

Review

Imaging of cell migration

Dirk Dormann and Cornelis J Weijer*

Division of Cell and Developmental Biology, School of Life sciences,
University of Dundee, Dundee, UK

Cell migration is an essential process during many phases of development and adult life. Cells can either migrate as individuals or move in the context of tissues. Movement is controlled by internal and external signals, which activate complex signal transduction cascades resulting in highly dynamic and localised remodelling of the cytoskeleton, cell–cell and cell–substrate interactions. To understand these processes, it will be necessary to identify the critical structural cytoskeletal components, their spatio-temporal dynamics as well as those of the signalling pathways that control them. Imaging plays an increasingly important and powerful role in the analysis of these spatio-temporal dynamics. We will highlight a variety of imaging techniques and their use in the investigation of various aspects of cell motility, and illustrate their role in the characterisation of chemotaxis in *Dictyostelium* and cell movement during gastrulation in chick embryos in more detail.

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Introduction

Directed cell migration is a fundamental property of many cells and plays an essential role during the development of most organisms. It is essential for gastrulation, the process where the endoderm and mesoderm take up their correct topological positions in the embryo, the formation and the wiring of the nervous system, and it plays a major role in the development of organs such as heart, lungs, kidneys and the intestinal tract. When during these processes cell movements are not properly controlled, it results in severe congenital defects and disease. Also in adult life, cell movement continues to play an extremely important role in processes such as wound healing and the functioning of the immune system. Cell migration is a highly complex process that is mediated by dynamic changes in the actin–myosin cytoskeleton, and

can be modulated by the microtubule system and associated motors and in some cells through interaction with the intermediate filament systems. Dynamic remodelling of the cytoskeleton is responsible for all cell behaviours and has to be precisely regulated in space and time, both in response to external as well as internal signals. It is now generally thought that localised actin polymerisation in the front of the cell provides a critical driving force for lamellopodia extension, while contraction of the back of the cell, a myosin-II-dependent process, is necessary for retraction of the rear but it also provides an additional driving force for the cell to move forward, through the generation of a hydrostatic pressure resulting in a cytoplasmic flow forward. Under some conditions, this may result in blebbing at the leading edge, in cases where the membrane dissociates from the underlying actin cortex (Pollard and Borisy, 2003; Ridley *et al.*, 2003; Affolter and Weijer, 2005; Vicente-Manzanares *et al.*, 2005; Langridge and Kay, 2006; Sheetz *et al.*, 2006). The intracellular components such as nucleus, mitochondria, endoplasmic reticulum and Golgi also all have to be transported during cell movement; however, the precise mechanisms coupling cell motility to movement of these organelles are so far unknown. It likely involves active transport of these structures along microtubules and the actin–myosin network. Cell movement not only requires the development of forces inside the cell but also requires these forces to be transmitted to the substrate to get traction. Furthermore, interactions with other cells are important and these contacts are mediated by specialised cell–matrix and cell–cell attachment complexes containing integrins and cadherins, respectively (Hynes, 2002; Nelson and Nusse, 2004; Giannone and Sheetz, 2006). These contacts also have to be very dynamic to allow translocation of the cell, which implies that they have to turnover rapidly. Since the cytoskeleton has such a vital function, the regulation of its activity is one of the major tasks the cell faces. This involves a very complex regulatory machinery of several hundreds proteins, which are the targets for extra- as well as intracellular signalling pathways. Understanding such a complex machine and one of its tasks, cell movement, is a major challenge, which will be impossible without a detailed knowledge of the components, their dynamics and insight in how these chemical reactions generate forces that ultimately do the work to result in cell movement (Dobereiner *et al.*, 2005; Giannone and Sheetz, 2006).

Understanding movement of single cells and cells in tissues requires the analysis of these complex processes under normal and perturbed conditions. Ideally one would like to measure movement and shape changes of cells and correlate these with the spatio-temporal dynamics of the cytoskeletal elements and the extra- and intracellular signalling pathways controlling these behaviours in isolated cells as well as in cells in tissues. Imaging is an excellent method to

*Corresponding author. Wellcome Trust Biocentre, Division of Cell and Developmental Biology, School of Life sciences, University of Dundee, Dundee DD1 5EH, UK. Tel.: +44 1382 345191; Fax: +44 1382 345783; E-mail: c.j.weijer@dundee.ac.uk

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obtain such data since sections through biological specimens can be readily collected at high spatial resolution and with high dynamic range and subsecond temporal resolution. Through the advent of genetically encoded labels, it is now becoming possible to label many of the cellular and cytoskeletal components, without interfering with their function (Zhang *et al.*, 2002; Miyawaki *et al.*, 2003, 2005; Chudakov *et al.*, 2005). In several systems these molecules can be expressed under the control of their own promoters, using knockin techniques, which ensures that they are expressed at physiological concentrations in the correct cell types and at the right stages of development. This allows visualisation of the dynamics of many of the key structural elements of the cytoskeleton, such as actin and actin binding proteins, myosin motors, microtubuli and associated motor proteins, microtubule binding proteins and intermediate filament components. Furthermore, it is possible to label proteins with tags that can at any given time later be modified with highly efficient fluorophores, which enables to perform pulse label experiments and follows the turnover of important cytoskeletal components (Chudakov *et al.*, 2005; Giepmans *et al.*, 2006). It has also become possible to visualise many of the signalling components that control the assembly/disassembly of the cytoskeleton such as PI3 kinase, PTEN, members of the family of Rho GTPases and their regulators (GAP's and GEF's), downstream targets such as members of the Wasp/Scar/Wave families and more structural components such as formins and components of the ARP2/3 complex (Ridley *et al.*, 2003; Lorenz *et al.*, 2004; Manahan *et al.*, 2004; Zigmond, 2004; Nakamura *et al.*, 2006). Activity can be measured through changes in the cellular distribution of these components, changes in intramolecular conformation or complex formation with other signalling molecules. Changes in distribution for instance from the cytoplasm to the membrane are simple to follow, while changes in conformational state or complex formation require more sophisticated measurements such as fluorescence resonance energy transfer (FRET) measurements or correlation spectroscopy (Stephens and Allan, 2003; Bacia *et al.*, 2006).

Imaging of fixed specimens

Much of our knowledge of the internal structure and organisation of cells comes from the investigation of fixed specimens, followed by detailed investigation using a combination of light microscopic and scanning and transmission electron microscopic techniques. These investigations have given a good understanding of the fundamental cytoskeletal components and their macro-molecular architecture. These studies have been instrumental for our understanding of the actin-myosin cytoskeleton, the microtubule network and the organisation of intermediate filaments (Pollard and Borisy, 2003; Mejillano *et al.*, 2004; Green *et al.*, 2005). Especially antibody staining of specific components in combination with light and electron microscopy has proven to be very powerful. The development of confocal and deconvolution microscopy has had an enormous impact on our understanding of cell structure through obtaining excellent three-dimensional (3D) images of cells at high spatial resolution (Wallace *et al.*, 2001).

Electron microscopy has also made great advances in imaging 3D structures. Especially recent developments of EM tomography and sectioning scanning electron microscopy

have yielded new insights in the 3D structural organisation of cells and now allow the collection of large-scale images at nm resolution (O'Toole *et al.*, 2002; Denk and Horstmann, 2004; Nicastro *et al.*, 2005). Another recent development is cryo-electron microscopy, which has given insight in the intracellular organisation of cells and their organelles (Baumeister, 2005; Nickell *et al.*, 2006). Although these techniques have not been applied extensively to investigate migrating cells in particular, it is to be expected that they will have considerable impact on our structural understanding of migratory cells, even if they only allow imaging of fixed specimens, which by default gives only a snapshot of the dynamic behaviour of a migrating cell. A technique that can be used to image surfaces of living and fixed specimens and covers size scales from the molecular to the cellular level is atomic force microscopy (AFM). In general, a cantilever is used to follow the 3D contours of the specimen under investigation and the deflection of the cantilever is measured with high precision (Simon and Durrieu, 2006). This method can also be used to probe the mechanical properties of the migrating cells (Radmacher, 2002; Laurent *et al.*, 2005; Park *et al.*, 2005; Trache and Meininger, 2005) as well as the strength of cell-cell adhesion and cell-substrate adhesion (Vedula *et al.*, 2005; Wu *et al.*, 2005).

