

Extended mechanical force measurements using structured illumination microscopy

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Abstract

Quantifying cell generated mechanical forces is key to furthering our understanding of mechanobiology. Traction force microscopy (TFM) is the most broadly applied force probing technology, but its sensitivity is strictly dependent on the spatio-temporal resolution of the underlying imaging system. In previous works, it was ~~shown~~demonstrated that increased sampling densities of cell derived forces permitted by super-resolution fluorescence imaging enhanced the accuracy and resolution of the TFM method. However, ~~previous~~these recent advances to TFM based on super-resolution techniques were limited to slow acquisition speeds and high illumination powers. Here, we present three novel TFM approaches that, in combination with total internal reflection (TIRF), structured illumination microscopy (SIM), and astigmatism, improve the spatial and temporal performance in either two-dimensional or three-dimensional mechanical force quantification, while maintaining low illumination powers. These three techniques can be straightforwardly implemented on a single optical setup offering a powerful platform to provide new insights into the physiological force generation in a wide range of biological studies.

Main text

Bioimaging is rapidly progressing with experimental strategies utilising physiologically relevant systems largely due to the establishment of new technologies. These cutting-edge imaging technologies underpin a significant proportion of bioscience research. One of the important questions in biophysics is understanding the mechanical forces generated by cells when they interact with their surrounding environment. It was recently reported that such forces are partially responsible for regulating the biological functions of cells [1-3]. During their life-cycle, living cells dynamically adjust their mechanics in response to external stimuli, owing to complex and intimate feedback between biochemical signal pathways and cellular force generating machinery, primarily the acto-myosin cytoskeleton. Consequently, this forms a previously underappreciated picture wherein cellular function relies on the mechanobiological context of the tissue micro-environment [4,5]. Understanding how mechanical forces present during such interactions influence cellular function is of primary importance for mechanobiology. The forces generated by cells can range from nano-Newtons to pico-Newtons per μm^2 , with temporal fluctuations ranging from milliseconds to hours [6]. The ubiquity of mechanical force generation to the function of cellular life imposes a requirement

on the biological sciences for tools and techniques that allow for the quantification of these forces at the relevant spatio-temporal scale. Techniques that allow for the quantification of biologically generated mechanical forces are likely to be key in providing a more complete understanding of the biophysical mechanism by which biological systems carry out their function.

