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Measuring the micromechanical properties of embryonic tissues

Nicolas R. Chevalier [a,*], Elodie Gazguez [b,c,d,1], Sylvie Dufour [b,c,d,1], Vincent Fleury [a]

[a] Laboratoire Matière et Systèmes Complexes, Université Paris Diderot/CNRS UMR 7057, 10 rue Alice Domon et Léonie Duquet, 75013 Paris, France
[b] UMR144, CNRS-Institut Curie, Paris, France
[c] Université Pierre et Marie Curie, UPMC, Paris, France
[d] Université Paris-Est, UPEC, Faculté de Médecine, Créteil, France

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ABSTRACT

Local mechanical properties play an important role in directing embryogenesis, both at the cell (differentiation, migration) and tissue level (force transmission, organ formation, morphogenesis). Measuring them is a challenge as embryonic tissues are small (μm to mm) and soft (0.1–10 kPa). We describe here how glass fiber cantilevers can be fabricated, calibrated and used to apply small forces (0.1–10 μN), measure contractile activity and assess the bulk tensile elasticity of embryonic tissue. We outline how pressure (hydrostatic or osmotic) can be applied to embryonic tissue to quantify stiffness anisotropy. These techniques can be assembled at low cost and with a minimal amount of equipment. We then present a protocol to prepare tissue sections for local elasticity and adhesion measurements using the atomic force microscope (AFM). We compare AFM nanoindentation maps of native and formaldehyde fixed embryonic tissue sections and discuss how the local elastic modulus obtained by AFM compares to that obtained with other bulk measurement methods. We illustrate all of the techniques presented on the specific example of the chick embryonic digestive tract, emphasizing technical issues and common pitfalls. The main purpose of this report is to make these micromechanical measurement techniques accessible to a wide community of biologists and biophysicists.

1. Introduction

It is becoming increasingly clear that the mechanical properties of biological tissues at all length scales, from cells to complete organisms, play an important role in determining key developmental events such as differentiation [1], migration [2], and organ [3–5] or body [6–8] formation. It is therefore important to have at hand methods that allow for a precise and quantitative determination of the mechanical properties (stiffness, viscosity, adhesion) of embryonic tissues. Measurements of these properties allow addressing a range of important biological issues, as we now illustrate by a few examples.

- Engler et al. [1,9] demonstrated that mesenchymal stem cells differentiate preferentially to neurons, muscle or bone tissue depending on the stiffness of the substrate on which they are cultured. Durotaxis [10,11], the phenomenon by which cells tend to migrate toward stiffer areas in 2D cultures, has revealed a fundamental mechanism of cell locomotion in mechanically heterogeneous environments. Extending these findings in vivo will require assessing the mechanical microenvironment of cells in their native environment, the tissue.
- Several diseases including fibrosis [12–14], collagen diseases [15] (e.g. brittle bone disease) or cancer [16] are associated to pathological modifications of normal tissue stiffness [17,18], and linked to the abnormal expression or functioning of extracellular matrix proteins involved in tissue mechanical integrity (e.g., collagen, elastin). Animal models are commonly used to better understand the molecular and cellular basis of these diseases. Characterization of the mechanical properties of these knockout or mutant embryonic tissues will better our understanding of the link between genetic mutation and the resulting pathological phenotype.
- Recent research has unveiled the importance of mechanics in the morphogenesis of organs and body structures. For example, neurulation in the early chick embryo [6], mesoderm specification in the drosophila [8], brain fold formation [19], have all been found to result from the mechanical buckling of tissues sheets. The formation of gut loops [4] has been explained as a mechanical coiling instability induced by internal stresses in a conjunctive tissue, the mesentery. Stiffening of organs and embryonic structures is an essential feature of the

* Corresponding author.
E-mail address: nicolas.chevalier@univ-paris-diderot.fr (N.R. Chevalier).
[1] Present address: INSERM U955, Equipe 6, F-94000 Créteil, France.

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morphogenesis of an embryo [20]. All of these investigations have relied on measuring the heterogeneous mechanical properties of tissues or tissue assemblies. The importance of mechanical forces and tissue deformability has long been recognized in the area of plant development [21] and is coming nowadays back to the forefront [22] with the advent of sophisticated characterization methods such as the atomic force microscope (AFM).

- The application of controlled mechanical stresses resembling those experienced in vivo during normal embryonic development is a powerful method to understand the link between mechanics and morphogenesis and improve protocols for ex vivo tissue and organ culture. It is well-known that culturing muscle strips requires applying a periodic longitudinal stress to the organ [23]. We are only starting to unveil the importance of mechanical stresses in the formation of other structures such as the lung [24], the heart [25] etc. Furthermore, the determination of the inotropic (contractile) effects on tissues (e.g. heart cardiomyocytes, gut smooth muscle) of signaling molecules and peptides such as Ca^{2+}, neurotransmitter agonists and antagonists is important for understanding their role in embryonic development and for regenerative medicine [26].