Imaging methods used to study cell migration

Wide field, deconvolution and confocal microscopy

The starting point for many investigations of cell movement is to characterise the behaviour of cells, by recording shape changes, direction and persistence of movement and the dynamics of subcellular processes such as extension and retraction of lamellipodia, pseudopods, filopodia and membrane ruffling (Soll *et al.*, 2003; Sepich and Solnica-Krezel, 2005). These investigations make extensive use of light microscopy methods, especially wide-field microscopy in either phase contrast or Nomarski Interference Contrast modes. Images are collected by CCD cameras as simple sections or as Z stacks. These time series then have to be analysed, which may involve image segmentation and reconstruction of 3D surfaces, followed by procedures to recognise particular behaviours and statistical analysis.

However, in order to understand cell behaviours, it is necessary to acquire a detailed understanding of the spatio-temporal control of the cytoskeleton. This can be achieved by following the dynamics of GFP tagged cytoskeletal components, making use of the great variety of fluorescent proteins with different spectral properties and biochemical characteristics now available (Zhang *et al.*, 2002; Miyawaki *et al.*, 2005; Shaner *et al.*, 2005). The combination of GFP tagged proteins with wide-field deconvolution microscopy and confocal microscopy, both techniques that have significantly improved resolution along the Z-axis, allows the 3D reconstruction of the cytoskeletal dynamics in isolated cells (Wallace *et al.*, 2001; Swedlow and Platani, 2002; Gerisch and Muller-Taubenberger, 2003). Furthermore, GFP tagging is now being used in large-scale genomic screens to define the behaviour of important signalling components, some of which are expected to play key roles in the control of the cytoskeleton. Screens that are performed include screen of all proteins containing phosphatidyl-inositol lipid binding domains or

screens investigating the translocation of protein kinases and phosphatases as part of their activation (Teruel and Meyer, 2000, 2002; Gilman *et al.*, 2002). Other methods of tagging molecules that hold considerable promise for the future are labelling of intracellular small epitopes (Adams *et al.*, 2002; Martin *et al.*, 2005; Giepmans *et al.*, 2006) and multicolour labelling of extracellular surface fusion proteins through suicide substrates that can be used for FRET studies (Vivero-Pol *et al.*, 2005; Meyer *et al.*, 2006).

FRAP/FLAP

In many instances it is of considerable interest to measure the exchange dynamics of molecules under investigation and deduce transport and flow properties from these measurements. A technique that is widely used is fluorescence recovery after photobleaching (FRAP), which measures the movement of labelled molecules back into a selected area after local destruction of the reporter molecule by high-intensity light irradiation (Stephens and Allan, 2003). FRAP has been used extensively to measure the exchange rate of proteins in specific structures in cells. A further development of this technique is FLAP, a technique in which populations of molecules of interest, for instance actin, are labelled with two distinct fluorophores. One fluorophore is bleached and by measuring the ratio between bleached and nonbleached molecules, it is possible to deduce not only the diffusion and flow into the bleached spot but also the diffusion and flow into other areas of the cell (Zicha *et al.*, 2003). Using this method it has been shown that delivery of actin to protrusions in the leading edge of migrating cells exceeds 5 $\mu\text{m/s}$, which would suggest that this delivery is faster than diffusion and is most likely flow driven.

There also appears much promise for the application of fluorescent proteins that can be photo-activated or inactivated by pulses of excitation light (Patterson and Lippincott-Schwartz, 2002; Habuchi *et al.*, 2005; Gurskaya *et al.*, 2006). This allows pulse label experiments to follow the behaviour of selected groups of labelled proteins especially their dispersal from the site of labelling and will be of great help in dissecting the dynamics of protein translocations during cell movement as has recently been performed for actin crosslink dynamics and their role in stress fibre formation (Hotulainen and Lappalainen, 2006) and the analysis of focal adhesion dynamics (Webb *et al.*, 2003).

TIRF

A method that is been used extensively to measure the dynamics of proteins associated with the plasma membrane is total internal reflection microscopy (TIRF), which allows measurement of molecules in close proximity to the membrane and when used in combination with high sensitivity detection can even result in the detection of the dynamics of single molecules (Yokota *et al.*, 2004). TIRF has been used extensively to investigate the dynamics of the actin myosin cytoskeleton, the targeting of adhesion sites by microtubuli and the dynamics of cell substrate adhesion and focal adhesion sites (Webb *et al.*, 2003). These measurements can in principle be supplemented by correlation spectroscopy measurements, which can deduce from the transit times of single molecules in a very small volume (typically a volume illuminated by the focal spot of a confocal microscope) whether it moves by diffusion or flow and whether it interacts with

other molecules (Bacia *et al.*, 2006). This is still a very experimental technique and has not yet been applied extensively to the investigation of cell motility.

Speckle microscopy

It was observed that the expression of limiting amounts of fluorescently labelled molecules, especially actin, results in a speckled appearance of these molecules in the polymerised (actin-) filaments (Waterman-Storer and Danuser, 2002). High-resolution imaging enables the analysis of the dynamics of these speckles and gives insights into the dynamics and flows of actin filaments in migrating cells. These experiments have given direct support for the retrograde flow of actin away from the leading edge to the base of the lamellipodia, where the flow reverses (Salmon *et al.*, 2002; Watanabe and Mitchison, 2002). The extent of retrograde flow has been shown to be dependent on the coupling of the cytoskeleton to adhesion sites and the rigidity of the substrate (Jurado *et al.*, 2005). These speckle methods are expected to be extremely useful to study and understand actin dynamics in migrating cells under normal and perturbed conditions.

FRET

FRET can be used to measure conformational changes in proteins and the dynamics of protein-protein interactions (Periasamy and Day, 1999; Harpur *et al.*, 2001). The use of FRET techniques to measure protein-protein interactions will become more important with the development of highly efficient FRET indicators (Miyawaki and Tsien, 2000; Kurokawa *et al.*, 2001; Truong *et al.*, 2001). Most experiments measure a change in FRET efficiency as a change in the ratio of the emission wavelength of the fluorescence donor to that of the acceptor. However in some cases, fluorescence lifetime measurements might be better suited, since these measurements are independent of the fluorophore concentrations (Harpur *et al.*, 2001). The drawback of these measurements is that they require more sophisticated equipment (fast pulsed lasers or modulated light) and the calculations needed to analyse the experimental data can be rather complex (Pelet *et al.*, 2004). FRET can be combined successfully with TIRF measurements and in extreme cases it is even possible to perform FRET measurements at the single molecule level. For instance, it has been shown recently that Ras conformation spontaneously fluctuates to enable the interaction with its various reaction partners (Arai *et al.*, 2006).

