumors. There is also a mixed histotypes that displays intermediate histological features between the two subtypes, with a mixed lobular and fascicular conformation, often accompanied by spiral bodies. The predominant histological structure of meningiomas is often described as "mosaic architecture," characterized by groups of tumor cells surrounded by collagen fibers. However, it is important to note that significant histological variations can occur depending on the specific subtype of meningioma [8].

To assess meningioma severity and complications, three different grades are used: benign (I), atypical (II), malignant (III). Often, the pathologist needs to rely on subjective evaluation based on his/her experience and qualitative parameters to make diagnosis and prognosis [9]. Timely diagnosis offers numerous advantages, including less invasive treatments, more therapeutic options,

higher chances of recovery, reduced patient impact, lower healthcare costs, and improved clinical outcomes [10].

The introduction of new tools and measurement methods that provide quantitative parameters represents a highly valuable support to the pathologists in their clinical decision-making. This project addresses the measurement of mechanical properties and topographical features of meningothelial tissue samples as quantitative biomarkers to assess the pathological state and differentiate between different histotypes.

The rationale relies on the fact the mechanical behavior and surface characteristics of tissues can provide insights into their (patho) physiology, and that this understanding has the potential to enhance diagnostic accuracy, improve treatment strategies, and enable a deeper understanding of the underlying mechanisms of diseases. This relies on the fact the understanding the mechanical behavior and structural characteristics of tissues at the micro- (i.e., cellular) scale and nano- (i.e., molecular) scale can provide novel and complementary insights in the fundamental mechanisms of tissue (patho) physiology.

The specific aim of this work is therefore to provide experimental evidence that histological analysis based on structural and mechanical parameters obtained at the micro- and nano-scale by means of atomic force microscopy (AFM) can provide complementary and clinically relevant quantitative information on different types of brain tumors [11].

Thanks to the collaboration with Professor Valerio Vellone (University of Genova) and his team at the Pathology Department of the Giannina Gaslini hospital, three different types of meningioma have been selected as the candidate samples to investigate with the proposed methods [12].

Specific AFM operating modes and probe types were first selected, as well as the most effective experimental conditions for investigating samples that had already undergone the standard histological sample preparation procedure. AFM measurements were then conducted, and the data were analyzed to address the sampling issues, aiming to obtain statistically significant results that could be used as biomarkers representative of a specific histotypes [13] [14].

2. Materials and Methods

2.1. Sample Preparation

Three different samples representing three different types of meningioma were analyzed: meningothelial, fibrous, and mixed. Various sample preparation techniques exist, depending on the specific experiment objectives. For digital image-based histological preparation there is a standardized and well-established process (since 1908). After surgical excision from the patients, tumor samples are sent fresh to the anatomical pathology unit. There, they are fixed in 10% buffered formalin for 12/18 hours and subsequently processed with an automated and standardized procedure for embedding in paraffin.

Routinely, paraffin blocks are sectioned at 3 μm thickness using a microtome, followed by deparaffinization, rehydration, and treatment with specific stains to highlight structures or cellular components. Hematoxylin-eosin staining, capable of coloring cell nuclei in bluish-violet (hematoxylin's basophilic components) and cytoplasm and reticular fibers of the extracellular matrix in pink (eosin's acidophilic components), is the most common one and used in for this work.

This process was performed automatically by the Tissue-Tek Prisma® Plus machine, which, through various steps involving high-concentration alcohol and Histo-C clearing agent, prepare the sample for the next step. Once the histological sections had been prepared, they were observed using digital microscopy [15]. For AFM analysis, we repeated the same standardized procedure described earlier, excluding staining.

However, to avoid substrate effects from indentation measurements, 20 μm -thick sections

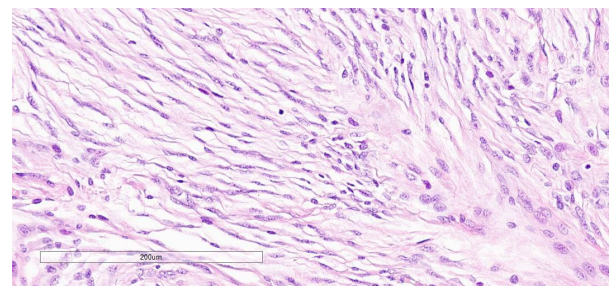


Figure 2.1 – fibrous sample digital microscopy

were cut and subsequently placed on a glass slide. Once the samples were appropriately prepared, AFM was used to scan the sampled surface.