The main challenges faced by scientists seeking to determine the mechanical properties of embryonic tissues are the small (μm to mm), soft (in the 10^2–10^4 Pa range) and visco-elastic (giving rise to time-dependent phenomena) nature of the material. In this paper, we present three different methods. The first two methods – uniaxial tensile testing and inflation – are based on applying tensile stress or pressure using elongated glass fibers or pipettes. They can be assembled at low cost and, although we focus on a particular organ – the embryonic gut –, they can be adapted to a wide variety of different tissue types and geometries. The principle of these methods have been elaborated by others [27,28]. Cantilevers in particular have been applied to a wide range of different biophysical problems like the elastic properties of the arterial wall [29], sarcomere contractility [30], embryonic epithelia [31] and cell monolayers [32]. The goal of this paper is to explain in simple terms how they work and to share our experience of some particular methodological issues: fiber fabrication, calibration, attachment to the embryonic tissue, strain and stress extraction using simple ImageJ plugins. In particular, we present an original and versatile tissue attachment technique using a hook formed at the end of the cantilever fiber. The goal of this report is to make these methods accessible to a wider community of biologists and biophysicists. The last part of this report is devoted to the use of the atomic force microscope to determine the elastic properties of embryonic tissue sections. We present a comprehensive AFM sample preparation protocol and nanoindentation maps on whole embryonic tissue sections. To our knowledge, the AFM maps we present are the first (1°) elasticity maps of native unfixed embryonic tissue, (2°) comparisons of AFM indentation results in formalin fixed and unfixed conditions on whole tissue sections, (3°) comparisons of the elastic modulus deduced by AFM with an independent measurement method, bulk uniaxial tensile testing.

2. Uniaxial tension test

The uniaxial tension test we present can be applied to tissue strips, tubes or more complicated geometries such as blastulas [6]. It is a miniaturized version of the tensile force setups that have been used by physiologists to measure muscle tone and contraction characteristics [33]. Typical forces that must be applied to embryonic organs to measure their linear elastic properties are in the range 0.1–10 μN. It is difficult to find commercially available force gauges suitable for this force range. Moreover, non-invasive attachment of the embryonic organ to the force gauge remains a major technical issue. Here we show how glass fiber cantilevers can be fabricated and used to both measure force and provide anchoring of the sensor to the embryonic tissue. Deformation is measured from particle image velocimetry analysis of movies of the organ as it is put under increasing mechanical tension. The measurement can be performed in a thermalized bath of culture medium or physiological buffer.

2.1. Fiber fabrication and calibration

The angular sensitivity (angular deflection of the fiber per unit force) of a cylindrical fiber of length L, diameter d and material elastic modulus E scales as $s \propto L^2/2Ed^4$. Maximizing the sensitivity of the fiber therefore requires making them as long and thin as possible. We fabricate such fibers by heating glass Pasteur pipettes with a flame and manually pulling on them. With some training, it is possible to fabricate fibers with very high sensitivities (typically up to 10°/μN). After pulling, bends can be added to the design of the fiber. As a general rule, the heavier parts of the pipette will be pulled down by gravity when heated, while the lighter ones (e.g. pipette end) will be drawn up by the convection currents of the flame. Fig. 1a shows a small hook that was formed by quickly applying heat with a Bunsen burner flame to the end of the fiber, causing it to curl. The final form of the hook can then adjusted by cutting with a silicon carbide blade.

After fabrication, the fiber can be calibrated by fastening small weights of known mass at the end of the fiber and measuring the resulting deflection (Fig. 1a). The weights we use are thin pieces of wire. The linear mass λ (g/m) of a long (∼1 m) piece of wire is first measured with a balance. For very sensitive fibers, we recommend using ultra-fine metal (e.g. copper) wire; thicker plastic or metal wire can be used for less sensitive fibers. We then cut small pieces of length l_i (if necessary measured using a binocular) resulting in a set of masses $m_i = \lambda l_i$. A hook or a stop (small ball of glue or molten glass) at the end of the fiber prevents the weights from slipping off the fiber during calibration. For very sensitive fibers ($s > 1°/\mu N$), the fiber and weights should be immerged in a liquid to abolish any perturbations from air currents and electrostatic forces. For fiber sensitivities <1°/μN, calibration can be performed in air. Fig. 1b shows a typical calibration curve, we found it to be linear up to at least 30° deflection. Once calibrated, fibers should be stored in a safe environment; they can be used for years if handled carefully.

2.2. Measurement setup

Fig. 1c shows the schematic setup of a uniaxial embryonic tissue tensile test. One should be especially watchful of the following issues:

1. Attachment of the tissue. One end of the tissue can be conveniently immobilized by lining the container bottom with a thin (∼1 mm) sheet of 1:50 Polydimethylsiloxane (PDMS, Sylgard silicon oil) so that the tissue can be pinned using ultra-fine (Ø = 50 μm) needles (Euronexia). PDMS is commonly used as a cell-culture substrate and is optically clear, so that the tissue can be illuminated from below. Attachment of the tissue to the cantilever depends on tissue geometry. For tissue strips, or tubes, such as the gut depicted in Fig. 1c–e, we have found that forming a small hook (Fig. 1a) by applying heat at the pipette end allowed for an easy, robust, and minimally invasive way of securing the organ to the fiber. For tissue sheets, at least two points of attachment are required. This can for example be