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Assay for characterizing the recovery of vertebrate cells for adhesion measurements by single-cell force spectroscopy

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ABSTRACT

Single-cell force spectroscopy (SCFS) is becoming a widely used method to quantify the adhesion of a living cell to a substrate, another cell or tissue. The high sensitivity of SCFS permits determining the contributions of individual cell adhesion molecules (CAMs) to the adhesion force of an entire cell. However, to prepare adherent cells for SCFS, they must first be detached from tissue-culture flasks or plates. EDTA and trypsin are often applied for this purpose. Because cellular properties can be affected by this treatment, cells need to recover before being further characterized by SCFS. Here we introduce atomic force microscopy (AFM)-based SCFS to measure the mechanical and adhesive properties of HeLa cells and mouse embryonic kidney fibroblasts while they are recovering after detachment from tissue-culture. We find that mechanical and adhesive properties of both cell lines recover quickly (<10 min) after detachment using EDTA, while trypsin-detached fibroblasts require >60 min to fully recover. Our assay introduced to characterize the recovery of mammalian cells after detachment can in future be used to estimate the recovery behavior of other adherent cell types.

1. Introduction

Specific adhesive interactions between cells and extracellular matrix (ECM) or between cells play crucial roles in cellular communication, tissue organization, embryonic development and wound healing. Accordingly, a wide variety of diseases are associated with impaired cell adhesion [1–4]. Animal cells sense and adhere to their extracellular environment via cell adhesion molecules (CAMs), which are typically transmembrane proteins. Specific interactions between CAMs and their extracellular ligands induce intracellular signaling pathways, which regulate the adhesive and mechanical properties of cells besides other cellular processes. CAMs are classified into different families, including integrins, cadherins and selectins [5–8]. To strengthen the cellular attachment to an extracellular substrate, multi-protein complexes anchor CAMs to the cytoskeleton. Key cytoplasmic adaptor proteins include talin, kindlin, vinculin and catenins [9–12]. Due to the general importance of cell adhesion, the interaction of CAMs and their ligands are studied extensively using various, yet mostly qualitative methods [13,14]. However, as these qualitative methods can provide helpful insights, describing the adhesive interactions of cells benefits greatly from measuring quantitative parameters such as cell adhesion forces, kinetics and energies.

Single-cell force spectroscopy (SCFS) offers the possibility to measure adhesive forces and energies of single cells adhering to a biotic or abiotic substrate, another cell or tissue [15,16]. SCFS methods are based on force sensing devices such as optical or magnetic tweezers, micropipettes, or atomic force microscopy (AFM) [14,17,18]. In these SCFS-based methods the cell is brought into contact with an adhesive substrate or another cell for a given contact time and then separated. While approaching and retracting the cell, the interaction forces are recorded and provide a quantitative measure of the adhesive interactions between cell and substrate. Among all currently available SCFS methods, AFM-based SCFS cov-

Abbreviations: AFM, atomic force microscopy; BSA, bovine serum albumin; CAMs, cell adhesion molecules; ConA, concanavalin A; ECM, extracellular matrix; GFP, green fluorescent protein; FD, force–distance; FCS, fetal calf serum; MYH9, myosin heavy chain 9; PAR, protease-activated receptor; SCFS, single-cell force spectroscopy

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ers the largest dynamic force range from ≈10 pN to ≈100 nN [16,18,19]. This wide range permits quantifying the adhesive force of an entire cell down to the adhesive force established by single CAMs. AFM-based SCFS attaches a single cell to the apex of a tipless AFM cantilever (Fig. 1). To facilitate cell attachment, the cantilever is coated either with a substrate-mimicking ligand (e.g., cell surface receptors or ECM proteins including collagens, laminins, or fibronectin), concanavalin A (ConA) to bind carbohydrates on the cell surface, antibodies, or an unspecific adhesive (e.g., CellTak, poly-L-lysine) [15,20–32]. The cantilever-bound cell is then approached either to a protein-coated substrate, another cell, tissue explant or biomaterial. After a pre-determined contact time, during which the cell is allowed to initiate adhesion, the cantilever is retracted until cell and substrate are fully separated. During the approach and retraction cycle cantilever deflection (e.g., force) and cell-substrate distance are recorded in so-called force–distance (FD) curves (Fig. 1C). Analysis of the FD curves provides several quantitative insights into the cellular interaction with the substrate. The approach FD curve provides insight into the mechanical properties of the cell being pressed onto the substrate [18,26,33–35]. The retraction FD curve provides the maximum detachment force, also called adhesion force, of the cell. However, two types of smaller unbinding events contained in the retraction FD curves correspond to the unbinding of single or clustered CAMs [15,16,18,19,36]. These unbinding events are frequently named rupture and tether events, and differ in the molecular scenarios leading to their emergence. In rupture events, the CAMs remain anchored to the actin-cytoskeleton and upon exposure of mechanical stress detach from their extracellular ligand [22,31,32,37–39]. If the anchorage to the cytoskeleton breaks before the CAM unbinds from the extracellular ligand or if the CAM has not been attached to the cytoskeleton in the first place, the CAM is pulled away from the cell cortex on the tip of a membrane tether [19,36,40]. In this so-called tether event, the tether is mechanically extended until the receptor-ligand bond breaks. The force required to extend a tether from the cellular membrane does not depend on the strength of the CAM-ligand bond but rather on mechanical properties of the cellular membrane (e.g., bending rigidity, viscosity, and tension) [40], the velocity at which the tether is extracted from the membrane, and on cell membrane attachment to the cortical cytoskeleton. In rare cases, tether extension from the cellular membrane terminates when the tether fails or if the receptor is pulled out of the membrane [40,41]. In the later separating phase between cell and substrate, the cell body is not in contact with the substrate anymore and tethers exclusively mediate cell adhesion [31]. The analysis of tether unbinding events can provide information on the lifetime of single CAM bonds, the mechanical properties of the cell cortex, and cell membrane tension [31,37,40,42–45].

