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REVIEW

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Probing the biology of cell boundary conditions through confinement of *Xenopus* cell-free cytoplasmic extracts

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Abstract

Cell-free cytoplasmic extracts prepared from *Xenopus* eggs and embryos have for decades provided a biochemical system with which to interrogate complex cell biological processes *in vitro*. Recently, the application of microfabrication and microfluidic strategies in biology has narrowed the gap between *in vitro* and *in vivo* studies by enabling formation of cell-size compartments containing functional cytoplasm. These approaches provide numerous advantages over traditional biochemical experiments performed in a test tube. Most notably, the cell-free cytoplasm is confined using a two- or three-dimensional boundary, which mimics the natural configuration of a cell. This strategy enables characterization of the spatial organization of a cell, and the role that boundaries play in regulating intracellular assembly and function. In this review, we describe the marriage of *Xenopus* cell-free cytoplasm and confinement technologies to generate synthetic cell-like systems, the recent biological insights they have enabled, and the promise they hold for future scientific discovery.

KEYWORDS

cellular reconstitution, compartmentalization, encapsulation, microfluidics, synthetic cell, *Xenopus* egg extract

1 | INTRODUCTION

For over one hundred years, *Xenopus* has been used as a model system to investigate organismal development and to characterize fundamental processes that regulate cell structure and function. *Xenopus* is unique among model organisms in that its eggs have broad utility for studies both *in vivo* and *in vitro*. When fertilized, eggs facilitate *in vivo* studies on the mechanisms guiding embryogenesis, whereas cytoplasm isolated from unfertilized eggs enables biochemical dissection of cell biological processes *in vitro*. Recently, *Xenopus* has also emerged as an important model organism for the study of human diseases; it has been particularly useful for identifying developmental mechanisms that are perturbed by genes mutated in patients (Duncan & Khokha, 2016). In this review, we highlight a new research area, cellular reconstitution, that takes advantage of *Xenopus* cytoplasmic extracts and confinement technologies to investigate how boundaries shape the spatial organization and activities of a cell.

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2 | UTILITY OF *XENOPUS* CYTOPLASMIC EXTRACTS TO DISSECT CELL BIOLOGICAL PROCESSES *IN VITRO*

Xenopus cell-free extracts are the cytoplasmic fraction that is isolated from intact eggs using centrifugation. This undiluted cytoplasm contains the protein milieu, organelles, and subcellular structures present in an intact cell, and is competent to carry out many processes associated with the cell cycle *in vitro* (Desai, Murray, Mitchison, & Walczak, 1998; Hannak & Heald, 2006; Murray, 1991). Although studies in extracts are often complemented by experiments performed *in vivo*, the extract system provides a number of unique advances over studies in tissue culture. Importantly, cytoplasmic extracts are more tolerant of manipulations to the levels of essential cellular proteins, enabling a more comprehensive analysis of vital cell biological processes. In contrast, studies of core cellular processes in live cells are difficult because knockdown of an essential gene often causes cell cycle arrest or cell death. Additionally, the cell cycle state of *Xenopus* egg extracts can be readily synchronized, which enables individual parameters to be varied in isolation of other confounding factors present in growing cells. The

