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Xenopus cell-free extracts and their contribution to the study of DNA replication and other complex biological processes

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ABSTRACT Here we discuss the important contributions that cell-free extracts have made to the study of complex biological processes. We provide a brief history of how cell-free extracts of frog eggs were developed to avoid many of the problems that can arise from the dilution and mixing of cellular components that typically occur when cell-free extracts are prepared. We briefly describe how *Xenopus* egg extracts have been fundamental to the study of many important cellular processes including DNA replication, cell cycle progression, nuclear protein import, nuclear assembly and chromosome organisation. We describe how, in particular, *Xenopus* egg extracts have made a major contributions to the study of DNA replication, by permitting the direct manipulation of proteins in a system that is extraordinarily faithful to the way that DNA replication occurs in the living embryo. Finally we consider how results obtained using *Xenopus* egg extracts are being translated to produce diagnostic reagents for cancer screening and diagnosis.

KEY WORDS: *Xenopus, frog, egg, cell-free extract, in vitro, DNA replication*

Why are cell-free extracts valuable?

Cell-free extracts have played important roles for many decades in both biochemistry and cell biology. An early and fundamental use of cell-free extracts was to provide the starting point for the purification of individual biochemical activities. This was typically achieved by following the biochemical activity whilst the extract was fractionated over several different chromatographic purification steps until only one protein remained. Nowadays cell extracts are frequently made as a starting point for analysis of protein and nucleic acids using any of the highly sensitive techniques available to molecular biologists, such as immunoblotting, mass spectrometry or DNA sequencing. The only major constraint on using extracts in these ways is that the components under study should remain intact and present during preparation of the extract and for example do not become degraded or insoluble.

Cell extracts are also a very powerful way of examining the potential interaction between cellular components in more complex biological systems. To address this sort of question, a typical approach is to prepare a cell lysate, and then use an affinity or fractionation step - commonly immunoprecipitation - to examine a potential physical interaction between two components such as the presence of a protein complex or the binding of proteins to DNA. The pitfalls of these types of experiment are well-known but not always avoided. For example inappropriate buffers used in making the lysate can force proteins together or separate them, so that the interactions occurring in the lysate are not those that occur in vivo. One reason why proteins might associate in a cell extract but not *in vivo* is that if they normally occur in different subcellular compartments that are mixed during extract preparation, they might encounter one another in a way that would not be possible in vivo.

Another major use for cell-free extracts, which is the main focus of the current review, is to examine the behaviour of complex systems that depend on the interaction of many different factors. The cell-free extract allows the identification and experimental manipulation of different components of the system in a way that is not possible in a living cell. Extracts have been used in this way to study many important biological processes, including transcription, translation, DNA replication, nuclear organisation and membrane trafficking. Cell-free extracts that perform these complex functions have to be prepared with great care in order to maintain the many activities involved, and this means that there are often a limited number of extract systems available to study a particular process. This review will emphasise in more detail the use cell-free extracts of *Xenopus* eggs and oocytes for the analysis of complex systems that depend on the interplay of many different factors, such as DNA replication.

One of the most successful early uses of mammalian cell-free

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extracts was for the study of protein synthesis and its control (Mathews and Korner, 1970; Pelham and Jackson, 1976). These studies resulted in identification of initiation factors and elongation factors for protein synthesis as well as identification of messenger RNAs. Reticulocyte lysates continued to make valuable contributions to these areas during the 1970s and 1980s. Ironically, a major challenge to their use for assaying mRNA came from microinjection studies using *Xenopus* oocytes (Gurdon *et al*., 1971). Because of their large size and availability in large numbers, eggs and oocytes of the frog *Xenopus laevis* have made important contributions to answering a wide range of questions in cell and developmental biology. The areas of their successes include control of the cell cycle and mechanism of cell cycle components, control of DNA synthesis and identification of components involved, assembly of chromatin and of the cell nucleus, and transport of macromolecules between the cytoplasm and the nucleus (see below).