Sample preparation was further completed by hydrating the histological sample for 30 minutes using a Phosphate Buffered Saline (PBS) solution, a buffer saline solution used to maintain the sample in a physiologically compatible environment. After measurements were taken, the sample was carefully cleaned using deionized water. This cleaning phase is crucial to remove any surface residues or contaminants that could affect subsequent measurements.

Subsequently, the sample was placed under a laminar flow hood. This structure is designed to ensure a sterile environment devoid of suspended particles, providing controlled airflow that prevents foreign particles from contaminating the sample. In this environment, the sample was allowed to completely dry.

Despite meningioma samples not consisting of fresh tissue, all measurements were conducted within a 48-hour period from the time of meningioma sectioning. This timing was carefully planned to ensure that the samples remain in an adequate state for AFM analysis, minimizing any changes. Every effort was made to preserve the integrity of the samples and obtain valid and representative data for the analysis of meningioma mechanical properties.

2.2. AFM Measurements

The measurements were conducted using a commercial AFM system (Bioscience AFM Nanowizard4, Bruker Corp.) coupled with an optical microscope (AxioZoom V.16, Zeiss) and were performed at room temperature in three different modes: Quantitative Imaging, force spectroscopy, and micro-rheology, each tailored to examine specific topographical and mechanical aspects of the samples:

- *Quantitative Imaging*: This mode allows for obtaining high-resolution images of the surface topography of the sample. This enables the identification of any differences in the surface structure of the tissue, such as roughness or cell shape. Furthermore, it can be used to generate maps of mechanical properties, such as sample stiffness. For these measurements, a commercial probe, the PFQNM-LC-CAL model (Bruker Corp.), with a paraboloidal tip and a tip radius of

70 nm, equipped with a cantilever with a known elastic constant of 0.073 N/m, was used. Three maps were acquired for each sample over a scanning area of 20x20 μm^2 while applying a maximum force set-point of 4 nN. This mode provided topography images that enabled the creation of a 3D representation of the surface and stiffness images, where contrast represents variations in stiffness that can be correlated with surface structure [16].

- *Force spectroscopy*: This mode aimed to measure the force interactions between the AFM tip and the meningioma sample. By allowing a detailed assessment of the forces acting between the tip and the sample surface, force spectroscopy can be used to study tissue stiffness, adhesion, and other mechanical properties.

To achieve the proposed objectives, two-commercial probes were employed:

1. *Biosphere B2000-FM*: This probe had a 2 μm radius, a cantilever with an elastic constant of 2.571 N/m, and applied a force of 500 nN to the sample at a velocity of 2 $\mu\text{m/s}$;
2. *SAA-SPH-5um*: This probe had a 5 μm radius, a cantilever with an elastic constant of 0.191 N/m, and applied a force of 20 nN to the sample at a velocity of 5 $\mu\text{m/s}$.

The first probe had greater stiffness and was used to explore the sample in-depth, providing detailed information about its mechanical properties. Thanks to its stiffness, combined with the smaller radius, it could penetrate deep into the sample, ranging from 300 nm to 400 nm, allowing us to obtain specific data about its composition and strength.

On the other hand, the second probe had lower stiffness and a significantly larger radius, making it suitable for more mediated measurements, considering the lateral geometric effects of the sample. Its lower stiffness and larger radius allowed for a more macroscopic measurement, evaluating the sample laterally with an indentation depth ranging from 50 nm to 150 nm.

Using the Biosphere B2000-FM, 30 maps were acquired on a randomly chosen fibrous histological sample. After observing that 3 maps were sufficient to characterize the sample, 3 maps were taken for each sample at two different time points: within 48 hours of paraffin removal and at a 2-month interval to assess aging effects.

