SB³C2023
Summer Biomechanics, Bioengineering and Biotransport Conference
June 4-8, Vail, CO, USA

ANISOTROPIC STIFFNESS MEASURED USING A TOROIDAL PROBE IN MESO LEVEL AND CELL LEVEL

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INTRODUCTION

Characterizing the mechanical properties of cells and biological tissues is of critical importance as the mechanical properties reflect the physiological state and integrity of tissues and may serve as hallmarks of diseases such as cancer, calcification, and atherosclerosis (1,2). While most biological tissues and many cell types are highly mechanically anisotropic, many studies, especially studies in microscale and mesoscale, assume samples to be mechanically isotropic often due to the technical difficulties in measuring anisotropic stiffness of cells and microtissues. This oversimplification does not accurately reflect the mechanical properties of cells and micro tissues and may bias the interpretation in tissue mechanics and mechanobiology.

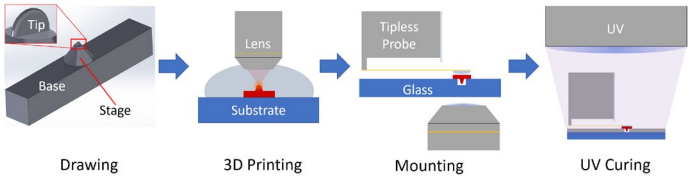
Indentation is one of the most common methods for measuring mechanical properties of biological tissues and cells. Conventional indenters are equipped with spherical, conical, or pyramidal probes which do not yield information related to mechanical anisotropy. Several recent studies have proposed solutions to this problem. However, these methods require complicated image analysis or samples to be prepared into specific shapes (3,4,5). Moreover, most studies are focused on macro level tissue properties; studies on the mesoscale and cell level are limited. A straight-forward indentation method for the measurement of anisotropic mechanical properties is needed.

Here, we propose a method to measure anisotropic stiffness of microtissues and cells by two indentations in orthogonal directions using our novel toroidal probe. Our preliminary results indicate that this approach is applicable in measuring anisotropic stiffness of aligned tissues and cells. This method will provide researchers with a simple and cost-effective means for measuring mechanical anisotropy of micro-scale samples.

METHODS

The mesoscale toroidal probe tip was designed to be 20 µm in cross-section radius and 100 µm in ring radius, the microscale probe tip was designed to be 2 µm in cross-section radius and 10 µm in ring radius. The tip was manufactured using a Naonoscribe GT two-photon 3D printer (Fig. 1). Briefly, the probe tip with supporting stage and base was created in CAD then translated to executable codes with printing parameters optimized for smoothness of the tip surface. A drop of negative photoresist resin was applied onto the cleaned substrate mounted on the device. In the printing, the two-photon laser scans over 3D space according to the code to polymerize the resin at the focal point. After printing, the base was detached from the substrate using a fine tweezer and flipped over under the microscope. A tipless probe was mounted to the nanoindenter (Optics11, Amsterdam), dabbed with UV-curing glue at the end of the cantilever, then lightly touched the bottom of the base. The glue was cured under the UV light for 30 minutes (Fig. 1). The probe was visually inspected using optical and scanning electron microscopy (Fig. 2).

Figure 1. A schematic brief procedure of manufacturing the toroidal probe tip



Chicken breast was used for meso-scale indentation study. The sample was dissected and transferred to a 100 mm-petri dish filled with PBS (Fig.3, left). Porcine aortic valvular interstitial cells (PAVICs)

were used in cell indentation study. To grow aligned cell monolayers, parallel scratches were made in 35-mm petri dishes, 100,000 cells were seeded. And the cells were cultured for 6 days to form an anisotropic monolayer (Fig.3, right).

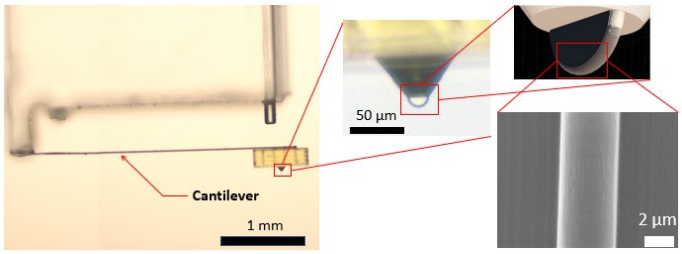


Figure 2. Optical and scanning electron microscopy images of microscale probe and its toroidal tip. The probes were manufactured in accurate size, shape, and smooth surface.

