Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Evaluation of the force and spatial dynamics of macrophage podosomes by multi-particle tracking

Amsha Proag [a,b], Anaïs Bouissou [a,b], Christophe Vieu [c,d], Isabelle Maridonneau-Parini [a,b,*], Renaud Poincloux [a,b,*]

[a] IPBS (Institut de Pharmacologie et de Biologie Structurale), UMR CNRS 5089, 205 route de Narbonne, F 31077 Toulouse, France
[b] Univ de Toulouse, UPS, F 31400 Toulouse, France
[c] CNRS, LAAS, 7 avenue du colonel Roche, F 31400 Toulouse, France
[d] Univ de Toulouse, INSA, F 31400 Toulouse, France

ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 29 August 2015
Accepted 1 September 2015
Available online xxxx

Keywords:
Podosomes
Macrophages
Multiparticle tracking
Force
Mechanosensing
Collective dynamics

ABSTRACT

Podosomes are submicron adhesive and mechanosensitive structures formed by macrophages, dendritic cells and osteoclasts that are capable of protruding into the extracellular environment. Built of an F-actin core surrounded by an adhesion ring, podosomes assemble in a network interconnected by acto-myosin cables. They have been shown to display spatiotemporal instability as well as protrusion force oscillations. To analyse the entire population of these unstable structures, we have designed an automated multi-particle tracking adapted to both topographical and fluorescence data. Here we describe in detail this approach and report the measurements of individual and collective characteristics of podosome ensembles, providing an integrated picture of their activity from the complementary angles of organisation, dynamics, mobility and mechanics. We believe that this will lead to a comprehensive view of podosome collective behaviour and deepen our knowledge about the significance of mechanosensing mediated by protrusive structures.

1. Introduction

Podosomes are actin-based protrusive structures that form constitutively at the ventral membrane of few cell types including macrophages, immature dendritic cells and osteoclasts. The physical structure of an individual podosome includes two components: a submicron-scale F-actin core, surrounded by a ring comprising many classical actin-based adhesion complex proteins such as vinculin, paxillin, talin and integrins [1]. Podosomes are involved in several functions including cell adhesion, mechanosensing, proteolysis of the extracellular matrix and three-dimensional migration [2–4].

The mechanosensitive properties of podosomes have recently been demonstrated by showing that they exert protrusive forces on the substrate [5,6] that increase with substrate stiffness [6]. Podosomes are highly dynamic: not only do they display rapid turnover [7,8], but their actin content, mechanical properties and force intensity vary over time [6,9–11]. Neighbours in particular seem to influence each other's mechanical and compositional dynamics in a synchronous manner [12]. Actually, podosome neighbouring cores have been shown to be interconnected by acto-myosin filaments [13–15], forming a network of cables that may play a role in maintaining spatial organisation and synchronising linked neighbours.

At a larger scale, podosomes form various arrangements, ranging from uniformly distributed patterns to so-called superstructures such as clusters, rosettes or belts [16]. In osteoclasts, podosomes organise into a sealing zone which has the ability to resorb bone. Thus, from a biological point of view, podosomes are dedicated to critical cell functions such as remodelling of the environment, cell adhesion and migration. From a mechanical point of view, podosomes are able to protrude in the matrix perpendicularly to the substrate and sense its rigidity. Given that specific podosome assemblies may be dictated by function requirements [2], we believe that at this stage of our knowledge on individual podosomes, we would gain to analyse them collectively.

Abbreviations: PFM, protrusion force microscopy; AFM, atomic force microscopy; TIRF, total internal reflection fluorescence.

* Corresponding authors at: IPBS (Institut de Pharmacologie et de Biologie Structurale), UMR CNRS 5089, 205 route de Narbonne, F 31077 Toulouse, France.

E-mail addresses: isabelle.maridonneau-parini@ipbs.fr (I. Maridonneau-Parini), renaud.poincloux@ipbs.fr (R. Poincloux).

http://dx.doi.org/10.1016/j.ymeth.2015.09.002
1046-2023/

The simultaneous tracking of multiple podosomes is useful for two reasons. Firstly, it makes it possible to determine the dynamics of their architectural and mechanical parameters, as well as the interdependence of these parameters over time. Secondly, population-scale data is a requisite for the study of the podosome network and its spatial organisation. Indeed, there is increasing evidence that podosome collective behaviour is important for podosome function, such as the unique osteoclast sealing zone structure that is crucial for bone degradation [2]. In macrophages, the spatial synchrony of individual podosomes [12] might be the very basis of the collective regulation of these structures, such as the one involved in sealing zone dynamics.

Multi-podosome identification and segmentation on static images using automated image analysis has led to measurements of ring composition and core geometrical parameters [15,17,18]. Fluorescence intensity variations have also been quantified on single podosomes [6,9,10]. However, until now, there was no available method enabling the measurement of the dynamic parameters of multiple podosomes over time. The major difficulty comes from the high instability of podosomes. Indeed, the aim is to follow mobile structures with fluctuating intensity, that possess the ability to emerge from the actin network or fade into it, to fission into two, or to fuse with their neighbours [8,19].