Force microscopy

An important question in understanding cell movement is how and where the motive and friction forces are generated and how individual molecules contribute to these. Methods have been developed to measure local forces associated with cell movement through the measurement of the deformation of elastic substrates spiked with fluorescent tracer beads (Dembo and Wang, 1999; Oliver *et al.*, 1999). When cells crawl over them, it is possible to deduce from an observed deformation field a force field using linear elasticity theory. These methods were originally developed for large cells such as fibroblasts, but they can be successfully adapted to cells of any size by adjusting the elastic parameters of the gels under investigation and can even be extended to tissues (Rieu *et al.*, 2005). Variations on these measurements use elastic patterned substrates consisting of arrays of elastic pillars. The

bending of individual pillars is then measured to get a local readout of the forces produced (du Roure *et al.*, 2005). These force measurement can be used in conjunction with other tools such as pico-second laser cutters that allow severing individual actin stress fibres and/or microtubules to investigate the role of these components in generating compressive and tensile forces in the cell (Kumar *et al.*, 2006). Other useful techniques that can be combined with the analysis of chemotactic cell movements are microfluidic devices that allow hydrodynamic forces to be exerted and precise generation and manipulation of signal gradients and even electrical fields that have been shown to be able to orient cell migration (Zhao *et al.*, 2002; Fache *et al.*, 2005; Diao *et al.*, 2006; Song *et al.*, 2006).

Two photon confocal microscopy

Many experiments are performed in cells on plastic culture dishes or on coated glass substrates, and such are not a very natural environment for most cells that normally are either embedded in a tissue such as an epithelial sheet or embedded in a 3D extracellular matrix. It will become more and more important to analyse cell behaviour in the context of its more natural environment (Webb and Horwitz, 2003). However, observing the behaviour of cells and their intracellular components in multicellular tissues poses special problems for imaging. In general, these samples are not as transparent and as flat as cells on coverslips and there is much more light-scattering in the sample. Another challenge for the future will be to apply these methods to the measurement of the dynamics of reactions underlying cell movement in the context of whole organisms and especially embryos. This is an area where confocal and deconvolution microscopes have been used extensively so far. Advances have been made in the observation of cells in tissues using two photon confocal microscopy, which due to the longer excitation wavelengths and reduced light scattering allows imaging of cells deeper into the tissue (Helmchen and Denk, 2002; Stephens and Allan, 2003; Helmchen and Denk, 2005). Two photon excitation happens normally only in the focus point of a high NA optical lens and thus results in a limited area of excitation, as a result a two photon microscope does not require a pinhole to reject out of focus emission light, all emitted photons can be collected since they are known to originate from a small confocal volume. The excitation by pulsed laser light at longer wavelengths, which is less subject to light scattering, results in a larger penetration depth than normal confocal microscopy (Helmchen and Denk, 2005). Two photon microscopy has therefore been used to study the migration of cells in organisms. Another advantage of using two photon confocal microscopy is that it can easily be adapted to study FRET through fluorescence lifetime imaging and exploit the principle of second harmonic generation to show structural elements of the extracellular matrix on which cells migrate. Collagen fibres, for instance, have been visualised to analyse the migration of tumour cells *in vivo* (Sahai *et al.*, 2005; Cox and Kable, 2006).

SPIM

Another technique that has been developed recently is Single Plane Illumination Microscopy (SPIM) (Huisken *et al.*, 2004; Keller *et al.*, 2006). This technique seems to be very useful for the imaging of large specimens with low magnification and

thus low numerical aperture (NA) lenses. SPIM uses a combination of plane illumination of the object with capture of the images using a cooled CCD camera at an angle of 90°. This significantly reduces the out of plane fluorescence, which results in reduced exposure of the sample to light and due to the use of a CCD to record the images results in an improved signal-to-noise ratio with a larger dynamic range. Since the pointspread function of the collection system is at right angles with the plane of illumination, the area from which fluorescence photons are collected becomes almost isotropic, which helps in 3D image reconstruction and quantitative measurements of fluorescence intensities in the 3D volume.

Fast imaging and spinning disk confocal microscopy

Other improvements that have to be made are to use imaging regimes that work both at high resolution and fast time scales to capture events such as very fast calcium waves associated with neutrophils polarisation and chemotaxis (Kindzelskii and Petty, 2003) or even rapid changes in the dynamics of the actin cytoskeleton as observed at high magnification during migration of fast moving cells such as *Dictyostelium amoebae* (Diez *et al.*, 2005). The speed problem is partly solved by the use of a spinning disc confocal microscope that excites many points simultaneously and makes faster data collection possible (Stephens and Allan, 2003). However there are still practical limitations to the process and the dynamic range of the recorded signals is typically small for fast data collection times, which generally require highly sensitive camera system that greatly amplify the signals recorded, resulting in considerable noise (Kindzelskii and Petty, 2003).

Finally, all these imaging experiments generally result in the acquisition of large quantities of 3D image stacks from many successive time points and thus generate large data sets that need to be properly annotated, analysed and stored, and there are still many challenges that need to be met before these problems are solved (Swedlow *et al.*, 2003; Goldberg *et al.*, 2005).

To illustrate how some of these techniques have been used to investigate the mechanism and role of cell movement in development, we will describe now the control of chemotactic cell movement in *Dictyostelium* and the analysis of cell movement during gastrulation in vertebrate embryos.

Imaging chemotaxis

One of the major questions in understanding cell movement is how external signals modulate the cytoskeletal machinery to result in translocation and movement. One of the mechanisms that cells use to move in a particular direction is to respond to gradients of chemicals, which they can detect through cell surface receptors. If the movement involves direction sensing followed by directed migration up or down the chemical gradient, this process is known as chemotaxis (Affolter and Weijer, 2005). Extracellular signals are detected by membrane receptors that have an extracellular ligand binding domain and an intracellular domain that is involved in signal transduction to signalling pathways that locally modulate the cytoskeleton to result in directed motion.

Chemotaxis in *Dictyostelium*

One of the experimental model systems that is used extensively to study chemotactic cell movement through the use of a variety of imaging techniques is the social amoebae *D. discoideum* (Weijer, 2004). *Dictyostelium* cells live as single amoebae in the soil and multiply through binary fission. Starvation triggers a developmental cycle in which hundreds of amoebae aggregate to form a multicellular structure, the slug that migrates away guided by environmental signals such as light and temperature gradients, which direct its migration to the surface of the soil. There the slug transforms into a fruiting body, consisting of a stalk supporting a mass of spores. Eighty percent of the cells differentiate into spores, while the remainder of the cells die and form the stalk (Figure 1A). The chemotactic aggregation process is known to result from chemotactic movement in the direction of cAMP waves initiated by the aggregation centre and relayed by surrounding cells outward. These waves trigger the inward movement of the amoebae during the rising phase of the waves, resulting in movement towards the aggregation centre (Weijer, 2004). Due to an adaptation process, the amoebae are insensitive to falling levels of cAMP, this prevents them from turning around and chasing the waves, once they pass, instead the cells wait for the next wave to come to guide them a little closer towards the aggregation centre. During movement cells are more polarised and exhibit a slightly elongated shape, in between waves, when they are not moving the cells appear more amoeboid in shape (Figure 1B; Supplementary Movie 1). This change in cell morphology can be detected at the macroscopic level by measuring small angle light scattering in monolayers of aggregating cells on an agar plate (Figure 1D, Supplementary Movie 2; Siegert and Weijer, 1989). The light-scattering changes are small but the differences can be made better visible by a rolling subtraction of successive images that amplifies the differences between the different time frames, and thus sensitively detects movement of the wave front (Figure 1E; Supplementary Movie 2; Sawai *et al*, 2005). The propagating waves are thus visible as alternating bands of moving and nonmoving cells. It has been shown by isotope dilution fluorography and a temperature-sensitive adenylyl-cyclase mutant that these optical density waves accurately reflect the dynamics of the underlying cAMP signalling (Devreotes *et al*, 1983; Patel *et al*, 2000). These waves can be even detected when the cells start to aggregate into aggregation streams (Figure 1F and 1G; Supplementary Movie 3).