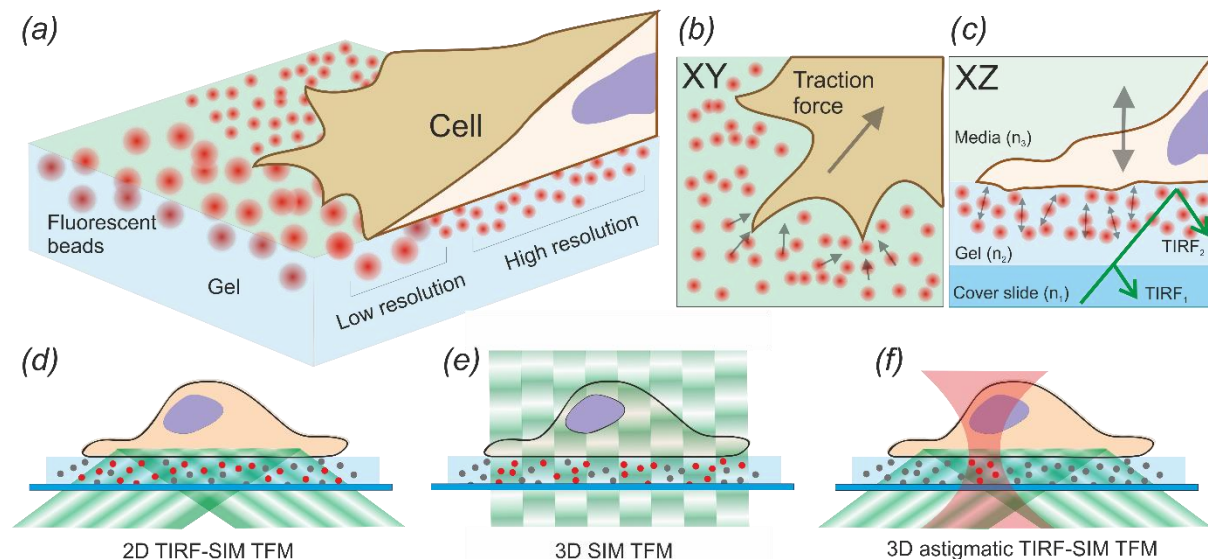


Figure 1. Schematic representation of **traction force microscopy (TFM)** experimental designs: (a) **general** overview and relation between the interplay of spatial resolution and bead density; (b,c) and (e) are lateral and axial views on cell edge and beads displacements during TFM measurements. TIRF₁ indicates standard TIRF at the interface between the coverglass and the upper medium. TIRF₂ indicates the novel TIRF method at the interface between the silicone gel and the medium in the absence of standard TIRF; (d) 2D TIRF-SIM TFM where the TIRF field is generated at the interface between silicone gel and cell media; (e) 3D SIM adapted for TFM measurements; and (f) wide-field astigmatic TFM combined with TIRF-SIM imaging for cell **visulisation-visualisation**.

This demand has led to the development of several force probing methods [5]. These techniques range from **Aa**tom**F**orce **M**icroscopy (**AFM**) which allows for both the probing of cellular mechanical properties and the quantification of local force generation, to more recently the use of DNA based force sensors, which allows mechanical force probing at the single molecule level [7,8]. **Traction Force Microscopy (TFM)** is perhaps one of the most widely used technologies, owing to its ease of implementation and its ability to recapitulate both the mechanical and biochemical environment of the cell [9,10].

In a typical TFM experiment, a transparent elastic substrate embedded with fiducial markers is formed on a cover slide. The surface of the substrate can be functionalised to biochemically mimic a cell's physiological environment, while the substrate stiffness can be tuned to mimic its biomechanical properties. Mechanical forces generated by live cells interacting with the elastic surface induce surface deformations, resulting in marker displacements (**Figure. 1a,b**). The direction and magnitude of the displacements, together with the mechanical properties the elastic substrate (Poisson's ratio and elastic modulus) are used to calculate the traction forces, which are typically at the 1 - 0.001 nN/μm² scale [5]. Notably, the units of modulus of elasticity are pressure units, as it defines the stress divided by strain, while stress is typically reported in units of pressure, and strain is dimensionless. The units of pressure is Pascal (Pa) and equals mechanical force production of one Newton per square metre. Because the area

of applied force production in the cellular environment is often not clear, it is beneficial to report stress instead of mechanical force production in units of pressure.

Optical microscopy has established itself as the method of choice. In principle, for TFM, but can be carried out at any optical microscope capable of imaging the fiducial marker beads. In recent years however Nevertheless fluorescence microscopy has dominated the field in the recent years, thanks to its high specificity, contrast and multi-channel readout. Using TFM coupled with fluorescence microscopy, morphological changes can be measured by labelling cells with dyes spectrally separated from the TFM fluorescent marker beads. In this way, the traction force map can be correlated with the cell ular function and behaviour.

The accuracy and resolution of forces quantified via TFM are determined depends on the by bead density, the resolution of optical system, and the acquisition speed. The spatial distribution of forces generated at cell-substrate interface in TFM can be both continuous and spatially concentrated in nature. Because of this, in order to capture the details within a given force distribution, the bead density must be sufficiently high, such that it is able to sample this distribution effectively. In theory, this means the spatial frequency of a given bead density must be double that of any details that exist within the force map exerted by the cell [10]. Crucially, conventional fluorescence microscopy is known to be diffraction limited. The smallest spot to which an optical system can focus light is known as the point spread function (PSF) and its lateral and axial dimensions are described by following equations respectively: $Res_{xy} = \lambda_{em}/(2NA)$ and $Res_z = \lambda_{em}/(NA)^2$, where λ_{em} is emission wavelength, and NA is numerical aperture of an objective lens [11]. Due to the wave properties of light the axial component is always several times larger than the lateral one. The PSF ultimately defines the highest resolution which can be potentially achieved with the given microscope. In other words, it demonstrates the ability to resolve two closely positioned objects, because objects located at distances smaller than the PSF would appear merged, as described by the Rayleigh criterion [12]. Owing to this, displacements within higher bead densities can only be reliably resolved when coupled with high spatial resolution imaging, indicating the great power of super-resolved fluorescence techniques to improve TFM (**Figure. 1a-c**). Complementary to the spatial resolution, the temporal resolution of the bead imaging must be sufficient to capture the temporal fluctuations inherent to cellular force generation, which often occur on the millisecond (ms) to second (s) time scale. Insufficient temporal sampling can lead to large errors in the estimation of force generation [10,13].

