Experiments inside a box lead to out-of-the-box ideas on cellular organization

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Abstract Microtubules are biopolymers that assemble from tubulin dimers into hollow tubes and play an important role in cellular organization. Their fascinating properties and variety of functions, like for example chromosome segregation, sperm propagation and polarity establishment, have made them a popular subject of study. In this perspective I focus on the contribution of minimal in vitro systems to our understanding of microtubule organization within the physical confinement of a cell.

Keywords Microtubules · Cytoskeleton · Positioning · Aster · Microfabrication · In vitro reconstitution

The first important milestone in the field of microtubule based in vitro systems was the reconstitution of microtubule growth by Weisenberg (1972). Tubulin had been purified for many years; however no one had managed to polymerize microtubules from tubulin subunits under physiological conditions. Weisenberg found out that calcium ions inhibit microtubule polymerization and imaged reconstituted fixed microtubules for the first time. Subsequently Mitchison and Kirschner did the next key experiment (Mitchison and Kirschner 1984). They grew microtubules, then diluted the tubulin concentration and subsequently measured the changes in the number and length distributions of microtubules with electron microscopy. Their data elegantly showed that a population of microtubules coexists in two dynamically unstable states, a process called “dynamic instability”. The direct proof of dynamic instability came from Horio and Hotani (1986).

They visualized the dynamic behavior of individual microtubules with dark field microscopy, followed up by Walker et al. (1988) who used video enhanced DIC microscopy.

After these first assays and observations, the field of in vitro reconstituted microtubule systems really took off. Many different functional assays were established. Some groups, for example, focused on the regulation of microtubules by microtubule associated proteins, such as XMAP215 and EB1 (Bieling et al. 2007; Brouhard et al. 2008; Kerssemakers et al. 2006; Kinoshita et al. 2001). Other groups built more complex microtubule structures such as asters or anti-parallel bundles (Bieling et al. 2010; Braun et al. 2011; Nedelec et al. 1997). The group of Marileen Dogterom, which I joined in 2004, specialized in the role of the physical confinement on microtubule organization in minimal model systems. This field began in 1996 when Elbaum et al. (1996) grew microtubules in a small confined space. They nucleated a bundle of microtubules in a small vesicle and observed how microtubule growth deformed the vesicle (Fig. 1a). As a next step they used a micropipette to increase the tension on the membrane and observed that as a consequence the microtubules buckled and formed a ring along the circumference of the vesicle. These experiments showed that changes in the physical confinement can lead to changes in microtubule organization.

Holy et al. (1997) used a different approach. Their intention was to mimic positioning of organelles such as the mitotic spindle or nucleus relative to the cell boundary in a minimal model system. They grew microtubule asters from glass beads that mimic a microtubule organizing center, in microfabricated chambers (Fig. 1b). These cleverly designed experiments demonstrated that microtubule assembly alone is sufficient to position asters. A

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Fig. 1 Cartoons of minimal systems to study microtubule organization within confined spaces. **a** A microtubule bundle (red) deforming a vesicle. **b** A microtubule aster grown in a microfabricated chamber. If the microtubules remain straight they can position a microtubule organizing center reliably. **c** If the microtubules grow too long they buckle and no longer faithfully position a microtubule organizing center. Scale bar indicates 10 μm

follow-up experiment revealed that for this mechanism to be robust microtubules need to remain straight. If the microtubules grow too long and buckle (Fig. 1c), the spatial information is lost and microtubule asters can no longer find the center (Faivre-Moskalenko and Dogterom 2002). Conversely, in plants, long buckled microtubules are important, because they form the cortical array (Mulder and Janson 2014). Cosentino Lagomarsino et al. (2007) grew microtubules in rectangular boxes to study the role of the mechanical properties of microtubules formation of this process. These experiments could reproduce the regularity of the plant cortical arrays, however failed to produce the critical transverse orientation of microtubules to the cell's long axis, showing the need for additional mechanisms.