Much attention has focussed on the mechanism of cAMP signal detection, which results in polarisation of the cells, localised activation of the actin myosin cytoskeleton and directed movement up the cAMP gradient (Manahan *et al*, 2004; Parent, 2004; Van Haastert and Devreotes, 2004). We

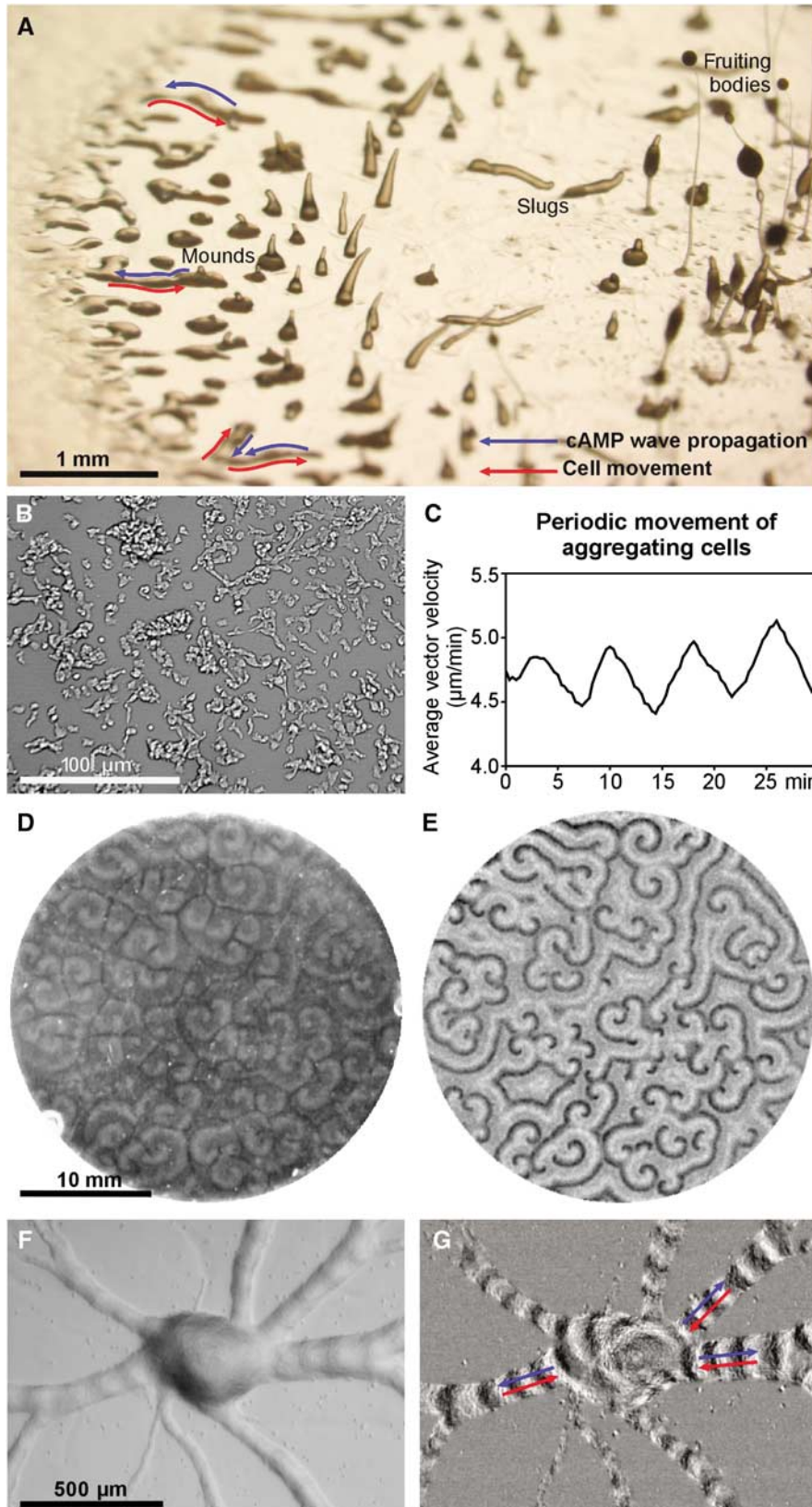
will now describe some of the imaging methods used to investigate chemotactic signalling pathways controlling the localised activity of the actin-myosin cytoskeleton to result in motion up cAMP gradients. cAMP is detected by seven transmembrane serpentine receptors. Sensing of a cAMP gradient by a moving cell results in localised PIP3 production, actin polymerisation and disassembly of myosin thick filaments at the leading edge, while the contraction in the back of the cell requires myosin thick filament assembly and motor activation (Figure 2A). Through monitoring the dynamics of GFP tagged cAMP receptors, it was shown that the receptors are homogeneously distributed in the membrane of cells chemotaxing in gradient of cAMP (Xiao *et al*, 1997). TIRF based single molecule analysis of fluorescent cAMP molecules has been used to measure the spatiotemporal dynamics of cAMP binding to the cAMP receptors (Ueda *et al*, 2001). Individual cAMP molecules stay bound only for a few seconds before dissociating again. cAMP molecules associate/dissociate faster in the front than in the back of the moving cells, which has been shown to reflect differences in coupling of the cAMP receptor to hetero-trimeric G proteins in the front and the back of the cell (Ueda *et al*, 2001). Binding of cAMP to the receptors triggers the activation of hetero-trimeric G proteins which dissociate into a GTP bound $G\alpha$ subunit and a $G\beta\gamma$ complex. FRET measurements between GFP tagged $G\alpha$ and $G\beta$ subunits have shown that the $G\alpha$ and $G\beta\gamma$ subunits stay dissociated as long as the receptor is stimulated (Janetopoulos and Devreotes, 2002) and that the gradient of cAMP is translated into a proportional gradient of G protein activation around the cell (Xu *et al*, 2005). The membrane bound $G\beta\gamma$ complex activates RasC, which also follows the gradient of activated cAMP receptors (Sasaki *et al*, 2004). Activated RasC in turn binds and activates PI3 kinase, which results in localised PIP3 production at the leading edge of the cell (Figure 2A and B). PIP3 production shows both amplification and adaptation (Janetopoulos *et al*, 2004). PTEN, a PIP3 phosphatase, which is membrane associated in unstimulated cells, dissociates from the membrane at the leading edge of the cell but stays membrane associated in the rear of the cell (Figure 2C). Single molecule measurements have shown that binding of CRAC (cytosolic regulator of adenylyl-cyclase) to PIP3 is highly dynamic (100 ms) and it accurately reflects the PIP3 levels (Matsuoka *et al*, 2006). Membrane binding of the PIP3 phosphatase PTEN has also been measured using single molecule imaging techniques. It has been shown that binding of PTEN to membrane binding sites of as yet unknown nature is also very dynamic. In the case of *Dictyostelium* PTEN, the dissociation rate is ~ 300 ms, sufficient to result in a visible membrane accumulation. In the case of mammalian PTEN, the binding duration is much shorter (~ 150 ms), which is not sufficient to result

Figure 1 Cell movement and cAMP wave propagation in aggregating *Dictyostelium* cells. (A) View of a *Dictyostelium* colony growing on a bacterial agar plate. Cells at the left are vegetative and feeding on bacteria, behind the feeding front the cells start to aggregate in aggregation streams. In the middle the aggregates develop into mounds and standing slugs and towards the right there are a few fruiting bodies, consisting of a stalk that supports a mass of spores. Blue arrows indicate the direction of wave propagation, while red arrows indicate the direction of cell movement. (B) *Dictyostelium* cells aggregating on a coverslip submerged in phosphate buffer 5 h after the onset of starvation. Cells communicate by propagating cAMP waves that cause the coordinated chemotactic response of cells towards the cAMP source. (C) Measuring the optical flow over the entire image and calculating the average vector velocity reveals the periodic movement of the cells in response to cAMP waves. (D) *Dictyostelium* cells that have been plated in a monolayer on agar show the characteristic macroscopic dark field waves that correspond to the relayed cAMP waves. (E) Subtraction of subsequent images highlights the differences between the images and enhances the visibility of the rapidly moving dark field waves. Same field of view as in (D). (F) Cells continue to move towards the aggregations centres/mounds in long streams. (G) Image subtraction shows again the propagated wave fronts that move away from the centre while the cells move in the opposite direction (see Supplementary Movies 1–3).

in visible membrane accumulation (Iijima *et al*, 2004; Vazquez *et al*, 2006).