TFM has been widely applied to study cell generated traction forces during adhesion [14]. These studies typically involved the steady state adherence of a cell to a TFM substrate, followed by its chemical removal. Imaging of the pre-stressed and relaxed state of the gel allowed for an accurate quantification of the applied cellular tractions. In such studies, the temporal resolution of the imaging system is not limiting since the gel is imaged in only two states. Alternatively, more dynamic studies can be performed, whereby substrate displacements are continuously monitored during for example cell migration [15]. The continuous evolution of the cellular force distribution means that the accuracy of such studies are highly dependent on the temporal resolution of the imaging modality. To maximise the acquisition speed, a widefield approach was typically applied, where using is camera detection unlike in scanning based methods such as confocal, the acquisition speed is only limited by the camera readout speed (up to few milliseconds) and sample labelling quality. The widefield approach demonstrates good results with respect to temporal resolution but can provide only limited sensitivity due to moderate spatial resolution, and it lacks an optical sectioning capability. With advances in scanning techniques, it became possible to perform confocal imaging at relatively high frame rates [16]. Using confocal microscopy combined with a dual bead approach, Sabass et al. were able to push the spatial resolution of TFM beyond previous limits [17,18]. Crucially, this opened pathways for the combination of super-resolution approaches and TFM, as an alternative approach to increase the bead density. The first super-resolution approach adapted for TFM, was stimulated emission depletion (STED) microscopy.

STED is a confocal based method, where superimposing a second doughnut shaped depletion beam over a conventional Gaussian excitation beam, results in an effective reduction in the PSF size and, therefore, improved resolution. The combination of STED and TFM permitted the use of bead densities 5-fold higher than conventional TFM, leading to a significantly improved sensitivity of the technique [10]. The primary drawback of STED-TFM is its reliance on pixel-by-pixel scanning, which increases acquisition times, limiting acquisition to slowly evolving biological system or sub cellular acquisition regions. Moreover, STED requires high illumination powers to achieve significant resolution enhancement, which further restricts the total acquisition times due to sample degradation. Spatial resolution enhancement has recently also been achieved by means of fluorescence fluctuation image analysis [19].

The above discussion was restricted to lateral forces detection, meaning imaging of only a single plane at the interface between cell and the substrate is performed. In that way only two-dimensional (2D) force maps can be generated. ~~However~~Nevertheless, during interactions with their environment, cells have been shown to demonstrate three dimensional (3D) motions resulting in out-of-plane stresses. Hence, the ability to follow both the directionality and magnitude of the applied forces at the cell-substrate interface is essential for complete understanding of cellular force generation (**Figure 1b,c**). Confocal based imaging techniques have good optical section capabilities so they can potentially be used for the 3D traction measurements, but due to the scanning speeds only slow processes can be observed.

The natural steps toward TFM improvement is the search for new imaging techniques, which can provide both high spatial and temporal resolution, and at the same time will not require very high illumination powers, ~~which are being~~ detrimental for live samples. Here, we present three novel TFM techniques, which are based on the same core microscope setup with only minor hardware modifications. The schematic representations of the principle behind each technique is shown on **Figure 1d,e,f**, and they are named 2D ~~total internal reflection structured illumination microscopy~~ (TIRF-SIM), 3D-SIM, and 3D astigmatic TIRF-SIM TFM, respectively. While the first approach targets rapid and high sensitivity measurements of lateral force, the latter two are designed to observe 3D force generation. The key feature is that all three techniques can be realised on the same optical setup, which provides great flexibility for potential applications. In this paper we will discuss the basics behind each approach together with their pros and cons and potential applications.