This approach was motivated by the fact that acquiring high-resolution indentation maps is a time-consuming process. Therefore, after performing 30 maps of 15x15 pixels on the fibrous histological sample, selected from different areas of interest, the statistical dispersion of elasticity values was evaluated. It was concluded that a statistics-based approach using only three maps with the same number of curves (i.e., elasticity values) randomly selected from the 30 measured maps maintained similar statistical properties in terms of mean value and dispersion. This allowed for reduced measurement times for the other two samples, which was an important constraint in the context of the thesis work. An additional three maps of 15x15 curves each for each sample at two different time points from paraffin removal were obtained:

- within 48 hours of rehydration: These maps were acquired shortly after paraffin removal, ensuring that the samples were still fresh and not significantly affected by aging processes;
- two months after rehydration: In this phase, three maps were taken for each sample, two months after paraffin removal. This time interval was chosen to assess the effects of aging on the samples and understand if there were significant variations in mechanical properties over time.

This choice of acquiring maps at two distinct time points allows for a thorough examination of how meningioma sample characteristics may vary over time and how this may affect measurements made through AFM, micro-rheology, and force spectroscopy.

With the SAA-SPH-5um tip, the geometric laterality of the sample was analyzed, using significantly larger maps of 100x100 μm^2 , consisting of 20x20 pixels.

The indentation curves were analyzed using the Sneddon model developed for spherical indenters, as described in *paragraph 3*:

$$F = \Phi(a) \equiv \frac{E}{1-\nu^2} \left[(R^2 + a^2) \operatorname{artanh} \frac{a}{R} - aR \right] \quad (3.1)$$

$$\delta = \Theta(a) \equiv a \operatorname{artanh} \frac{a}{R} \quad (3.2)$$

The combined use of two probes with different characteristics, one with a 2 μm radius and higher stiffness, and the other with a 5 μm radius and slightly reduced stiffness, provided a comprehensive and detailed insight into the mechanical properties of meningiomas.

- *micro-rheology*: this mode was employed to analyze the mechanical and rheological properties of meningiomas at the microscopic level. It allowed for the measurement of tissue response to controlled dynamic deformations and applied forces, assessing its elasticity and viscosity [17] [18]. This mode was conducted using the Biosphere B2000-FM probe described earlier. The maximum loading force applied was fixed at 500 $n\text{N}$. With this force, an indentation depth ranging approximately between 200 nm and 400 nm was achieved, depending on the intrinsic mechanical differences of each sample. These indentation curves were also analyzed using the model developed for spherical indenters.

Three maps were generated for each sample, each with dimensions of 10x10 μm^2 and divided into a 4x4 pixel grid. This procedure was repeated for both the fresh and two-month-aged samples to compare their mechanical characteristics. Additionally, 14 different frequencies were tested on a logarithmic scale to comprehensively assess the mechanical properties of the samples. The curve analysis was performed using the instrument's commercial software (*JKSPM data processing*), which provided values for Storage Modulus (E'), Loss Modulus (E''), Shear Storage (G'), and Shear Loss (G'').

3. Results

3.1. Imaging

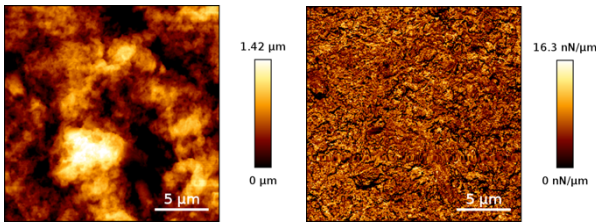


Figure 3.1 and 3.2 – fibrous histological sample

By performing scans on areas measuring $20 \times 20 \mu\text{m}^2$, it was possible to identify cellular components within the mixed and meningothelial samples. This presence is confirmed by the stiffness images of the sample shown in Chapter 5.1 of the thesis. However, despite this cellular presence, the distribution of stiffness in the fibrous histological sample was found to be lower compared to the other histological samples. Regions of interest were carefully selected, comprising entirely neoplastic areas devoid of preparation artifacts or native structures.