Before indentation, the chicken breast was lightly pressed by a tweezer to avoid floating. Indentation speed was set to be 10 µm/s until 50 µm max indentation depth. For the cell sample, the cell culture media was changed to complete L-15 medium to maintain a stable pH. The cells were aligned to the probe under a Nikon TS-100 inverted microscope. Indentation speed was set to 1 µm/s, with 500 nm maximum indentation depth. The force-indentation curves were recorded and fit to the Hertzian model for elliptical contact, in which the effective Young's modulus (E*) is calculated by

$$E^* = \frac{3F}{4d^{3/2}R_e^{1/2}} f_2^{3/2}(e) \qquad (1)$$

where d is indentation, F is force; $R_e = \sqrt{R_1 R_2}$ is the equivalent radius of the probe tip; $f_2(e)$ is an empirical function of the eccentricity of the ellipse (~1 for an aspect ratio of 5) (6). Two chicken breasts and one PAVIC monolayer were indented at multiple sites along (0°) and across (90°) the fiber direction. For PAVICs, the indentation data of top 50% stiffness was filtered to rule out the samples affected by substrate effect. Statistical comparisons were made using t-test, $p < 0.05$ is considered significant.

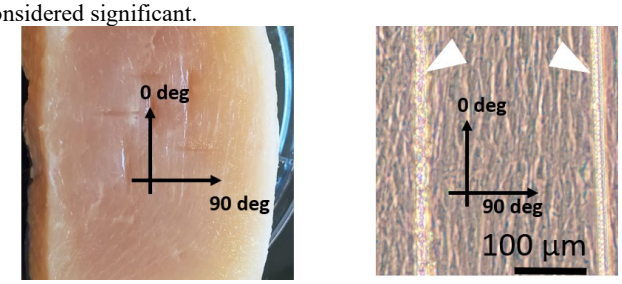


Figure 3. Anisotropic biological materials tested. Left: chicken breast shows clear fiber orientation. Right: PAVICs align in the direction of the scratches (arrowheads) to form an aligned monolayer.

RESULTS

As predicted, chicken breast samples have a higher effective Young's modulus when the long axis of probe is perpendicular to the fiber direction of the chicken breast (90°) compared to the indentation in parallel direction (0°) on both samples ($p<0.05$). In the two samples, the moduli across the fiber direction were 3.45 ± 2.12 kPa, and 3.70 ± 2.17 kPa, while the moduli at 0° were 2.35 ± 1.27 kPa and 2.17 ± 1.28 kPa (Fig. 4, left).

Similarly, for the indentation test on PAVICs, we found a trend toward higher modulus at 90° (7.22±2.01 kPa) than at 0° (5.84±1.86 kPa) ($p=0.07$, n.s.) (Fig. 4, right).

DISCUSSION

Measuring the anisotropic properties of biological materials is not readily possible with indentation testing, especially at the cell level. Previous studies trying to resolve this problem are mostly limited to macro samples, and commonly require high-resolution live imaging to measure the non-uniform strain field or samples of specific shapes (3-5). The one study to date reporting a measure of cell anisotropy using indentation requires live confocal microscopy and inverse finite element analysis (4) Here, we present a method to measure the mechanical anisotropy of cells and tissues by two orthogonal indentations without the need for microscopy or sample of specific shapes which allows for rapid measurement of samples *in vivo*. Importantly, our method can be performed by most of the existing indenters and AFMs at a low cost. The cost of each probe tip is less than $5 for the mesoscale probe, and $40 for the micro probe.

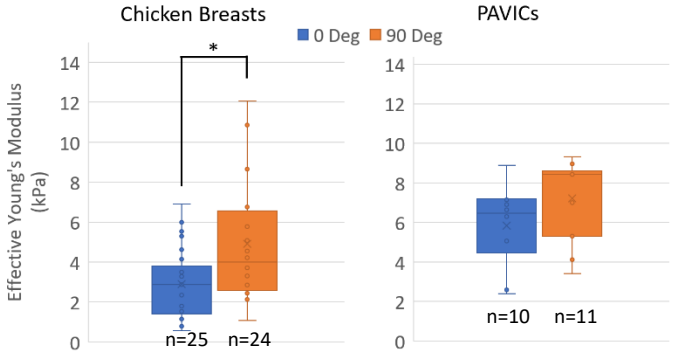


Figure 4. Effective Young's modulus of a chicken breast (left) PAVICs (right) measured in two orthogonal directions (*p<0.05).

In this study, we used chicken breast and PAVICs as the samples. Both samples show higher effective Young's modulus across the fiber direction than along the fiber direction. This result is consistent with previous studies (4,7). While indentation on chicken breast shows significant difference in two directions, the difference is not significant in the case of PAVICs. This may reflect a different degree of anisotropy of PAVICs and chicken breast or simply the low sample size in these pilot tests. Probes of higher anisotropy ratio may be needed for samples with lower degree of anisotropy.

One limitation of this study is that the modulus is calculated based on Hertzian contact theory which assumes materials isotropy and linear elasticity. To our knowledge, there is no analytical solution for the indentation problem on anisotropic materials. In the next step of the study, we plan to apply FE modeling to extract the mechanical parameters based on the indentation curves either with inverse methods or with forward modeling and machine learning.

In conclusion, we created a novel method for measuring anisotropic stiffness of biological samples. This method is simple, cost-effective, and can measure small samples down to the cell level. We expect this method will be of use to researchers in biomechanics, biophysics, and mechanobiology.

ACKNOWLEDGEMENTS

This work was supported by the NSF (CMMI 1761432) and ARMI BiofabUSA (T0137).

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