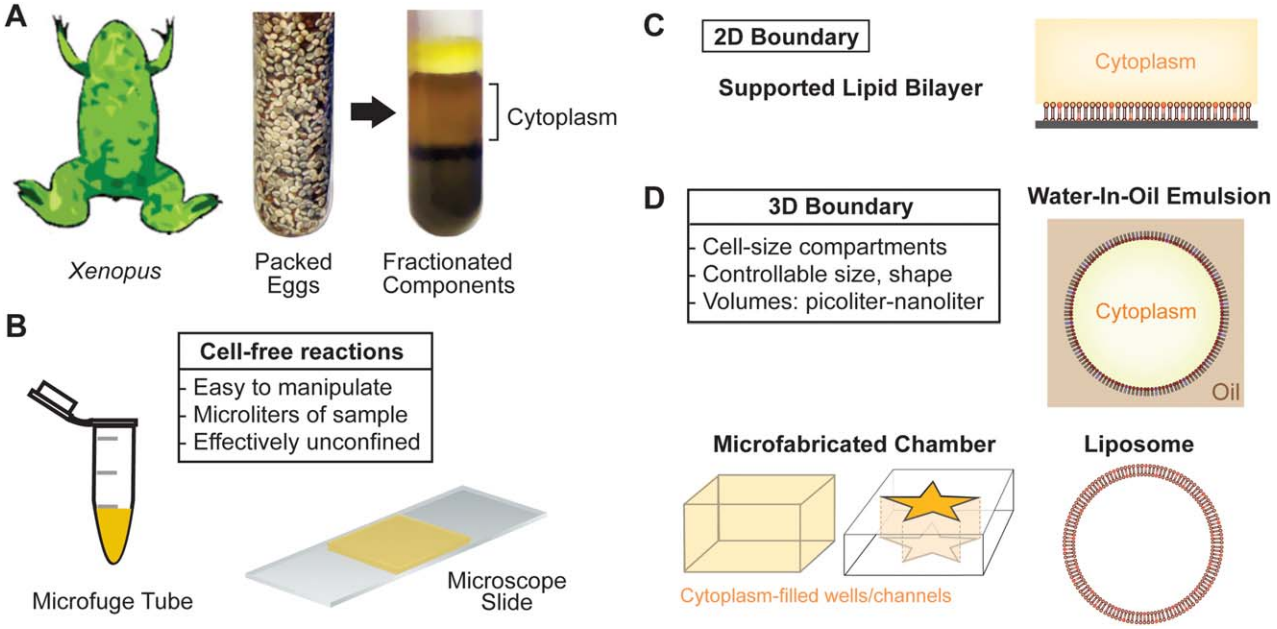

FIGURE 1 *Xenopus* cell-free egg cytoplasmic extracts and strategies for 2D confinement or 3D encapsulation. (A) Eggs collected from the frog, *Xenopus laevis*, are fractionated to generate cell-free cytoplasm. (B) The cytoplasmic extract is capable of carrying out complex cell biological processes *in vitro* in the absence of cell boundaries. Microliter volumes of cytoplasm are often activated in test tubes and imaged on microscope slides. (C) Configuration for confinement of cellular reactions to a supported lipid bilayer surrounded by cytoplasm. The process of interest is initiated at or signals to a two-dimensional membrane whose composition is controllable. (D) Encapsulation of cytoplasmic extract reactions within cell-like boundaries. The stiffness of these boundaries varies: PDMS wells (rigid), water-in-oil emulsion (intermediate, lipid monolayer), or liposomes (soft, lipid bilayers). Importantly, compartment dimensions can be specified to encapsulate cell-size volumes of material; from picoliters to nanoliters

system also lends itself to molecular investigation—the extracts are biochemically accessible and the availability of milliliter quantities of pure cytoplasm provides a means of fractionating and identifying the proteins that drive fundamental cellular activities.

For more than three decades, studies using cell-free extracts isolated from *Xenopus* eggs have produced groundbreaking molecular insights on the cell cycle control system, DNA replication and repair, chromosome condensation, transcriptional regulation, and regulated assembly or disassembly of cytoskeleton, kinetochores, organelles, and other subcellular structures. Early studies focused on DNA replication and chromosome condensation in *Xenopus* extracts (Blow & Laskey, 1986; Hutchison, Cox, Drepaul, Gomperts, & Ford, 1987; Lohka & Maller, 1985), guided by initial work using cell-free preparations from the frog, *Rana pipiens* (Lohka & Masui, 1983, 1984). The use of *Xenopus* egg extracts has facilitated a multitude of seminal discoveries including cyclin-based regulation of the cell cycle (Murray & Kirschner, 1989), and centrosome replication (Hinchcliffe, Li, Thompson, Maller, & Sluder, 1999), characterization of the DNA damage response pathway and cell cycle checkpoints (Cupello, Richardson, & Yan, 2016; Guo, Kumagai, Wang, & Dunphy, 2000) and microtubule branching (Petry, Groen, Ishihara, Mitchison, & Vale, 2013). Furthermore, fractionation and purification of proteins from egg extracts led to the identification and characterization of proteins such as microtubule regulators, XMAP215, XKCM1, and TPX2 (Shirasu-Hiza, Coughlin, & Mitchison, 2003; Tournebize et al., 2000; Walczak, Mitchison, & Desai, 1996), and the actin nucleator Arp2/3 (Welch, Iwamatsu, & Mitchison, 1997). These studies have provided key insights into the regulation of cytoskeletal dynamics. Additionally, *Xenopus* extracts maintain preeminence as a vertebrate model system for building functional subcellular structures *in vitro*; most notably assembly of the metaphase spindle and interphase nucleus (Desai, Murray, Mitchison, & Walczak, 1998; Heald et al., 1996; Kapoor, Mayer, Coughlin, & Mitchison, 2000; Merdes, Ramyar, Vechio, & Cleveland, 1996; Murray, 1991). This has led to important discoveries, such as the characterization of the bifunctional role for the Ran GTPase in interphase nuclear transport and assembly (Zhang & Clarke, 2000) and as a controller of chromatin-mediated microtubule nucleation (Kalab, Weis, & Heald, 2002; Ohba, Nakamura, Nishitani, & Nishimoto, 1999; Tsai et al., 2003).