2D TIRF-SIM TFM

The TIRF-SIM microscope architecture was first developed by Dong et. al. and the exact technical details can be found elsewhere [20,21]. The setup features a ferroelectric spatial light modulator (SLM) which replaces the conventional grating used for SIM. The SLM generates a diffraction pattern, from which the first orders are selected with a spatial mask and focused on the back focal aperture of the objective. The beams interfere with each other generating the structured illumination pattern. The pattern helps to highlight higher frequency components within the sample and double the spatial resolution compared with conventional techniques. SLM can very quickly switch between grating modalities (up to 1kHz), which together with high speed cameras allows significantly improved acquisition times up to few ms per colour per frame. In that case final temporal performance is no more limited by the hardware, but rather by sample brightness and photo-stability. To enhance the axial resolution performance, the SIM in that setup is combined with ~~total internal reflection microscopy~~ (TIRF) approach. TIRF takes advantage of the so-called critical angle, when light hits the interface between high to low refractive indexes media, at a certain angle it does not propagate through, but gets refracted at the interface. The small portion of light nonetheless goes beyond the interface in the form of an evanescent field, with a depth in a range of 100-300 nm that can be tuned by the incidence angle. That approach is used to restrict the effective PSF in the axial direction, creating optical sectioning which is 3-5 times smaller than the axial confocal resolution. In this setup, diffraction orders from the SLM hit the sample at the critical angles,

and consequently a SIM pattern is generated in TIRF modality. In that case, nine raw images are needed to reconstruct the final super-resolution image, leading to super-resolution imaging at an acquisition rate of 1kHz making TIRF-SIM an ideal candidate for live cell imaging and fast dynamics observation.

In a typical TIRF experiment, the total internal reflection is generated between the top cover-glass and the cell medium (**Figure. 1c**, see TIRF₁). Applying TIRF-SIM technology for TFM means that unlike in normal scenarios cells are deposited on the top of the elastic substrate, shifting the imaging plane 10-20 microns away from cover glass. Such depth is unachievable for the TIRF field. Gutierrez et al. proposed a novel approach: if a gel refractive index is chosen closest to the glass ($n_1=1.52$), using for instance silicone gel ($n_2=1.49$), it is possible to find the angles at which TIRF is generated on the interface between the gel and the cell media ($n_3=1.33$), as is schematically demonstrated in Figure 1d (see TIRF₂) [22]. This idea can be further extended by coupling with a SIM modality. In that case, it is not only the TIRF angle that should be experimentally matched to gel thickness but also the flatness of the gel surface becomes critical, as it needs to be smooth enough not to disrupt the SIM pattern. SLM based microscope architectures provide great flexibility in tuning the incidence angles by changing the grating patterns on the ferroelectric display. Hence, the TIRF depth and angle can be selected to match experimental needs. With TIRF-SIM TFM, Barbieri et al. [23] were able to achieve over 2.5-fold spatial and 10-fold temporal resolution enhancement in planar cellular force probing versus conventional modalities, making TIRF-SIM TFM a powerful tool for studying cells-interface interactions.

3D SIM TFM

TIRF-SIM TFM demonstrates great resolution performance, but due to the nature of the TIRF modality it is restricted to a single imaging plane, meaning only lateral displacements can be recorded, and hence only a two-dimensional force map can be reconstructed. As discussed, many biological processes take place in three dimensions, therefore there is a need to find techniques suitable to measure volumetric displacements with high precision. To this end, the SIM technology can be extended to a 3D mode by adding an additional excitation beam, creating structured patterns in the axial dimension resulting in an optical sectioning capability.