To accomplish this task, we sought to design automated multi-particle tracking adapted to following every podosome in a cell. Indeed, automated tracking has become a powerful method to quantify moving objects by measuring parameters of motion, with increased precision and in a larger number as compared to manual tracking. Precision is efficiently obtained as each particle may be localized at subpixel resolution by image analysis, leading to nanoscale fluctuation measurements [20]. More importantly, number is an essential factor in collective systems where simultaneous measurements on multiple particles are required to take interactions into account [21].

In this manuscript, we describe an algorithm developed to follow over time a population of moving, unstable particles throughout their fluctuations. We detail how podosome are identified and tracked, how their formation and disappearance events are taken into account, and the limitations of the technique. Then, we successively report the measurements of podosome architecture, spatial organisation, compositional and spatial dynamics and protrusion force measurements that this method allows. Finally, we provide an overview of the podosome characteristics measured with this approach.

2. Material and methods

2.1. Cell preparation

Human macrophages were differentiated from primary monocytes isolated from the blood of healthy donors, as previously described [22]. After 7 days of differentiation, macrophages were detached with trypsin-ETDA (Invitrogen) and plated at 2.5×10^4 cells cm^{-2} on glass coverslips or on Formvar®-coated grids in RPMI 1640 containing 10% foetal calf serum. Time-lapse fluorescence experiments used Lifeact-mCherry and paxillin-GFP as probes. Monocyte-derived macrophages were detached using trypsin–EDTA and transfected with the corresponding plasmids 4 h before observation using a Neon™ MP5000 electroporation system (Invitrogen) with the following parameters: two 1000 V, 40 ms pulses, with 0.5 μg DNA each for 2×10^5 cells. Cells were then plated in a cover-glass-bottom Lab-TeK™ observation chamber (Nunc, Roskilde, Denmark).

2.2. Immunofluorescence imaging of fixed cells

Macrophages plated on glass coverslips for 3 h were unroofed using distilled water containing cOmplete™ protease inhibitors (Roche) and 10 μg/mL phalloidin (Sigma–Aldrich) at 37 °C for 30 s, and then the cells were flushed 10 times before being fixed with a 4% paraformaldehyde solution in Phosphate Buffer Saline (Fisher Scientific). Samples were stained with Texas Red®-X-phalloidin (Invitrogen, 1/500) for F-actin, and anti-vinculin antibody (M3191, Sigma, 1/500) and an AlexaFluor® 488-coupled secondary antibody for vinculin. Nine images at 100 nm interval were acquired on each field with a 100×/1.45 objective mounted on a Nikon Eclipse Ti-E and an Andor sCMOS Neo camera in high dynamic mode and in global shutter. These images were processed by nonlinear deconvolution as described previously [12]. Briefly, the ImageJ software *Deconvolution Lab* [23] was used with a total variation regularization determined from each image using the method described by Laasmaa and co-workers [24]. The point spread function (PSF) used for inversion was determined as an average of 30 experimental PSFs.

2.3. Time-lapse total internal reflection fluorescence microscopy

Live macrophages plated in Lab-Tek™ chambers were imaged using a TIRF microscopy setup (Olympus FV1000) so as to provide a high spatial resolution. Imaging was carried out using an inverted IX-81 microscope with a 60× immersion objective (NA = 1.49) and an Orca-R2 camera (Hamamatsu). A 'Box-Cube-Brick' set (Life Imaging Systems) served to stabilize temperature (37 °C) and CO_2 pressure (5%). Image sequences were acquired every 2 s to follow podosome dynamics. Preliminary processing includes registration and bleaching correction using the *StackReg* [25] and *Bleach Corrector* ImageJ plugins. Podosome tracking and further analyses were then performed with the algorithm detailed in Section 3.1.

2.4. Protrusion force microscopy (PFM)

Protrusion height measurements were performed as previously described [6,12]. Briefly, atomic force microscopy (AFM) measurements were performed using silicon nitride cantilevers (MLCT-AUHW, Veeco Instruments) with a nominal spring constant of 0.01 N m^{-1} mounted on a NanoWizard III™ AFM (JPK Instruments) coupled to an inverted optical microscope (Axiovert 200, Carl Zeiss). To ensure reproducibility in force application, the cantilever sensitivity and spring constant were calibrated before each experiment using the JPK Instrument software using the thermal noise method [26]. To prepare Formvar® sheets, ethanol-cleaned glass slides were dipped into a Formvar® solution of 0.5% (w/v) ethylene dichloride (Electron Microscopy Science) for a few seconds and the solution was emptied from the film casting device using a calibrated flow. A Formvar® film was detached from dried slides by contact with water and was left floating at the surface. Acetone-washed 200-mesh nickel grids (EMS) were arranged on the floating film, picked up coated with the film onto another glass slide and then air-dried. To evaluate the thickness of the Formvar® sheet, the border of the Formvar® that remained on the glass slide after removing the grids was imaged in contact mode by AFM.

For AFM analysis of living cells seeded on Formvar®-coated grids, a temperature-controlled chamber was used (Petri dish heater™, JPK Instruments) and the culture medium was supplemented with 10 mM HEPES (pH = 7.4) (Sigma–Aldrich). Images were recorded in contact mode in liquid at scanning forces lower than 1 nN. The pixel resolution was between 128 and 256 px