Constraints on cell-free extracts

In order for it to be a valuable tool for studying a complex process, a cell-free extract should support the process under study in a way that mimics as closely as possible what happens in the intact cell. This is often hard to achieve. To make the extract 'cell-free' so that components can easily be manipulated, the very minimum is that the plasma membrane has to be ruptured or dissolved. This is typically achieved by using detergents or by mechanical forces, both of which may then lyse or disrupt structures or protein complexes within the cell that are important for the process under study. Cells are highly organised structures, containing many insoluble or large structures that co-ordinate biological activities, such as mitochondria, endoplasmic reticulum, Golgi or the nucleus. When making an extract, the consequences of eliminating these structures (eg mixing cytoplasm and nucleoplasm) need to be clearly appreciated. To make a soluble extract, proteins often need to be removed or eluted from larger structures so that they become available for experimental investigation. For example, in order to study transcription factors or other DNA-bound proteins, they may need to be eluted from chromatin into the extract, unless the cell has a large excess of these proteins free in the cytoplasm or nucleoplasm. The interior of a cell is a densely-packed jumble of proteins, nucleic acids and other macromolecules in very close proximity to each other with very little free water. In typical cell extracts, however, the cellular components are significantly diluted when compared to the intact cell, which can have profound implications for the ability for some complex processes to proceed. A good example of this is nuclear assembly in *Xenopus* egg extracts, the efficiency of which is highly sensitive to dilution. The dilution that occurs when cell-free extracts are made introduces an ionic aqueous environment that can be very different from that of the intact cell. This can have profound effects on complex biological reactions, so the components of the diluting buffer need to be chosen with care. For example, whilst the physiological intracellular cation is potassium at ~150 mM, the main intracellular anion comes from negatively-charged amino acids on proteins and the concentration of chloride is much lower than that of potassium (10-80 mM, varying between cell types). In cell-free extracts, chloride is typically added to buffers at concentrations higher than exist in vivo, and is inhibitory to many reactions. For example, concentrations of chloride above ~75 mM disrupt the Mcm2-7 heterohexamer and prevent it being loaded onto DNA in the licensing step of DNA replication.

Value of *Xenopus* eggs for making cell-free extracts

Xenopus eggs have been extraordinarily valuable for a range of studies using cell-free extracts (Fig. 1). One crucial advantage of *Xenopus* eggs or oocytes as sources of cell-free extracts has been the ease of microinjecting molecules into the intact cell to guide in vitro, cell-free, studies and to validate the results that emerge from them. Cell-free assays have a major advantage over microinjection studies in that they facilitate fractionation by providing more convenient assays for fractionated molecules. However, microinjection provides a gold standard for validating cell-free results.

Eggs are large single cells and therefore easy to lyse without extraction components such as detergents that are potentially inhibitory. Furthermore they can be lysed gently by centrifugation minimising the shear forces involved in mechanical homogenisation procedures. Centrifugation also provides a convenient means of sub-cellular fractionation of the resulting homogenates. The large maternal stockpile of materials required for rapid cell division and development following fertilisation provides rich sources of enzymes and regulatory factors. Proteins involved in cell proliferation are extremely well represented. This same stockpile means that only minimal transcription and translation are required for the rapid series of cell divisions that follow fertilisation. Therefore these pre-packaged cell division machines have been particularly valuable in studies of various aspects of cell proliferation and basic cell biology, though their value for studying gene expression has been more limited.

Early attempts at homogenising *Xenopus* eggs and oocytes to make cell-free extracts were made in the mid-1970s (Benbow and Ford, 1975; Gandini Attardi *et al*., 1976; Mattoccia *et al*., 1976). Benbow and Ford added *Xenopus* liver nuclei to eggs that had been homogenised in a blender and observed tritiated dTTP incorporation as well as possible replication bubbles in the electron microscope (Benbow and Ford, 1975). Gandini Attardi added SV40 DNA to oocyte extract and observed complex forms in the electron microscope which they interpreted cautiously as the products of either replication or recombination (Gandini Attardi *et al*., 1976). Mattocia *et al.,* used the same procedures as Gandini Attardi and fractionated a relaxing activity which we would now

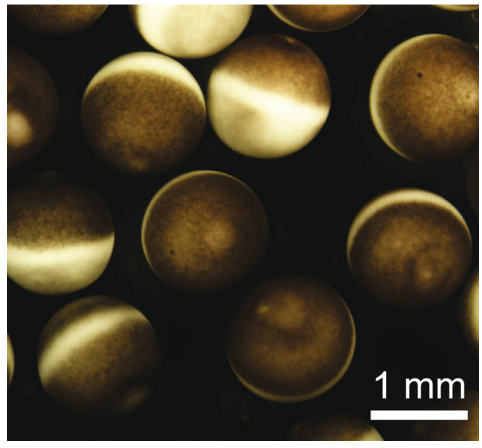

Fig. 1. *Xenopus* eggs. *A photograph of de-jellied* Xenopus laevis *eggs. Scale bar, 1 mm. Courtesy of Jolanta Kisielewska, University of Plymouth.*