The preparation of cytoplasmic extracts from eggs of *Xenopus laevis* has been described thoroughly in previous work (Desai et al., 1998; Good, 2016; Hannak & Heald, 2006; Murray, 1991). Briefly, ovulated female frogs provide a source of thousands of unfertilized eggs which can be packed, crushed, and fractionated using centrifugation (Figure 1A). Typically, one milliliter of the middle cytoplasmic layer is collected and supplemented with protease inhibitors and an energy regeneration mixture. The large volume of extract enables many reactions to be carried out in parallel and provides ample material for biochemical manipulation. Cytological reactions are often performed in a standard microfuge tube or imaged live on a microscope slide (Figure 1B).

3 | VARIANTS OF THE CANONICAL EGG EXTRACT SYSTEM

In addition to the traditional crude extracts prepared from *Xenopus laevis* eggs, new cytoplasm systems have been described which expand the scope of cell biological and developmental processes that can be characterized *in vitro*. For example, high speed extracts (HSEs) are prepared by clarifying crude extracts using ultracentrifugation, and these extracts provide an ideal system to investigate chromosome assembly or disassembly (Maresca & Heald, 2006). Furthermore, cell-free extracts can be prepared from eggs of the frog *Xenopus tropicalis* (Brown et al., 2007). These extracts form subcellular structures that are smaller than those found in egg extracts of *Xenopus laevis*, and thus can be utilized to identify factors that control intracellular size scaling (Helmke & Heald, 2014; Levy & Heald, 2010; Loughlin, Wilbur, McNally, Nedelec, & Heald, 2011). Importantly, extracts are no longer limited to the egg stage; cytoplasm from early blastula embryos at various stages of development has been shown to function *in vitro* (Good, 2016; Good, Vahey, Skandarajah, Fletcher, & Heald, 2013; Wilbur & Heald, 2013; Wuhr et al., 2008). This embryo extract system enables characterization of a specific cell biological process at varying stages of early embryogenesis, and may provide insights on how developmental regulation impacts intracellular function.

An important consideration is whether *Xenopus* egg extracts provide unique benefits that cannot be achieved using other cell-free systems. Extracts from prokaryotes (e.g., *E. coli*) and eukaryotes (e.g., wheat germ), along with rabbit reticulocytes and mammalian cell extracts have been widely used for protein synthesis *in vitro* (Bernhard & Tozawa, 2013; Endo & Sawasaki, 2006; Zemella, Thoring, Hoffmeister, & Kubick, 2015). However, the prokaryotic systems cannot be used for eukaryotic cell biological studies and yeast extracts are not suitable for most cytological imaging. Notably, yeast extracts have been successfully used to reconstitute protein transport across ER–Golgi (Baker, Hicke, Rexach, Schleyer, & Schekman, 1988) but cannot be used to build large cytoskeletal structures and organelles that closely mimic those found in vertebrate species. In short, none of these simple extract systems have been broadly established for cell biological applications. Additionally, unlike *Xenopus* egg extracts, the preparation of cell extracts from cultured cells, including mammalian tissue culture, leads to dilution of the cytoplasm. Intriguingly, recent work demonstrated the preservation of cell cycle activity *ex vivo* in cytoplasm aspirated from a single *Drosophila* embryo (Telley, Gaspar, Ephrussi, & Surrey, 2012). However, only a few nanoliters of cytoplasm can be collected from an embryo using this method, and the centrifugation of thousands of embryos does not produce an active, cycling cytoplasm. Therefore, the *Drosophila* embryo system is not suitable for most standard biochemical analyses. In summary, given the limitations of other systems, *Xenopus* egg extracts remain the gold standard for characterizing most cell biological processes *in vitro*.

4 | LIMITATIONS OF 'TEST TUBE BIOCHEMISTRY'

Although undiluted *Xenopus* cytoplasm closely mimics the chemical composition of the egg or embryonic blastomeres, it lacks the structure and spatial organization that define a living cell. For example, studies using *Xenopus* egg extracts are typically carried out in microfuge tubes, requiring tens of microliters of cytoplasm, whereas the typical somatic cell volume is often less than ten picoliters. This difference of six orders of magnitude in the amount of cytoplasmic material has the potential to confound the interpretation of cellular activities that are studied *in vitro*. Additionally, cells have a defined architecture, including a cell boundary—the plasma membrane—which dictates the volume and shape of cell, controls protein subcellular localization, and provides mechanical feedback to internal processes. These and other variables may lead to diverging results in cells and test tubes (Minton, 2006). Therefore, to fully characterize a cell biological processes in a more cell-like context it is necessary to confine or encapsulate *Xenopus* cytoplasmic extracts. This experimental strategy is often described as 'cellular reconstitution' (Liu & Fletcher, 2009). In the following sections we describe recent studies that explored how boundaries regulate subcellular processes and structures, including cytoskeletal architecture and dynamics, organelle growth, and intracellular signaling.