Although SCFS measurements and other methods applied to characterize cell adhesion provide quantitative and qualitative insights into cell adhesion, a drawback is that adherent cells must first be detached from culturing flasks in order to characterize their adhesion to a given substrate. Cells are commonly detached with trypsin and/or ethylenediaminetetraacetic acid (EDTA) [27,46,47]. Although some CAMs, such as α2β1 integrin [14], are trypsin resistant, other CAMs such as cadherins are sensitive to trypsin cleavage [48]. Furthermore, other proteins involved in the initiation of

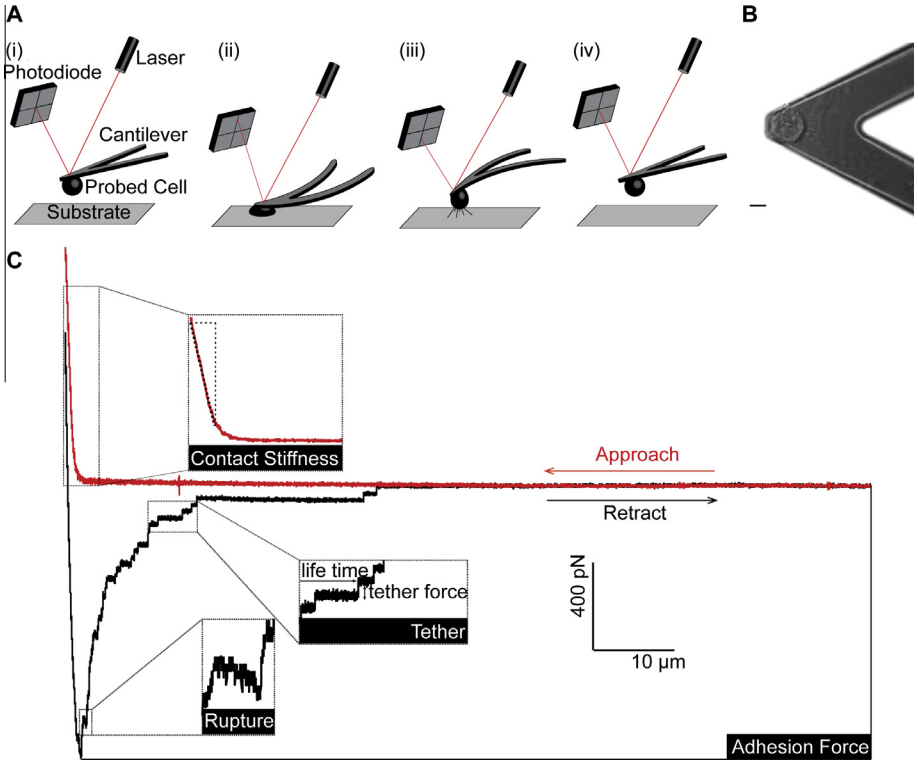

Fig. 1. Scheme of AFM-based SCFS. (A and B) To use a single cell as a probe it is bound to a concanavalin A (ConA)-coated tipless AFM cantilever (scale bar, 10 µm). (A) (i and ii) The cantilever is approached onto a protein-coated substrate until a preset contact force is reached. After a defined contact time (ii), the cantilever is retracted until the cell is fully separated from the substrate (iii and iv). During approach and retraction, the cantilever deflection and thus, the force acting on the cell is recorded in force–distance (FD) curves. (C) FD curves show different features: In the approach FD curve (red) the cantilever deflection measured upon pressing the cell onto the substrate correlates with the stiffness of the cell and is called contact stiffness [33]. The retraction FD curve (black) records the adhesion force of the cell, which represents the maximum downward force deflecting the cantilever and thus the maximum force needed to detach cell and substrate. After recording the maximum adhesion force, single receptor unbinding events are observed. Rupture events are recorded when the CAM-ligand bond of a cytoskeleton-linked CAM fails. Tether events are recorded when a membrane tether is extruded from the cell membrane with the CAM at its tip (tethers). In the latter case attachment of the CAM to the cytoskeleton is either too weak to resist the mechanical stress applied or non-existent [19,40,41].