Localised PIP3 production at the leading edge of the cells results in localised activation of downstream signalling path-

ways, which involve activation of Rac which—through the activation of the Wasp and Scar/Wave complexes—ultimately results in actin polymerisation (Blagg *et al*, 2003; Blagg and Insall, 2004; Ibarra *et al*, 2005). *De novo* actin polymerisation



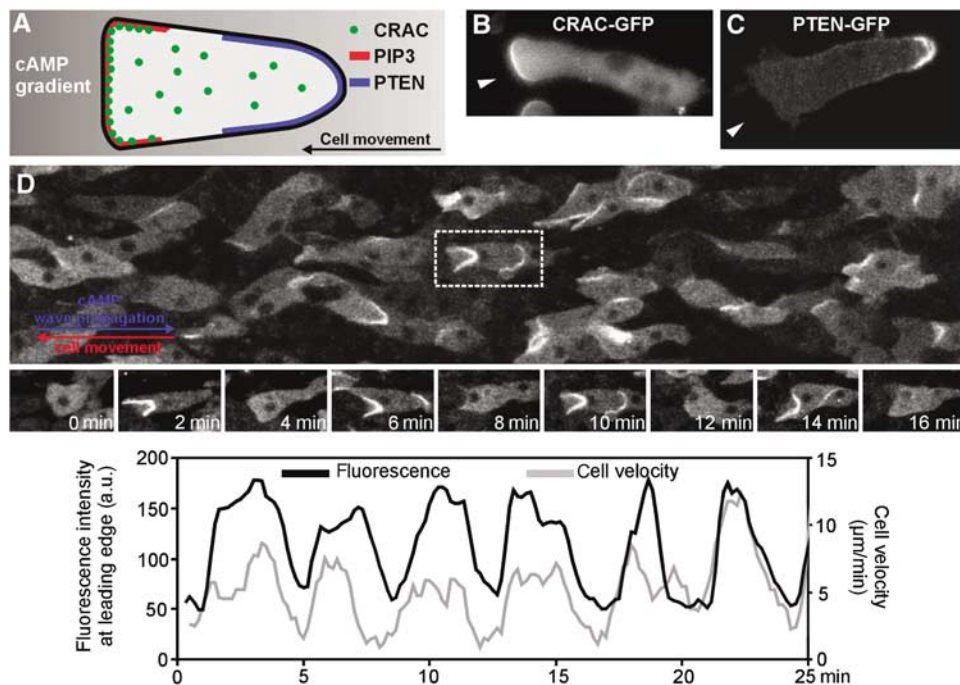


Figure 2 Periodic translocation of PIP3 binding PH domains to the leading edge. (A) Schematic diagram of a cell migrating from right to left in a gradient of cAMP. The process of signal detection involves binding of cAMP to cell surface receptors, reflecting the gradient in cAMP. This results in localised PIP3 production in the leading edge (red), which acts as a docking site for PIP3 specific PH domain containing proteins such as CRAC (green). The PIP3 gradient is the result of the translocation of PI3 kinase to the front and dissociation of the PIP3 3-phosphatase PTEN (blue) from the front, while remaining membrane bound in the back where it degrades PIP3. (B) Cell moving chemotactically in a cAMP gradient from right to left showing the binding of GFP tagged CRAC to the front of the cell. (C) Cell moving chemotactically in a cAMP gradient from right to left showing the binding of GFP tagged PTEN to the plasma membrane in the rear of the cell. (D) Cells moving in an aggregation stream. Cell express the PIP3 binding PH domain of CRAC fused to GFP, which translocates to the leading edge in response to the external cAMP waves. The red arrow shows the direction of cell movement and the blue arrow shows the direction of signal propagation. Snapshots of the highlighted cell taken at 2-min intervals show the changes in membrane translocation of CRAC-GFP and cell shape as cAMP waves sweep across the cells in the aggregation stream. The graphs show the fluorescence changes at the leading edge (measured in a 10×10 pixel window at the anterior plasma membrane), indicating the periodic production of PIP3 at this site which also correlates with the changes in cell velocity (see Supplementary Movie 4).

is required for further accumulation of PI3 kinase at the leading edge of the cell and further amplification of PIP3 production at the leading edge of the cell (Sasaki *et al*, 2004). Analysis of the cytoskeleton in moving cells has shown that actin polymerisation is highly dynamic. Using TIRF microscopy it can be seen that actin filaments are generated and remodelled everywhere in the cell, not only at the leading edge (Bretschnieder *et al*, 2004; Diez *et al*, 2005). Furthermore, experiments that investigate the dynamics of actin polymerisation after recovery from latrunculin treatment show that the kinetics of actin polymerisation behaves as an excitable system and often results in the formation of actin polymerisation wave fronts (Gerisch *et al*, 2004). These waves arise from random centres in the cell and propagate along existing and forming filaments. Wave speeds recorded are in the order of $0.5 \mu\text{m/s}$ and there is evidence that the propagation of the waves may depend on Myosin I activity. It is thought that this highly dynamic organisation of the actin cytoskeleton is required for a rapid response to changing extracellular information; furthermore, it is hypothesised that the propagating actin waves in particular could prime the system for a fast chemotactic response (Diez *et al*, 2005).

There have been extensive studies in which cell behaviour has been studied in natural waves of cAMP, revealing highly complex behavioural changes. During their migration up the chemotactic gradient cells stick out pseudopodia and filo-

podia that point up the gradient (Heid *et al*, 2005). These responses are modulated by internal cAMP concentrations through the activation of cAMP-dependent protein kinase (Zhang *et al*, 2003; Stepanovic *et al*, 2005).

Cell movement during multicellular development

It is becoming apparent that all stages of the *Dictyostelium* life cycle are controlled by chemotaxis of individual cells to propagating waves of cAMP. These optical density waves can also be visualised by measuring the translocation of the PH domain containing protein CRAC, which is required for activation of the aggregation stage adenylyl cyclase (Insall *et al*, 1994). At the population level, it can be seen that CRAC translocates to the leading edges of the cells in response to the cAMP waves and that this results in propagation of waves of CRAC-GFP translocation, thus providing a readout for the cAMP signalling system at the single cell level (Figure 2D; Supplementary Movie 4; Dormann *et al*, 2002a, b, 2004).