There are a variety of strategies for generating boundaries to confine extract reactions to a two-dimensional membrane or within a micron length scale compartments, a subset of which have been discussed previously (Vahey & Fletcher, 2014). Historically, studies seeking insights on the chemical and physical impacts of boundaries relied on forming lipid bilayers on top of a solid support (Mueller, Rudin, Tien, & Wescott, 1962; Richter, Berat, & Brisson, 2006; Tamm & McConnell, 1985). Because supported bilayers can promote specific associations of proteins on the membrane surface, they can be used to assess interactions between intracellular components and the cell boundary. More recently, research on the impacts of cell boundaries has been extended by three-dimensional compartmentalization. This includes confinement of protein solutions within microfabricated channels and chambers containing a rigid boundary, and encapsulation within cell-size emulsions or liposomes (Griffiths & Tawfik, 2006; Martino & deMello, 2016) whose lipid composition can mimic that of the plasma membrane. Variations on these techniques have applications for reconstituting complex cellular processes *in vitro* in a cell-like configuration, and provide a novel method for manipulating the microenvironment of fundamental cell biological processes.

4.1 | 2D CONFINEMENT OF *XENOPUS* CYTOPLASMIC REACTIONS: INVESTIGATING SUBCELLULAR ASSEMBLY AT THE CELL MEMBRANE

Liposomes and supported lipid bilayers (SLBs) can be used in conjunction with *Xenopus* cytoplasmic extracts to reconstitute cellular processes that normally localize to the plasma membrane. For example, actin-dependent structures, such as filapodia, are nucleated by components concentrated on the membrane. Additionally, many signaling proteins, including GTPases, contain an amphipathic helix, lipidated peptide sequence or lipid-interacting domain, that promotes their association with membranes. Thus, the presence of a 2D bilayer is a

minimal requirement to recapitulate the spatial segregation of cytosolic and membrane-associated factors found in a cell.

Recently, it was shown that phosphoinositide containing SLBs promote the assembly of filapodia-like structures from the surface of the bilayer in the presence of *Xenopus* high speed extracts (Lee, Gallop, Rambani, & Kirschner, 2010). The authors demonstrated that these dynamic structures were nucleated from Arp2/3 complexes containing N-WASP, which associated with $PI(4,5)P_2$ in the membrane. Additionally, they demonstrated a temporal order of recruitment: the first wave consisted of Toca-1, N-WASP, and Arp2/3, followed by a second wave of actin and formins. More recently, this method has been extended to allow additional membrane formulations (Walrant, Saxton, Correia, & Gallop, 2015), which should enable characterization of how membrane properties, such as fluidity and lipid clustering, influence actin polymerization.

The combination of supported bilayers and *Xenopus* egg extracts has also been used to characterize cytokinesis signaling from regions of overlapping antiparallel microtubules to the cell membrane (Nguyen et al., 2014). The authors assembled centrosome-like asters, which recruit components of cendralspindlin and the chromosome passenger complex, proximal to a phosphoinositide-containing SLB. They demonstrated that, proximal to regions of overlapping microtubules, this assembly was sufficient to induce the recruitment and activation of RhoA and additional cleavage furrow proteins. This system provides a modular framework with which to dissect the molecular activities and spatial constraints required for cell division *in vitro*. The specialized requirements for stabilizing SLBs in the presence of *Xenopus* egg extracts have recently been described (Field, Groen, Nguyen, & Mitchison, 2015; Nguyen, Field, Groen, Mitchison, & Loose, 2015).

These studies, which leverage a 2D boundary, provide unique insights that could not have been derived from bulk egg extracts in a test tube and represent a clear step toward the long-term goal of reconstituting an intact cell. However, although these studies utilize a bilayer composition that mimics the plasma membrane, they lack the three-dimensional constraints and limited cytoplasmic volumes present in a cell. In the next section, we describe techniques for 3D encapsulation.

4.2 | 3D ENCAPSULATION OF CYTOPLASMIC EXTRACTS IN CELL-LIKE COMPARTMENTS

As with many previous advances in biology, the generation of micron-sized compartments required adoption of technological advances in the physical sciences. Specifically, encapsulation of cells and other biological components was facilitated by applying photolithography (Whitesides, Ostuni, Takayama, Jiang, & Ingber, 2001) to development of microfluidics technologies for generating tunably-sized emulsions (Garstecki, Fuerstman, Stone, & Whitesides, 2006; Link, Anna, Weitz, & Stone, 2004; Thorsen, Roberts, Arnold, & Quake, 2001; Utada et al., 2005).

Initially, a number of important studies demonstrated the feasibility of encapsulating purified proteins, including tubulin, and centrosomes in micropatterned compartments (Faivre-Moskalenko & Dogterom, 2002; Holy, Dogterom, Yurke, & Leibler, 1997). This was further extended by adding motor proteins to the compartment boundary (Laan et al., 2012). More recently, microtubules and motor proteins, and actomyosin assemblies have been characterized inside cell-size emulsion droplets (Baumann & Surrey, 2014; Miyazaki, Chiba, Eguchi, Ohki, & Ishiwata, 2015; Sanchez, Chen, DeCamp, Heymann, & Dogic, 2012). However, these studies are limited because many complex cellular structures, including the centrosome, nucleus and mitotic spindle, cannot be reconstituted using purified components.