3.2. Nanoindentation

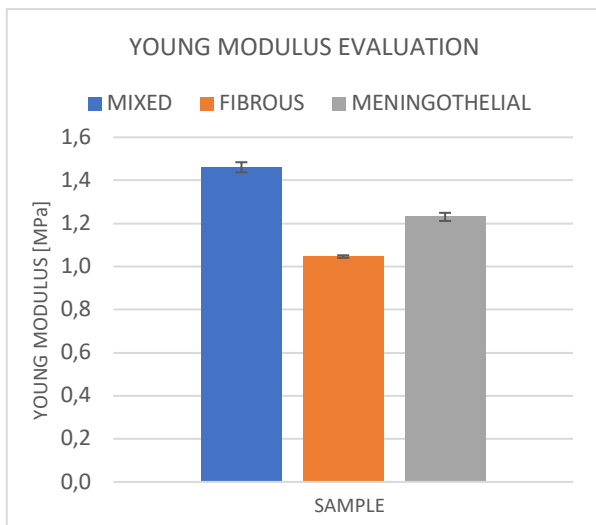


Figure 3.3 – with Biosphere B2000-FM probe.

In the figure, the results of the analysis are presented after using the Biosphere B2000-FM probe, enabling us to explore the sample with an indentation depth of approximately 400 nm. The outcome revealed a reduced Young's modulus of the fibrous histological sample compared to both the mixed and meningothelial samples.

In the subsequent image (3.4), there is an analysis with the SAA-SPH-5um probe, which allowed for an exploration of the sample's lateral characteristics.

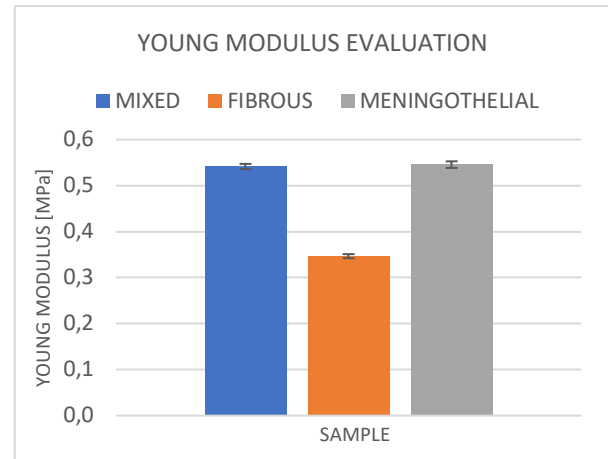


Figure 3.4 – with SAA-SPH-5um probe.

The results showed a lower Young's modulus in the fibrous histological sample compared to the other two types, namely the mixed and meningothelial samples.

3.3. Micro-rheology

For the microrheological analysis, the Biosphere B2000-FM probe was employed. The results of this analysis, as exemplified in Figure 3.5, demonstrate that the values of the analyzed moduli E' , E'' , G' , and G'' are lower in the fibrous histological sample, followed by a higher modulus in the meningothelial sample, and finally, the highest modulus in the mixed sample. It is important to emphasize that this trend consistently held true at every analyzed frequency. This suggests that differences in mechanical properties among the various histological samples were robustly observed across various frequency scales, providing a comprehensive view of the intrinsic differences among the samples.

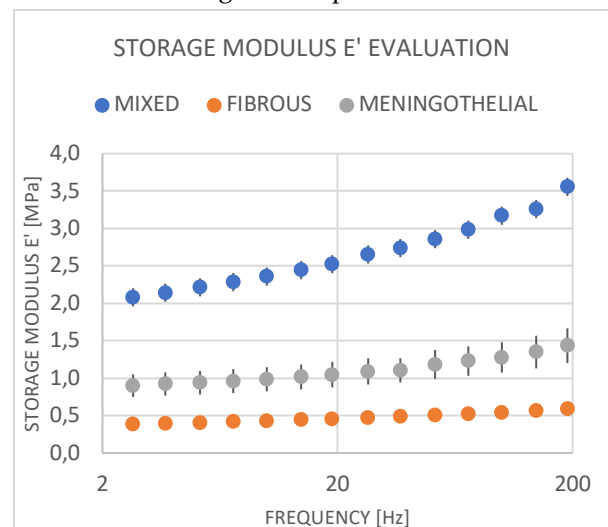


Figure 3.5 - with Biosphere B2000-FM probe.