With minor modifications, the TIRF-SIM setup can be converted to 3D SIM. This requires uploading appropriate patterns to the SLM, changing the spatial mask to allow an extra illumination beam to pass through and also changing the objective from TIRF mode to one which has a refractive index matching the imaging media. This is a crucial parameter for 3D SIM, as the structured pattern is easily distorted by a refractive index mismatch. For conventional 3D SIM it is a matter of selecting an objective and immersion media to match with the sample refractive index. To achieve an axial resolution enhancement, 3D SIM requires 15 raw images to be taken at each plane for reconstruction, making the approach roughly 1.5 times slower versus TIRF-SIM, but it still ranges within a few hundreds of milliseconds per colour per frame, much faster result compared with scanning techniques. Confocal spinning disk microscopy can provide comparable imaging speeds, but at the price of diffraction limited resolution. By applying matching the substrate refractive index to the cover glass, like in TIRF-SIM mode, it became possible to perform TFM by using 3D-SIM [13]. Optimisation of the substrate thickness was an important step, as it was necessary to ensure it was sufficiently thick to avoid mechanical influence of underlying cover glass, but thin enough to minimize the optical aberration distortions. Consequently, 3D-SIM can be applied for the quantification of bead displacements at the upper surface of the 20 μm thick silicone gel. A two-colour 2 μm stack was acquired every 10s with using z step size of 142nm, so roughly 14 slices per stack [13]. Despite speeds being slower than TIRF-SIM approach, 3D SIM offers a unique capability of visualizing 3D forces with high sensitivity. Therefore, 3D SIM offers a great compromise for fast and high spatial resolution, with reduced photo-bleaching. This, together with availability of such microscopes at the market, makes it a great candidate for TFM studies.

3D astigmatic TIRF-SIM TFM

As we have mentioned previously, due to wave properties of light, the PSF is highly anisotropic. It is significantly stretched along the optical axes, which makes the axial resolution 2-4 times worse than the lateral one. Even super-resolution approaches, such as STED or SIM suffer from that drawback, and demonstrate much reduced spatial resolution enhancement in axial direction. In the previous section we have discussed an alternative way to enhance that resolution by restricting the imaging depth via the TIRF modality, which indeed can constrain the effective PSF to 100 nm in the z direction. But in that case only a single plane at the interface between substrate and sample can be visualised. Other techniques like 3D SIM, or confocal spinning disk can provide optical sectioning, but the axial resolution in that case will be at the range of several hundreds of nanometers. The technological breakthrough was developed for single molecule localisation microscopy (SMLM) techniques. SMLM is a group of wide field detection super-resolution methods, which can provide very high lateral resolution improvement down to a few tens of nanometers, thanks to the fitting of single emitters over multiple data frames. Nevertheless, the axial resolution for a long time was a challenge, and TIRF mode was used as a compromise for the data acquisition. To extend SMLM to 3D imaging, a so-called PSF engineering approach was introduced. By intentionally distorting the PSF, creating an axial aberration pattern the PSF shape can be correlated with the focus depth, and therefore the axial location of the individual emitter. There are a variety of PSF engineering methods, such as biplane detection, astigmatic imaging, and the double-helix PSF [24]. They all rely on the same basic principle and only differ by the shapes of the generated PSF. With an engineered PSF it is possible to achieve axial resolutions down to a few tens of nanometers and obtain isotropically super-resolved SMLM images. The only drawback of SMLM techniques is the acquisition speeds; they require enough statistical data for image reconstruction which means hundreds or thousands of images need to be acquired. Even though individual frames are recorded at ms speeds, cumulative times of obtaining a data set extends to minutes range. That makes SMLM not suitable for TFM. But the idea of engineered PSF can be applied for recording axial beads displacements. Fluorescent beads are multiple times brighter than single molecules, so the PSF pattern is very easily detectable. Li et al. applied the principle of astigmatic PSF for traction force measurements [25]. To adapt the TIRF-SIM setup for this technique, the wide-field and the TIRF-SIM illumination modalities are combined with the wide-field path used for the beads channel. Here, in the described imaging platform, the long working distance cylindrical lens is added before the beads channel camera at the detection path. The cylindrical lens introduces the necessary PSF aberrations, so the z-position of individual markers can be re-assigned to their actual position with respect to the focal depth. To ensure correct correlation of PSF shape with the focal depth, calibration of the system needs to be performed before the measurement. To visualise the sample, the TIRF-SIM illumination modality is used. Hence, the 3D astigmatic TIRF-SIM TFM utilises the engineered PSF approach for the beads channel, and TIRF-SIM illumination at the gel to media interface (same as in TIRF-SIM TFM approach), allowing the basal plane of the sample to be visualised at high spatial resolution. The bead data is analysed using a maximum likelihood estimation approach [26], allowing the bead height to be determined with a localisation accuracy of about 9 nm laterally and 22 nm axially [25]. The astigmatic bead data has a dynamic range of ~1 μm axially which can be captured in as little as 10 ms, with no need for axial scanning. This allowed force information to be quantified within a 1 μm volume every second improving the temporal resolution compared to scanning approaches, but also significantly reduces phototoxicity and photobleaching [25].