The first studies to encapsulate *Xenopus* egg extracts in cell-like compartments focused on the architecture and dynamics of compartmentalized cytoskeletal assemblies. For example, Jimenez and colleagues demonstrated formation of microtubule asters and actin filaments inside aqueous-in-oil emulsions droplets stabilized by a nonlipid surfactant (Jimenez et al., 2011). Subsequent experiments exploited Ran-mediated microtubule aster formation inside similar droplets (Hoffmann et al., 2013). In a separate study, the authors quantified actin flow inside emulsion droplets (Pinot et al., 2012), and demonstrated that a polymerized actin network can promote subcompartmentalization (Colin, Bonnemay, Gayrard, Gautier, & Gueroui, 2016). Other researchers have confined *Xenopus* egg extracts in micropatterned wells or channels to characterize contraction of microtubule networks (Foster, Furthauer, Shelley, & Needleman, 2015).

A major benefit of confinement is that it reveals the contribution of boundaries to regulation of subcellular activities. For example, the boundary can function as a passive organizing platform that dictates subcellular anchoring of specific proteins or activities. Additionally, it defines the physical dimensions of a cell, and thus volume, of the cytoplasmic material present. Further, it can act as a mechanical barrier that resists osmotic swelling and cytoskeletal growth. The following studies provided important new insights on cell biological processes by exploiting *Xenopus* egg extracts and 3D encapsulation techniques to overcome previous experimental limitations.

One prediction of confinement is that cytoskeletal polymers, assembled *de novo*, will experience a resisting force if the steady state size of the unencapsulated structure exceeds the dimensions of the compartment. Pinot and colleagues found that the size of the compartment-a cytoplasm-in-oil emulsion-impacted aster assembly; below a threshold size the lipid monolayer boundary mechanically restricted microtubule growth, causing buckling and reorganization of the canonical aster structure (Figure 2A) (Pinot et al., 2009). This work is in agreement with previous studies using purified components that demonstrated that a boundary force will resist microtubule polymerization, leading to centering of an unanchored radial structure such as a microtubule aster (Faivre-Moskalenko & Dogterom, 2002; Holy et al., 1997).

A cell boundary also provides a platform to spatially restrict subcellular assembly, including the cytoskeleton. For example, the cell cortex is composed of many proteins, including actin filaments that are

