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Generating and characterizing the mechanical properties of cell-derived matrices using atomic force microscopy

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ABSTRACT

Mechanical interaction between cells and their surrounding extracellular matrix (ECM) controls key processes such as proliferation, differentiation and motility. For many years, two-dimensional (2D) models were used to better understand the interactions between cells and their surrounding ECM. More recently, variation of the mechanical properties of tissues has been reported to play a major role in physiological and pathological scenarios such as cancer progression. The 3D architecture of the ECM finely tunes cellular behavior to perform physiologically relevant tasks. Technical limitations prevented scientists from obtaining accurate assessment of the mechanical properties of physiologically realistic matrices. There is therefore a need for combining the production of high-quality cell-derived 3D matrices (CDMs) and the characterization of their topographical and mechanical properties. Here, we describe methods that allow to accurately measure the young modulus of matrices produced by various cellular types. In the first part, we will describe and review several protocols for generating CDMs matrices from endothelial, epithelial, fibroblastic, muscle and mesenchymal stem cells. We will discuss tools allowing the characterization of the topographical details as well as of the protein content of such CDMs. In a second part, we will report the methodologies that can be used, based on atomic force microscopy, to accurately evaluate the stiffness properties of the CDMs through the quantification of their young modulus. Altogether, such methodologies allow characterizing the stiffness and topography of matrices deposited by the cells, which is key for the understanding of cellular behavior in physiological conditions.

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1. Introduction: definition, terminology and rationale for studying cell-derived matrices (CDMs)

Interactions between cells and the extracellular matrix (ECM) are key and vital drivers of embryonic development and adult organ homeostasis. Mis-regulation or impairment of this process leads to a plethora of disorders including developmental malformations, fibrosis and cancer, among others. *In vivo*, cells naturally encounter cell-derived extracellular matrices (CDMs). The interaction of cells with the surrounding ECM is essential for their proper function, as well as the maintenance of tissue architecture and homeostasis. The ECM provides a physico-chemical scaffold in which cells adhere, anchor and function, shaping tissue architecture and homeostasis. The ECM provides also a signaling support which regulates cellular functions such as growth and survival [1,2]. Cells have developed a large repertoire of receptors capable of binding to the ECM [3,4]. They provide a physical link to the ECM and allow them to transduce signals emanating from the ECM by adapting their behavior to the properties of this complex microenvironment. Noteworthy, cell behavior is affected by the composition, the topography and the mechanical properties of the ECM [5]. In addition, ECM is a potent reservoir of growth

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factors that can drastically affect cellular functions [6]. Finally, the ECM is commonly deregulated and disorganized in diseases such as cancer [7].

It is therefore of utmost importance to mimic as much as possible the reality by developing *in vivo*-like 3D CDMs. By definition, *in vitro* CDMs are ECM molecules that are deposited and organized by the cells themselves. They are eventually decellularized and represent 3D composites of ECM proteins. CDMs have been developed for many years and not surprisingly have led to several terminologies: cell-free matrix, cell-secreted matrix, cell-derived matrix, acellular matrix, decellularized ECM or decellularized matrix, immobilized ECM surface, cell-driven matrix. Here, for the sake of simplicity, we will use a common nomenclature, which is cell-derived matrix (CDM). When derived from a tissue, they can be named cell-free-organ-derived ECM (ex: cell-free-bone marrow-derived ECM) or tissue-derived matrices (TDMs).

Such matrices, when used as substrate for other cells, allow the characterization of a plethora of cellular functions with high physiological relevance and which drastically differs from culturing cells on artificial 2D substrates *in vitro* [8]. They further allow mimicking *in vivo* situations since ECM does not function merely as an inert structural role but rather plays an active role in the control of cell growth and differentiation [9]. In addition, CDMs allow to reproduce *in vitro* the micro-heterogeneity of the basement membranes, or more generally of the ECMs, that is known to regulate cellular functions or to establish niches suitable for the growth of stem or progenitor cells. They further allow taking into account the tissue-specificity of the ECM and thus to better replicate and mimic cell-specific features of the ECM architecture. They naturally allow to present associated bioactive factors such as growth factors and are advantageous for the culture of anchorage-dependent cells that fail to attach properly to available substrates, or that are not capable to produce their own matrix. They also provide a way to study the synthesis and asymmetrical deposition of the ECM. Finally, they are perfectly suitable for the characterization of their mechanical properties (stiffness and viscoelastic properties) and allow thus to study their biomechanical contribution to cell behavior.