Once the cells have aggregated the aggregates become hemispherical and the cells start to differentiate into the precursors of the stalk and spore cells. This is characterised by cell type specific gene expression, which can be followed by expression of different GFPs under the control of cell type specific promoters. The prestalk cells now sort out from the prespore cells through more efficient chemotaxis, resulting

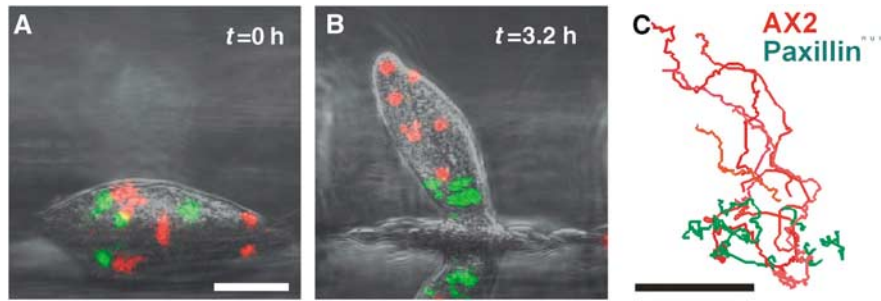


Figure 3 Cell sorting during slug formation. (A) Side view of a mound of wild-type cells (red) and fluorescently labelled PaxB^{null} cells. Scale bar: 50 μm. (B) As the mound transforms into a slug, the dispersed paxB^{null} cells are restricted to the posterior of the forming slug. (C) The corresponding cell traces of paxB^{null} cells (green, $n = 7$ cells) and Ax2 cells (red, $n = 7$) indicate reduced motility and directionality of paxB^{null} cells during slug formation. Scale bar: 50 μm (see Supplementary Movie 5).

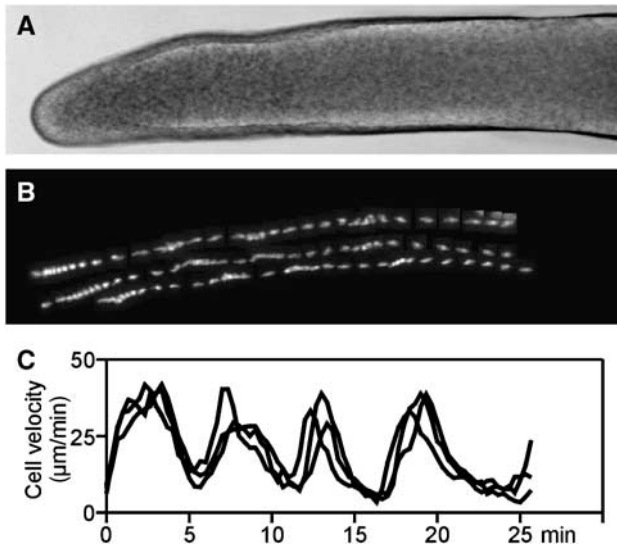


Figure 4 Periodic cell movement at the slug stage of development. The cAMP waves that coordinate cell movement in the slug are initiated in the slug tip (A: brightfield image of a slug) and relayed along the length of the slug causing periodic surges in cell velocity as can be seen in the tracks of the three GFP labelled cells (B) and in the corresponding velocity plots (C) (see Supplementary Movie 6).

in them accumulating towards the tip of the mound. Since *Dictyostelium* cells move fast (up to 20 μm or two times their own length/minute), it is necessary to take images frequently and we normally take image stacks every 5 or 10 s. These are normally viewed from the side, which allows us to plot the trajectories of the sorting cells (Figure 3; Supplementary Movie 5).

There is good evidence that cAMP wave propagation still controls the movement of the cells in the *Dictyostelium* slug. Optical density waves can be seen to propagate from the tip of the slug towards the back of the slug. Measurement of the movement of cells in the slugs has shown that this movement is periodic with a period of around 5 min (Figure 4; Supplementary Movie 6). Analysis of signalling has shown that the periodic movement is also controlled by propagating cAMP waves. PIP3 is produced in the leading edge of the cells and PTEN localises in the back of the slugs as in the earlier stages of development. This gives rise to the idea that the polarity of cell migration is still controlled by localised PIP3 signalling (Dormann *et al.*, 2002a, 2004). In later develop-

ment, the movement of the cells becomes also heavily dependent on contacts with the extracellular matrix. Deletion of molecules involved in mechano-transduction all result in defects of development, notable examples are paxillin, talin and Myosin II (Patterson and Spudich, 1996; Tsujioka *et al.*, 2004; Bukharova *et al.*, 2005). In the slug stage, the cells are dependent on the formation of focal adhesion-like plaques, these can be observed in great detail in TIRF microscopy where events occurring close to or at the plasma membrane are recorded especially well (Figure 5; Supplementary Movie 7). Direct observation of the actin myosin cytoskeleton shows that actin filaments are formed at the leading edge and that there is relatively little evidence for retrograde flow of actin. The myosin thick filaments are assembled in the trailing half of the cells. These filaments can be easily visualised by TIRF microscopy (Figure 5, Movies 8 and 9).

Force microscopy of chemotaxing cells

We have measured forces produced by chemotactically migrating *D. amoebae* (Figure 6). The advantage of *Dictyostelium* is that they move chemotactically and that mutants in elements of the cytoskeleton and cell substrate adhesion machinery as well as in the signalling pathways that control them are available. It appears that the most of the force is generated to control particular cell behaviours, like holding on to the substrate and detaching the back of the cell from the substrate. Furthermore, these measurements have confirmed the role of myosin II in the contraction of the back of the cells, but also show that it is involved in controlling the number of filopodia in the front of the cell (Figure 6). This type of analysis is expected to give important new insights into the biomechanics of cells movement.

Other mechanical properties that have been measured using imaging are the bending stiffness of the cell cortex. In these experiments, part of a cell membrane is sucked into a capillary and measurement of the curvature of the membrane under these conditions gives an indication of the bending stiffness of the membrane and associated cytoskeleton. Experiments of this type in *Dictyostelium* showed the importance of talin in determining the bending stiffness (Merkel *et al.*, 2000). In general, it is to be expected that force measurements will become more important and that several techniques that are able to measure the rheological and other mechanical properties of cells are going to be combined with

imaging experiments (Dobereiner *et al*, 2005; Park *et al*, 2005; Giannone and Sheetz, 2006).

Cell movement during gastrulation

There is more and more interest in trying to investigate the movement of cells in the context of a tissue or even a whole organism. It is especially important for our understanding of development where cell movement plays a major role in all stages of development especially

during gastrulation (Solnica-Krezel, 2005). Gastrulation has been investigated in great detail in frog, fish, chick and mouse embryos (Keller, 2005). We will describe here some results obtained in mapping out the movement patterns of mesoderm cells during gastrulation in the chick embryo and movement of cells in the epiblast during streak formation.

During gastrulation in the chick embryo, a structure called the primitive streak is formed in the central midline of the embryo. Cells in the epiblast move towards the primitive streak where they undergo an epithelial to mesenchymal transition, ingress into the streak and move out in the space between the hypoblast and the epiblast. Cells migrate over long distances before they re-aggregate to form distinct structures such as somites alongside the central midline and flanked by lateral plate mesoderm. By using a combination of transfection and grafting techniques, the migration patterns of the mesoderm cells leaving the primitive streak cells have been mapped out in detail (Yang *et al*, 2002). The tracks of the cells are visualised not by following all cells individually, which would be difficult due to extensive movement occurring in the embryo but by successive superposition of the images, giving rise to the formation of individual cell tracks (Figure 7; Supplementary Movie 10). We have shown that cells from the anterior streak initially migrate outward, and when the regression of the node begins and the node has regressed past them, they migrate back towards the midline where they form somites. Cells migrating out from the middle streak show a very similar migration pattern, initially they migrate out and then they migrate back in towards the midline but stay more lateral position and form lateral plate mesoderm. Cells in the posterior streak migrate out and form extra-embryonic mesoderm and haematopoietic cells that form blood islands and blood vessels. These data show that the movement of many cells can be visualised. In this case, cell tracks are generated by segmentation and logical addition of this information in the overlay of the image. We have proposed that some of the migration patterns result from chemo-repulsion (migration out of the streak) and chemo-attraction, the movement of the mesoderm cells back in towards the central midline in response to growth factors of the FGF and VEGF family (Yang *et al*, 2002). The mechanisms underlying signal detection and polarised activation of the cytoskeleton are still unknown. It has recently been proposed based on the analysis of movement of cells in tissue culture