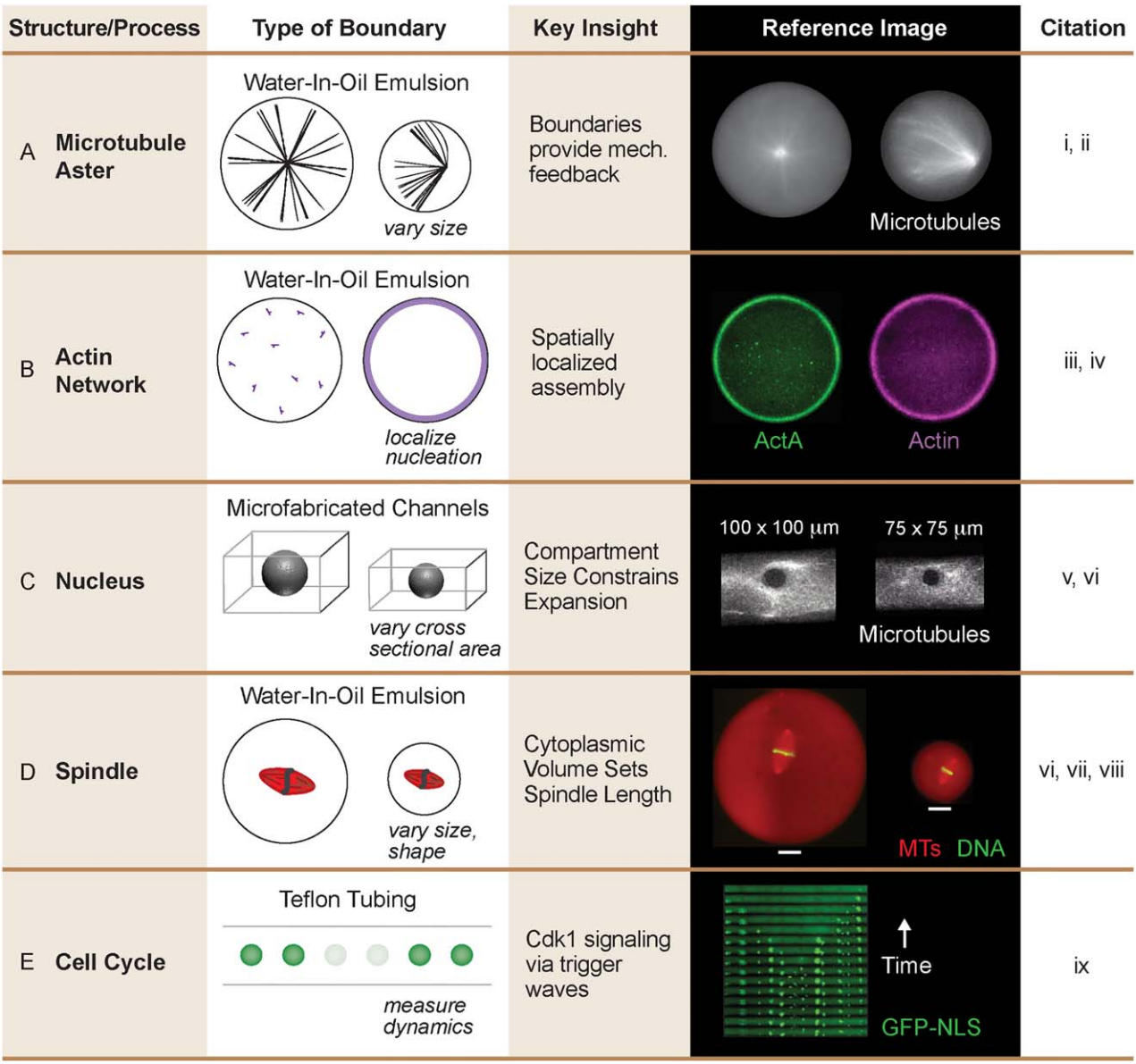

FIGURE 2 Encapsulation of *Xenopus* cytoplasmic extracts in microfabricated chambers and cell-like compartments. (A) Growth of radial microtubule asters is restricted by the droplet boundary in cytoplasm-filled water-in-oil emulsions. Below a threshold emulsion size, the microtubule growth causes the aster structure to buckle. (B) Spatial control of actin nucleation. Boundary-localized ActA initiates cortical actin polymerization in a cytoplasm-filled water-in-oil emulsions. (C) Nucleus growth scales with the cross-sectional area of the microchannel in which it is confined. (D) Metaphase spindle length is regulated by cytoplasmic volume. This scaling effect, in water-in-oil emulsions, is independent of compartment geometry. (E) Cell cycle duration is dependent on spatial position within cytoplasm-filled tubing. Citations: i: Pinot et al. (2009), ii: Jimenez et al. (2011), iii: Shah et al. (2014), iv: Pinot et al. (2012), v: Hara & Merten (2015), vi: Good et al. (2016), vii: Good et al. (2013), viii: Hazel et al. (2013), ix: Chang & Ferrell (2013). Images courtesy of Z. Gueroui, K. Keren, C. Merten, J. Ferrell.

nucleated by membrane-localized factors, such as the complex containing N-WASP and Arp2/3. The dynamic actin network that assembles near the plasma membrane is crucial for generating cell polarity, inducing cell motility, and providing stiffness to the cell cortex. To investigate the spatial organization and biophysical properties of actin assembly, Shah and colleagues anchored the bacterial protein ActA to the boundary of emulsion droplets filled with *Xenopus* cytoplasmic egg extracts (Abu Shah & Keren, 2014; Abu Shah, Malik-Garbi, & Keren, 2015). When unanchored, ActA promoted actin polymerization throughout the droplet. However, when ActA was localized to the droplet boundary it nucleated an actin shell that resembles a minimal cell cortex (Figure 2B). Intriguingly, the authors determined that this actin meshwork is dynamic, polarizes, and can generate force-features that mimic the properties of actin networks in cell.

Cell size is tightly regulated and cell volume varies only twofold during the cell cycle of most symmetrically dividing cells grown in culture (Ginzberg, Kafri, & Kirschner, 2015). In contrast, cell size reduces dramatically during early embryo development, a period after egg