2. Preparation of CDMs

The ultimate goal of developing and using CDMs is to allow the cells to produce and deposit their own ECM and then to use extraction procedures to remove the cell layer but, importantly, leaving the underlying deposited-ECM intact, free of cellular debris and firmly attached to the culture dish. While it is unfortunately impossible to clearly identify optimal experimental conditions for producing CDMs of high-quality, multiple laboratories over the world have developed a wide range of protocols with the idea of optimizing the methodology: this includes priming of the substrate before seeding of the cells, usage of various cell types, culture timings and conditions (such as presence of cytokines, growth factors, oxygen tension) and cell extraction protocols. All these parameters will strongly influence the composition, the organization, and the mechanical properties of the matrices, and thereby modulate differentially the phenotype of cells seeded on them. Importantly, cells will secrete and deposit the ECM molecules basally as a function of time. Intuitively, the more cells are seeded in a confluent state, the higher the amount of ECM should be deposited on the surfaces by cells that were seeded on. Yet if cells are too confluent, detachment and/or contraction of CDMs can occur.

The common feature of each workflow is to achieve efficient lysis of ECM-producing cells. For this purpose, several scenarios have been developed [10,11]. While some experimental strategies rely on physical detachment of cells using freeze–thaw cycles (formation of intracellular crystals that disrupt cell membrane and cause cell lysis), the most-commonly used methods are based on chemical or enzymatic approaches (Fig. 1):

- *Removal of cells using alkaline buffers:* They are used to solubilize the cytoplasmic components of the cells as well to remove nucleic acids (RNA, DNA). The most common used is ammonium hydroxide (NH_4OH); yet this molecule may dissociate glycosaminoglycans from collagens [10].
- *Removal of cells using detergents:* (i) non-ionic detergents with the most commonly used being Triton X-100 (disruption of lipid–lipid and lipid–protein interactions); Tween-20 or NP-40 have been also used but to a lesser extent; (ii) ionic detergents such as sodium deoxycholate (DOC) that will disrupt protein–protein interactions and solubilize cytoplasmic and nuclear membranes. Combined use of Triton X-100 with NH_4OH is often realized.
- *Removal of cells using denaturating agents:* Such as urea.
- *Removal of cells using hypotonic treatment:* Osmotic shock with a hypotonic solution such as deionized water or low ionic strength solution is used to lyse the cells.
- *Removal of cells using chelating agents:* EDTA or EGTA for disrupting the cell–cell junction. The advantage here will be that EDTA inhibits metalloproteases and therefore may contribute to ECM preservation [11].
- *Removal of cells using enzymatic methods:* Enzymatic detachment of the cells from the deposited ECM can be done using trypsin; the major drawback is the possible damage of the remaining ECM and its bound factors.

Which method to use is a rather difficult assumption/choice to make and one should carefully consider the existing protocols, which we will review in the subsequent sections, according to cell subtypes.

2.1. Endothelial cell-derived matrices

Generation of matrices derived from endothelial cells have led to several protocols detailed below. They are historically the first cells that had been used to produce CDMs and have led to multiple observations. ECM produced by cultured endothelial cells closely resembles the sub-endothelium *in vivo*, in its morphology and molecular composition. Endothelial CDMs have been used to study the adhesion- and growth-promoting properties, but also as substrate to assess cell differentiation potential (for a review see [12]). Importantly, cells maintained on CDMs produced by corneal endothelial cells proliferated rapidly and no longer required FGF to reach confluence [13]:

a. *Original method using detergents:* The first description of a technique that allows isolating ECM deposited by cells in culture was from Gospodarowicz's team [13,14]. The original technique used only Triton X-100 to prepare CDMs from bovine endothelial cells. Briefly, once the cultures became confluent (ordinarily within 6 days) the media were renewed, and the cultures were further incubated for 6 days. After washing with PBS, cells were exposed for 30 min to 0.5% Triton X-100. In this condition, only a few cytoskeletons and nuclei were associated with the intact ECM (as defined by a thick layer of amorphous material) that coated the entire dish as shown by scanning electron microscope (SEM). The remaining nuclei and cytoskeletons were removed either by pipetting [15] or by adding subsequently 0.025 N NH_4OH (2–3 min exposure) to the CDM [16].

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