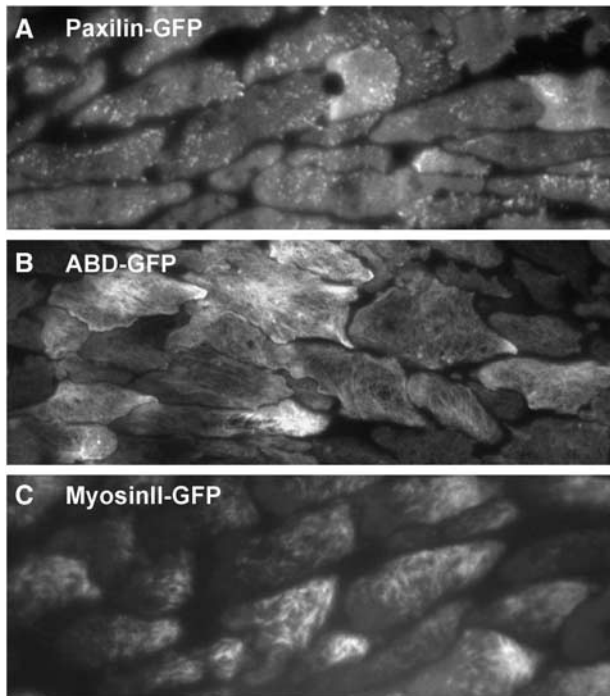


Figure 5 Visualising the localisation of cytoskeletal components using TIRF microscopy. (A) PaxillinGFP accumulates at focal adhesion sites that remain stationary once they have been formed. Shown is the ventral membrane of cells moving in aggregation streams under TIRF illumination (see Supplementary Movie 7). (B) Using the f-actin specific ABD-GFP, the dynamics of the actin cytoskeleton can be studied in great detail. Filamentous actin structures are clearly visible in the cortex of the slug cells (see Supplementary Movie 8). (C) MyosinII-GFP marks the cellular localisation of myosin II, which is involved in the contraction of the cell posterior through interaction with the actin cytoskeleton. As can be seen MyosinII-GFP filaments are absent from the leading edge of slug cells (see Supplementary Movie 9).

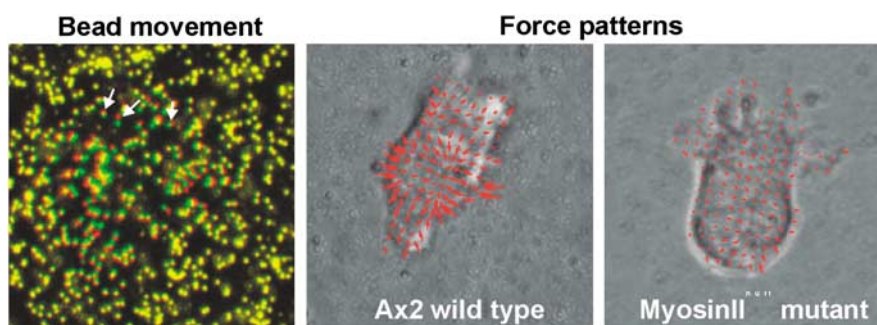


Figure 6 Measuring forces in migrating cells. The forces cells exert on their substrate can be deduced from the analysis of the displacement of fluorescently labelled beads that are embedded in an elastic substratum over which the cells move. Beads that have been moved by a migrating cell are indicated by the red and green colours (arrows), while stationary beads further away from the cell appear in yellow. The calculated force patterns are shown for a wild-type Ax2 cell and a MyosinII^{null} cell both moving in a cAMP gradient.

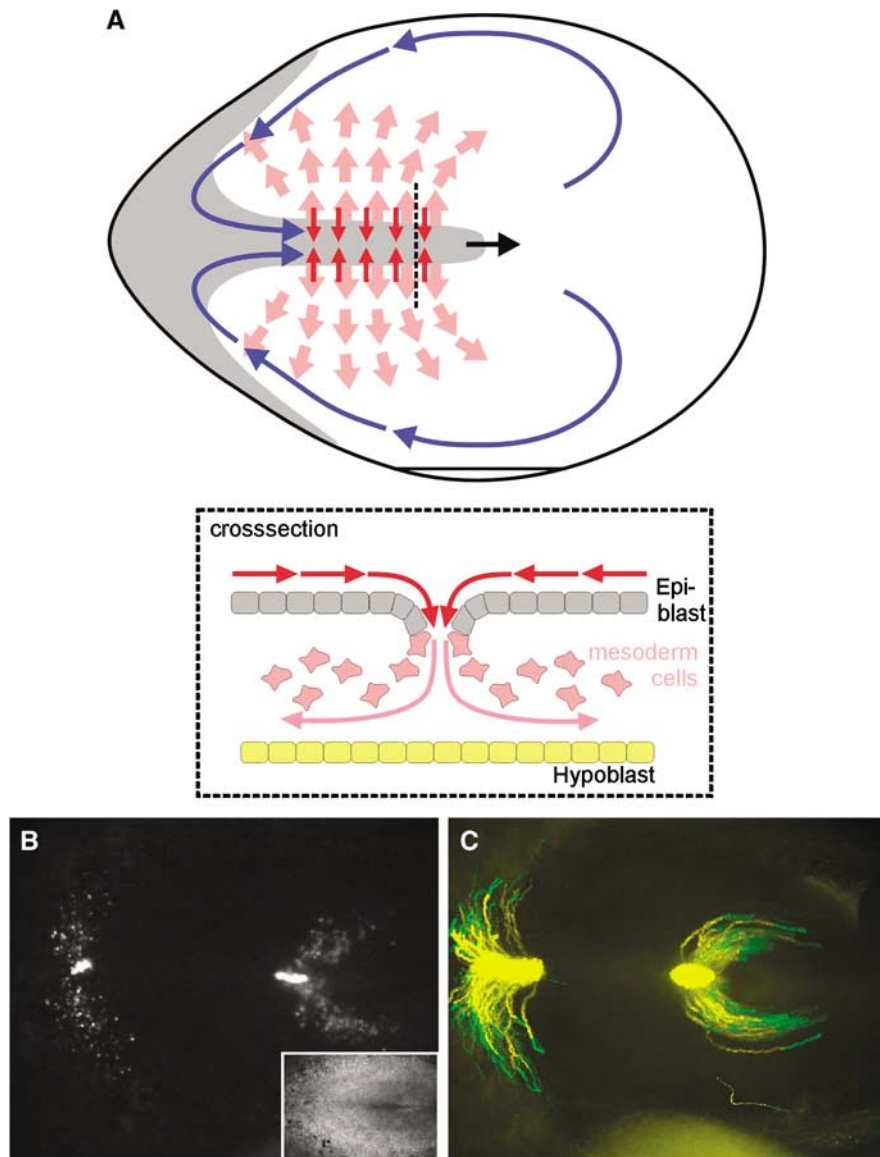


Figure 7 Migration of mesoderm cells in a HH4 chick embryo. (A) Schematic diagram of a chick embryo. Cells in the epiblast migrate (red arrows) to the forming primitive streak. There they undergo an epithelial to mesenchymal transition and move (roza arrows) in the space between the epiblast and hypoblast (insert), where they migrate over large distances to form mesodermal structures such as somites, lateral plate mesoderm and blood islands. The streak itself forms from the posterior end of the embryo towards anterior, involving two counter rotating cell flows of cells in the epiblast that merge at the site of streak formation and indicated schematically by the blue arrows (see Figure 8 for experimental data). (B) GFP expressing cells that have migrated from a graft in the posterior and middle primitive streak, inset with corresponding brightfield image of the same chick embryo. The tip of the primitive streak points to the right. (C) Cell tracks of the cells shown in (A) over a 6-h period, the last 2 h of migration are shown in green (see Supplementary Movie 10).

that local detection of a signal could result in a local extension of a cell process that then results in altering the cells migration trajectory, and that there is no need for a large-scale integration of the signals (Arriemerlou and Meyer, 2005).