Microscopy	resolution	Illumination	TFM
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approach	Spatial (nm)		Temporal (s)	power (W/cm ²)	sensitivity (bead/μm ²)
	lateral	axial			
TIRF	300	100-300	0.1	20-100	1
confocal	200-300	600-800	0.1 – 10	10 ² -10 ⁴	1
STED	80-100	300	1 - 10	10 ³ - 10 ⁸	2.2 - 5
2D TIRF-SIM	80-100	150-300	0.1	20-100	15-20
3D SIM	90-100	300	1 – 5	20-100	1
3Da TIRF-SIM TFM	200-300	20	0.1	20-100	0.3

Table 1. Fluorescent based microscopy TFM approaches. The temporal resolution is estimated based on one single colour channel for a single image frame. All the SIM modalities refers to a super-resolved frame (9 raw images for 2D SIM, and 15 raw images for 3D SIM). The 3D SIM temporal resolution was calculated considering a 3D stack of 14 z-slices [13].

Discussion

We have presented three complementary TFM techniques. With minor modifications all of them can be built upon the same super-resolution TIRF-SIM setup. The TIRF-SIM TFM demonstrates unprecedented acquisition speeds, which together with high sensitivity makes it an ideal candidate for studies focused on lateral forces. The 3D SIM approach demonstrates slightly slower acquisition times, but can provide optical sectioning capabilities, so the volume of interest can be selected and 3D force map reconstructed. Lastly, 3D astigmatic TFM coupled with TIRF-SIM imaging allows for rapid volumetric force reconstruction together with enhanced SIM quality visualisation of sample at the basal plane. Table 1 summarises the discussed TFM approaches, comparing ranges of their spatial and temporal resolution from the optical system perspective, as well as the illumination powers and the TFM sensitivity [10,13,22,23,25]. As we have mentioned, the precision of forces detection is directly related to number of markers which we can reliably track from the given volume. Notably, a grid with regular sub-diffraction spacing could yield even better trackability of the fiducial marker beads. To simplify comparison between approaches, ~~here~~ we defined TFM sensitivity as highest beads density achievable per μm². Noticeable, each of the approaches demonstrate stronger and weaker performances for different parameters. Consequently, these approaches represent a complimentary suit of techniques that can be tuned to the biological system of interest. For example, to study 2D substrate interactions of a rapidly migrating cell, TIRF-SIM-TFM would be the ideal technique. Conversely, 3Da-TFM lends itself to the study the nano-scale axial forces present during immune cell surface interactions occurring on the sub-second time_{scale}.

The presented range of high performance TFM techniques and the previous advances in high-resolution TFM [9,10,13,27] users have the ability to balance high spatio-temporal acquisition with the requirements of the biological sample, finally making sensitive force probing suitable to live-cell measurements. Notably, SIM has become established as the primary live-cell

imaging super-resolution modality and is now widely used in this capacity. We therefore anticipate that 2D-TIRF-SIM-TFM and 3Da-TFM will provide researchers with a technology that is straightforward in its implementation, allowing new insights into the physiological role of force generation in a wide range of biological systems.

Authors' contributions

K.K., H.C.Y., L.B. and M.F. designed, structured, and wrote the article and its intellectual content.

Competing interests

The authors have no competing interests.

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