fertilization in which cells divide without growing (Newport & Kirschner, 1982; Wuhr et al., 2008). A fundamental question is whether alterations in cell size also impact intracellular assembly or cellular function. A number of recent studies have begun to address this question, utilizing a cellular reconstitution approach that overcomes the challenges associated with manipulating cell size *in vivo*. This approach relies on the encapsulation of *Xenopus* cytoplasmic extracts in cell-size compartments, followed by analyses of how the sizes of intracellular structure vary as a function of compartment dimensions. As a first order approximation of cell boundaries, Hara and colleagues analyzed nucleus growth inside PDMS channels of varying cross-sectional area (Hara & Merten, 2015). They identified a minimal nuclear domain—a volume of material—below which nucleus growth rates began to slow. A closer approximation to a cell is a water-in-oil emulsion, with a spherical shape and lipid monolayer boundary that can be specified to mimic cellular dimensions and the lipid composition of the cytoplasmic leaflet of the plasma membrane. This system, when coupled with *Xenopus* cytoplasm, allows variation of cell diameters from 10s to 100s of microns, which mimics the size and composition of blastomeres present in blastula-stage embryos. Additionally, the shape of these emulsions can be controlled by compressing them or confining them in microfabricated channels or chambers of varying size (Good, 2016). An exciting recent discovery is the ability of the mitotic spindle, whose steady state size is relatively invariant in unencapsulated egg extracts (Brown et al., 2007), to sense and adapt to the size of the compartment in which it is assembled. Two recent studies demonstrated that cytoplasmic volume sets metaphase spindle length *in vitro*, and this scaling closely matches the size scaling present in early embryo development (Good et al., 2013; Hazel et al., 2013). These results suggest that the physical dimensions of a cell regulate spindle size through a limiting pool of cytoplasmic components. These experiments utilized a passivated boundary rather than the full complement of plasma membrane lipids; therefore, these studies were carried out in the absence of a cell cortex. Thus, it remains possible that an interaction between astral microtubules and the cell cortex could further modulate spindle length, particularly during anaphase.

Theoretically, a cytoplasm-filled liposome surrounded by a fluid bilayer membrane would most closely resemble an intact cell. However, technological advancements are necessary to enable vesicle size control and improve the stability of these membranes, which are destabilized in the presence of *Xenopus* egg cytoplasm (unpublished data). One promising approach is the use of microjets for encapsulation. Pulsatile jetting of a solution will deform a planar lipid bilayer to generate a liquid-filled vesicle (Richmond et al., 2011; Stachowiak et al., 2008; Stachowiak, Richmond, Li, Brochard-Wyart, & Fletcher, 2009). Microfluidic approaches for generating vesicle-like double emulsions may also succeed, although a concern is that residual oil or solvent present in the membrane limits their function as true bilayers. Additionally, formation of cytoplasm-filled vesicles using swelling or electroformation approaches is not feasible; the encapsulation efficiency is very low and osmolytes disrupt liposome formation. At present, microfluidic droplet formation provides the best combination of stability, size-control, and throughput for generating cell-like compartments filled with undiluted *Xenopus* cytoplasmic extracts.

Not all intracellular processes scale with cell size. For example, in some organisms the timing of the cell cycle is invariant during early embryogenesis (Carvalho, Desai, & Oegema, 2009; Newport & Kirschner, 1982). However, a size-invariant process poses a conundrum for very large cells. As cell size increases, signals initiated from the lumen of a cell should take longer to diffuse to the cortex. Chang and colleagues investigated this problem by encapsulating cycling *Xenopus* egg extracts containing sperm nuclei in a Teflon tube (Chang & Ferrell, 2013). Normally, the period in which unencapsulated *Xenopus* extracts traverse the cell cycle is constant, averaging approximately 30–40 min, tied to the expression or activation of cyclins and Cdks. However, when the cytoplasm is encapsulated in a tube, individual nuclei assemble and dissemble at distinct rates. This result is linked to spatial position within the tube because neighboring nuclei are more closely synchronized than distal nuclei. When nuclei in the tube are visualized as a kymograph, a wave behavior appears (Figure 2E), and these waves travel rapidly, at a rate that enables signaling from the centrosome to the cortex during the first cell division in the giant cells of the *Xenopus* embryo. The authors propose that these trigger waves result from interlinked positive and negative feedback loops regulating Ckd1 activation (Chang & Ferrell, 2013). Importantly, Cdk1 signals are transmitted more quickly than the rate of simple Brownian diffusion of activated Cdk1 between two distal sites.

Collectively, the encapsulation studies described in this section have generated exciting new insights on the biology of a cell. Importantly, these breakthroughs would not have been possible using unencapsulated egg extracts because they lack cell boundaries. Furthermore, many of the chemical and physical perturbations performed in these studies are not tolerated by live cells and therefore cannot be carried out *in vivo*.

5 | FUTURE DIRECTIONS

5.1 | Expanded toolkit for protein recruitment to compartment boundaries

To mimic a cell, cytoplasm must be encapsulated within compartments of appropriate size containing boundaries that at least partially mimic the lipid and protein composition of the plasma membrane. At present, the evolution of encapsulation technologies has far out-paced development of chemical strategies that are required to build a synthetic cell cortex and insert membrane proteins into a synthetic lipid bilayer. Alternatively, a simpler strategy is to recruit individual proteins to the head groups of lipids that comprise a compartment boundary, such as the lipid monolayer present in water-in-oil emulsions or the lipid bilayer present in liposomes (Stachowiak et al., 2012). Conceptually, this strategy would enable proper spatial positioning of peripheral membrane proteins; in the best case scenario, it could also be used to anchor the cytosolic domain of transmembrane proteins to the boundary, enabling formation of higher order cortical assemblies. However, achieving this goal requires the development of chemical tools that are compatible

with cytoplasmic extracts and enable constitutive or inducible recruitment of proteins to a compartment boundary.