Formation of the primitive streak

Many movements occurring during embryogenesis involve deformations of epithelial cell sheets. Formation of the primitive streak in the chick embryo is one such process that can be readily observed in time lapse bright field images. Using a modification of an optical flow technique, it is possible to reconstruct the cell flow patterns in the epiblast of the embryo quantitatively (Figure 8; Cui *et al*, 2005). It can be

seen that the cells in the epiblast move in highly regular coherent rotational flow patterns that merge at the side of streak formation. The flows bring the mesoderm cells that differentiate at the boundary between the area opaca and area pellucida into the central midline of the embryo, where they will form the primitive streak. Through transfection of hundreds of individual cells in the epiblast with GFP, it is possible to follow their displacements in time by calculating their movement trajectories (Figure 8). All cells follow the highly streamlined flow patterns, the mechanistic basis of which is still unknown. These measurements nicely confirm the flow patterns deduced from the flow analysis. The mechanism by which the cells move is so far also not known and it is to be expected that they move on a basal membrane, which they

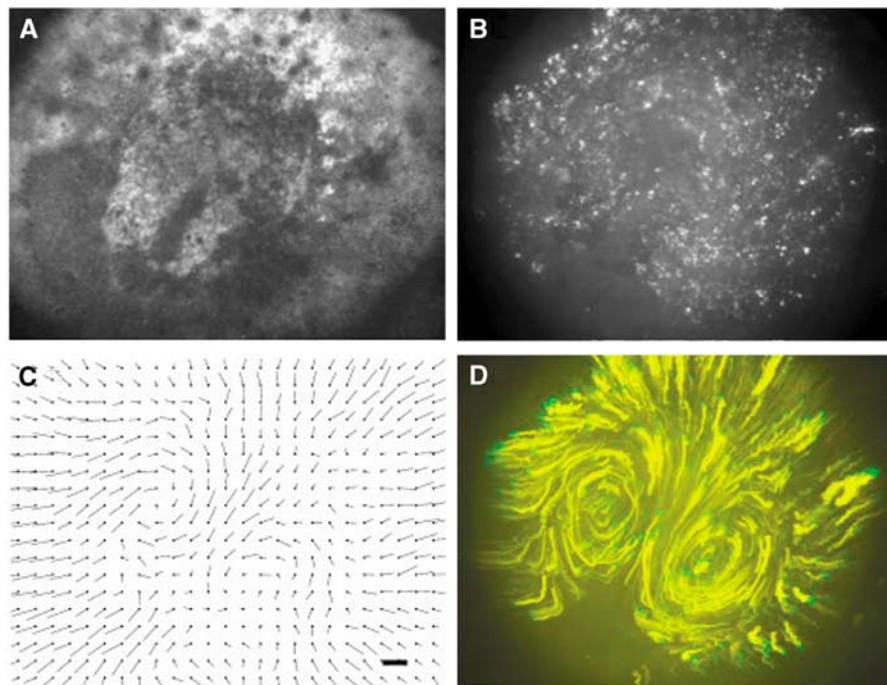


Figure 8 Movement of tissue in the epiblast of a prestalk chick embryo. (A) Bright field image of an HH1 stage chick embryo, with a streak extending halfway over the epiblast. (B) The same embryo as shown in (A) (9 h after transfection with GFP), every white dot is a single cell in the epiblast. (C) Vector velocity field calculated from a brightfield sequence as shown in (A), the marker bar indicates a velocity of 1 $\mu\text{m}/\text{min}$. (D) Traces of the fluorescent cells shown in (B), the traces were calculated over a time period of 6 h, the cell migrate in the direction yellow to green, the green track shows migration over the last hour (see Supplementary Movie 11).

themselves secrete, possibly involving chemo-attraction and chemo-repulsion, something which has to be confirmed by high magnification imaging of the behaviour of individual cells in the sheet.

Data analysis

These specific examples show some of the questions regarding the control of cell movement of individual cells that are being investigated with imaging techniques. There has been a considerable effort in the development of techniques to track cells in space and time. Most of these techniques use simple segmentation algorithms and define areas of interest that shift with the movement of the cell. These methods work well provided that there is high enough contrast in the images and that the data sampling rate is such that the cells do not move out of the area of interest between data points (Rietdorf *et al*, 1996; Dormann *et al*, 2002b). More sophisticated approaches that have been used to follow the movement of cells involve adaptive chain algorithms, which can follow the cells outline and provide quantitative information to link changes in cell shape with translocation of fluorescent proteins (Dormann *et al*, 2002a). In the future, these methods can be expanded to work as adaptive volumes in which it will be possible to follow the 3D shape changes by cells and correlate their behaviour with changes in the underlying biochemistry as detected by changes in fluorescence of GFP tagged proteins. One of the main difficulties in tracing cells is that they often merge and then separate again and it is still relatively difficult to follow these cells automatically in 3D. This will require computer vision techniques,

where cells are described by a mathematical model taking into account parameters such a shape, characteristic change in shape, size velocity, texture followed by a learning process where cells are identified and then followed over time.

Another completely different approach to measure the flow of information in a stack of images is not to try to recognise objects in the image, but simply follow the flow of information in the system under the assumption that the same is essentially invariant during the time interval of two successive frames as shown above for the flow of cells during formation of the primitive streak. There are many methods available as discussed in (Miura, 2005). The temporal local optimisation method (TLO) has been used successfully to measure the dynamics of tissue flows in the epiblast of the developing chick embryo during streak formation (Figure 8) (Cui *et al*, 2005). It has also successfully been used to measure tissue flows in migrating slugs of *D. discoideum* (Abe *et al*, 1994; Siegert *et al*, 1994; Dormann *et al*, 1997). This technique can readily be extended in 3D and should prove useful in characterising dynamics of movement in complex images.

Outlook and promise

Cell movement is a process that requires detailed understanding of the spatio-temporal control of the underlying chemistry ultimately leading to the local activation of the cytoskeleton. At the moment, the majority of studies are still concerned with the investigation of cell behaviour of individual cells and much progress is being made to uncover the role of all components from the receptors that receive the

signals to downstream signalling reactions to the dynamics of individual cytoskeletal components. The methods being used are widefield microscopy often combined with deconvolution techniques and confocal microscopy. These techniques are being used to look at changes in localisation of proteins and can even be used in large-scale genomic screens to define the behaviour of for instance all PH domain containing proteins. The use of FRET techniques and fluorescence correlation spectroscopy to measure protein–protein interactions will become more prevalent. Photo-activatable fluorescent proteins should become widely used to investigate the flow of molecules in moving cells and certainly will be a useful expansion of the already widely used FRAP (FRAP technique). These measurements are complemented by more specialised techniques such as TIRF, which in the extreme case allows the measurement of the dynamics of single molecules of receptors on the surface and the inside of the

cell membrane. It is to be expected that these measurements will become more important as new probes are developed.

The challenge will be in the future to see whether these techniques can be used to measure the dynamics of reactions underlying cell movement in the context of whole organisms such as embryos. This is an area where confocal and deconvolution microscopes have been used extensively so far, but techniques like SPIM could become very important.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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