3.4. Measurement protocol validation

In this work, the primary issues addressed were those that might arise when proposing the use of this AFM method for clinical diagnostics. Specifically, the focus was on assessing the representativeness of the obtained parameters and understanding how they were influenced by the sample preparation process.

To assess the homogeneity or heterogeneity of the samples at the probed scale (with the lateral scale determined by the probe dimensions, which are on the order of a few micrometers in this study), one of the three specimens was sampled in 30 randomly selected $15 \times 15 \mu\text{m}^2$ areas, specifically in regions marked as "of interest" by the pathologists. This process involved collecting a total of 6,750 indentation curves and analyzing the statistical distribution of the corresponding 6,750 elasticity values.

The distribution histogram of such values showed a single peak could be well fitted by a gaussian. Interestingly, when compared with the distribution obtained from other (independent) indentation measurements taken on the sample in three different areas (for a total of 675 curves), a similar mean value and standard deviation were observed. Based on this observation, it was assumed that the number of measurements conducted on the three specimens represented their elastic properties.

The second concern, which involved examining how sample preparation influenced the analysis results, considered the effects of sample aging following the removal of paraffin and the subsequent rehydration process.

A study was conducted two months after the removal of paraffin, utilizing the Biosphere B2000-FM probe, following the same experimental conditions as previously described.

In force spectroscopy, it was found that the Young's modulus of the fibrous histological sample is lower compared to that of the meningotheial sample. An absolute increase in Young's modulus was observed between the samples analyzed within 48 hours and those analyzed after 2 months. This increase was associated with an overall stiffening of the mechanical response of the samples with aging. In

this case, the mixed sample exhibited lower stiffness compared to the other samples and a lower Young's modulus than in the analyses within 48 hours. This phenomenon suggests that aging may have caused a simultaneous hardening of the samples along with an increase in fragility in the analyzed areas of the mixed sample.

In the micro-rheological mode, it was found that the Young's modulus of the fibrous histological sample remains lower compared to that of the meningotheial and mixed samples, confirming the observations made during the analysis within 48 hours. Additionally, there was an increase in all values of E' , E'' , G' , and G'' .

4. Conclusions

Different results were obtained based on the chosen techniques:

- **Nanoindentation:** From sampling of samples in different regions at two penetration depths, it became evident that the fibrous histological sample exhibit a significantly lower Young's modulus compared to the meningotheial and mixed histological samples.
- **Micro-rheology:** The values of calculated indices, including Storage Modulus (E'), Loss Modulus (E''), Shear Storage (G'), and Shear Loss (G''), consistently show lower values for the fibrous sample compared to the others, regardless of the frequency.
- **Aging effects:** Through the observation of histological samples, repeating the same spectroscopy and micro-rheology measurements, a significant increase in stiffness was observed compared to "fresh" samples, i.e., analyzed within 48 hours from paraffin removal. Specifically, the fibrous histological sample exhibited a lower Young's modulus for both force spectroscopy and micro-rheological measurements compared to the meningotheial histological sample. The mixed histological sample showed signs of aging, suggesting a possible increase in intrinsic fragility.

These results collectively indicate a marked variation in the mechanical properties of histological samples in relation to their composition and some effect of being exposed to humid air (aging). The lower stiffness of the fibrous sample compared to other sample types suggests intrinsic differences in nanostructure and tissue composition, and aging appears to significantly influence their mechanical response. Different histological types of meningioma have shown significantly diverse biophysical characteristics. Histological appearance does not fully explain the differences observed in these analyses, which need to be correlated with the molecular characteristics of the cytoskeleton of different cell types, the expression of intercellular adhesion molecules, and interactions between the cytoskeleton and the extracellular environment.

The results obtained from these analyses, though preliminary, indicate an encouraging initial stride towards the development of new tools and methods for histological sample classification. These advancements hold the potential for practical applications in meningioma pathology and, more broadly, within the realm of clinical diagnostics.

5. Bibliography

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