All of the above experiments employed the interaction of microtubule arrays with microfabricated chamber walls that were inert. In this case, microtubule polymerization is the driving force for centering of a microtubule aster or microtubule buckling. In small living cells, such as the fission yeast *Saccharomyces pombe*, these microtubule based pushing forces have indeed been shown to be important in nuclear positioning (Tran et al. 2001). However in larger cells, such as the first cell stage *Caenorhabditis elegans* embryo, elegant laser-cutting experiments demonstrated a prominent role for pulling forces (Grill et al. 2001). How these pulling forces organize the cellular interior though remained unclear. We decided to design a quasi 2D minimal in vitro experiment to address the role of pulling forces. We again employed microfabricated chambers but now we introduced pulling forces by specifically binding dynein motor-proteins to a gold layer

in the microfabricated chamber (Fig. 2a), a technique pioneered by Romet-Lemonne et al. (2005). Native dynein is a large 1.2 MDa minus-end directed processive motor protein, which used to be notoriously hard to work with. Fortunately for us, Reck-Peterson et al. (2006) had just elegantly truncated this large protein to its minimal functional form and thoroughly characterized it, reducing it to a much better behaved protein (Gennerich et al. 2007). So we grew microtubule asters from purified centrosomes in dynein functionalized chambers and observed the dynamics and positioning of the microtubule aster with fluorescence light microscopy (Fig. 2a). By comparing the difference between chambers with and without dynein, we could focus on the role of pulling forces. We were very surprised to find that microtubule asters position more reliably in the presence of pulling forces than in their absence (Laan et al. 2012).

To explain this observation we built a simple mechanical model. This model predicted that as long as microtubules grow in an isotropic manner from a centrosome, pulling forces do not improve positioning: in brief, they cancel each other out, resulting in a zero net force at every position in the microfabricated chamber (Fig. 1c top). However, our simple in vitro experiment revealed that the distribution of microtubules growing from a centrosome in a microfabricated chamber is not isotropic: growing microtubules reorient themselves when they touch the chamber boundary resulting in an anisotropic distribution (Fig. 1c). The pulling forces arising from this distribution no longer cancel out and lead to a sum centering force. We convinced ourselves of the accuracy of our model by varying the shape of the microfabricated chamber in the model as well as in our in vitro experiments (Pavin et al. 2012). So, is the mechanism that we found in vitro important in vivo? It is too early to confidently answer this question with yes, however it has become clear that the key ingredient, re-orientation of microtubules due to interaction with the cell boundaries is present in living cells (Picone et al. 2010).

So what lies ahead? The field has split in two directions. The first direction consistently follows the minimal approach. For example, the Dogterom lab is currently growing microtubules in vesicles coated with dynein (Fig. 3a). These geometries more closely mimic the three-dimensional properties of cells. In addition, dynein is bound to the membrane so it can diffuse, allowing for a positive feedback between the microtubule distribution and dynein concentration (Laan et al. 2012). In the second research direction the complexity is increased much more dramatically. Pinot et al. (2009) for example, also grew microtubule asters in droplets, using cellular extracts instead of purified components. Nevertheless, the positioning of those asters is in agreement with predictions

Fig. 2 Pulling forces reliably position a microtubule aster in a microfabricated chamber. **a** Cartoon of the top and side view of the experiment where a microtubule aster (red) is grown from a centrosome (grey) in a microfabricated chamber with dynein (green) specifically bound to a gold layer (yellow). **b** Fluorescent microscopy

image of a microtubule aster in a microfabricated chamber with dynein. Scale bar indicates 10 μm . **c** Cartoon showing the mechanism by which reorientation of the microtubule distribution leads to a net centering force by pulling forces

Fig. 3 Cartoons of complex systems confined in droplets or micro-fabricated chambers. **a** Microtubule aster grown in a droplet coated with dynein. **b** Spindle grown from cytoplasm extracted from living cells in a droplet. **c** Fission yeast grown in microfabricated channels, microtubules are indicated in red. **d** Sea urchin embryo grown in star shaped chamber. All scale bars indicate 10 μm

of our mechanical model, making the use of extract an interesting intermediate between minimal systems and living cells. Recently, even functional spindles were grown from cytoplasm in small droplets (Fig. 3b) (Good et al. 2013; Hazel et al. 2013) showing that the size of a mitotic spindle scales linearly with the size of the confining compartment. Interestingly, spindle size scaled with volume rather than with the shape of the compartment.

As a most extreme opposite of a minimal model system, whole cells were grown in microfabricated structures to physically change their cell geometry. In 2008 already

Terenna et al. (2008) grew fission yeast in microfabricated channels and showed directly how cell shape and microtubule organization are coupled and control cell polarity. The most dramatic changes in cell shape were created by Minc et al. (2011). They grew sea urchin embryos in a variety of different confinements, among which a star shape (Fig. 3d) and found that the nucleus always sits at the center of mass of a confining geometry.

It is fascinating to see how much progress has been made since the first microtubules were reconstituted in vitro in 1972 and first asters were grown in small cell like boxes in 1997. I look forward to seeing which level of complexity can be achieved with minimal model systems: you need to add a lot of components to start approaching the complexity of the cytoplasm! So at what level will the minimal approach meet the cellular approach? And what will the middle ground look like?

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