To date, synthetic lipid–protein interactions have been utilized to recruit tagged proteins to the surface of lipid monolayer or bilayer boundary through the incorporation of non-native lipids. For example, NTA-lipid has been widely used to induce recruitment of His-tagged proteins to one leaflet of liposomes and supported lipid bilayers (Gizeli & Glad, 2004; Stachowiak et al., 2012). Additionally, biotinylated lipids have been used to recruit oligomeric assemblies of streptavidin and biotinylated proteins to the boundary of water-in-oil emulsions (Vleugel, Roth, Groenendijk, & Dogterom, 2016). Covalent chemistry can be utilized to overcome weak protein–lipid interactions. Chemical couplers have also been paired with various classes of dendrimers (Percec et al., 2010; Turnbull & Stoddart, 2002), which can be used to form vesicle-like structures. However, the robustness of these strategies has only been established using purified proteins, not cytoplasmic extracts. Further testing is necessary to determine whether they will function in the context of a cytoplasm-filled, cell-like compartment. One localization mechanism that has proven compatible with *Xenopus* extracts is to label proteins with a strongly lipophilic dye, such as bodipy (Abu Shah et al., 2015). By doing so, it is possible to non-specifically localize a protein to the water–oil interface of an emulsion droplet. However, we recommend caution with such an approach and suggest that orthogonal chemical dimerization strategies offer an optimal path forward.

For cells to function properly, proteins must be recruited to the plasma membranes at defined intervals of time and in specific spatial patterns. To recapitulate spatiotemporal regulation of protein localization inside cell-like compartments, light-inducible protein dimerization has emerged as the tool of choice. Recent studies have leveraged optogenetics to control the spatial localization of proteins and organelles within the cell (Ballister, Aonbangkhen, Mayo, Lampson, & Chenoweth, 2014; Ballister, Ayloo, Chenoweth, Lampson, & Holzbaur, 2015; Guntas et al., 2015; Strickland et al., 2012; van Bergeijk, Adrian, Hoogenraad, & Kapitein, 2015), and induce protein assembly at the plasma membrane (Levskaya, Weiner, Lim, & Voigt, 2009; Wagner & Glotzer, 2016). Additionally, there has been limited development of *in vitro* optogenetic tools (Hallett, Zimmerman, Yumerefendi, Bear, & Kuhlman, 2016), which are expanding the types of manipulations that could be implemented for cellular reconstitution. We envision the inducible control of boundary-localization within an extract-filled compartment. This requires a configuration in which one-half of a light-inducible dimerization system is localized to the compartment boundary, while the protein of interest is fused to the other half of the optogenetic pair. In the optimal scenario, it would be possible to trigger recruitment of proteins to compartment boundaries or subcellular structures within a time scale of seconds. Additional practical considerations will likely have to be considered to achieve this goal.

5.2 | Applications to disease

The cellular reconstitution strategies described in this review provide a unique experimental framework to investigate molecular mechanisms that underlie human diseases, particularly those associated with improper sizing or spatial organization of a cell. For example, spindle positioning and chromosome segregation are critical for cell fate determination and organismal development. Importantly, mutations responsible for neurological diseases and some cancers are linked with defects in spindle orientation (Noatynska, Gotta, & Meraldi, 2012). Additionally, for a cell to function properly it must tightly regulate its dimensions and the sizes of its intracellular organelles (Ginzberg et al., 2015; Levy & Heald, 2012); deregulation of cell or nucleus size is associated with cellular senescence and diseases such as cancer (Li et al., 2015; Mitsui & Schneider, 1976; Sastre-Garau et al., 2004; Zink, Fischer, & Nickerson, 2004). To dissect mechanisms that contribute to these diseases it is necessary to control the dimensions and composition of the cell boundary; a feat that is not often possible *in vivo*. Therefore, investigating intracellular size control in cell-like compartment *in vitro* may provide paradigms for the contribution of organelle size misregulation to disease progression.

Xenopus cell-free cytoplasmic extracts are an emerging platform for screening small molecules that inhibit essential cellular pathways, including those involved in embryonic development and cancer progression (Broadus, Yew, Hann, & Lee, 2015; Hang et al., 2012; Hardwick & Philpott, 2015; Sackton et al., 2014; Thorne et al., 2010). In contrast to screens in cell culture systems, *Xenopus* cytoplasm can be used to analyze the phenotypes of drugs whose uptake are restricted in live cells. Additionally, cell-free cytoplasm can be used to differentiate between effects that are broadly cytotoxic versus those that inhibit a specific cellular process. Intriguingly, extract encapsulation may be useful in combination with drug screening to identify molecules that affect nucleus size and shape (Hara & Merten, 2015), which are indicators of cancer progression (Zink et al., 2004).

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