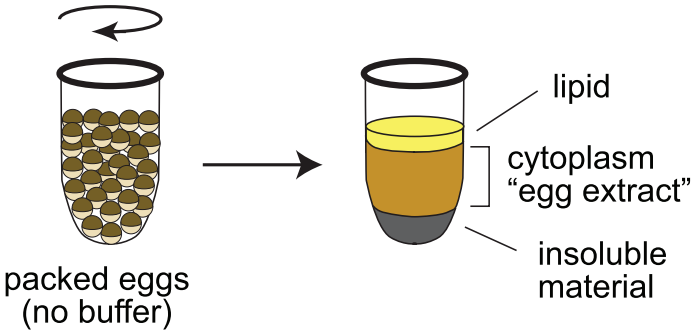

Fig. 2. Making *Xenopus* egg extracts. *Cartoon of the method of making* Xenopus *egg extracts ('low seed supernatants') according to the original protocol of Lohka and Masui. Dejellied eggs are packed into a centrifuge tube and any excess buffer is removed. Eggs are then lysed by relatively gentle centrifugation (~20,000* g*). This separates the egg contents into a floating lipid plug, an insoluble pellet and in between, the cytoplasmic fraction.*

recognise as DNA topoisomerase 1 and also endonuclease activities (Mattoccia *et al*., 1976). While there may have been genuine contributions of DNA replication in these studies, nevertheless the extent of recombination or repair of DNA following endonuclease attack of templates is now known to be a problem that confounds these early studies.

Another attempt to seek evidence for DNA replication in cell-free extracts of *Xenopus* eggs failed to find replication, but did find extensive nucleosome assembly activity, leading to a series of publications on chromatin assembly (Laskey *et al*., 1977; Laskey *et al*., 1978; Earnshaw *et al*., 1980). The first nucleosome activity that was isolated from egg extracts was nucleoplasmin, which was able to bind histones and transfer them to DNA in vitro. The term molecular chaperone was coined to describe that function (Laskey *et al*., 1978). However, we now know that only histones H2A an H2B are bound to nucleoplasmin *in vivo* (Dilworth *et al*., 1987). The other two core histones H3 and H4 are bound to other acidic proteins N1/N2. The functions of nucleoplasmin and N1/N2 were identified by fractionation of *Xenopus* egg extracts. A puzzling problem encountered in these early studies of nucleosome assembly by nucleoplasmin or the combination of nucleoplasmin with N1/N2 (Kleinschmidt and Franke, 1982; Kleinschmidt *et al*., 1985; Dilworth *et al*., 1987) was its relatively slow assembly rate compared to the extremely short cell cycles of early embryos. This paradox was resolved by the discovery that DNA replication accelerated chromatin assembly in egg extracts substantially (Gaillard *et al*., 1996).

Studies of nucleoplasmin had two important spin-offs. First they revealed that nucleoplasmin has an important role in decondensing sperm chromatin at fertilisation (Ohsumi and Katagiri, 1991; Philpott *et al*., 1991; Philpott and Leno, 1992; Leno *et al*., 1996). Second, attempts to confirm that nucleoplasmin was indeed a nuclear protein produced the initially controversial result that import of nuclear proteins into the nucleus is not by free diffusion and selective retention of only nuclear proteins as was previously thought, but was by signal mediated selective import of only nuclear proteins (Dingwall *et al*., 1982; Robbins *et al*., 1991). The initial studies of nucleoplasmin import into nuclei were made by microinjection of proteins and protein fragments into the cytoplasm or the nucleus of intact oocytes. However, fractionation to identify the import factors called importins or karyopherins was made from egg extract, using permeabilised mammalian cells as the nuclear targets for import (Gorlich *et al*., 1994; Gorlich *et al*., 1995b; Gorlich *et al*., 1995a).

Amphibian egg extracts were exceptionally important in analysing the control of the cell cycle and the assembly of nuclear structures after mitosis. Lohka and Masui made critically important contributions to analysing the control of the cell cycle. Initially they used cell-free extracts from eggs of the frog *Rana pipiens*, though in subsequent studies they used *Xenopus* as the source of eggs. They introduced several crucial procedures that increased the value of egg extracts dramatically (Fig. 2) (Lohka and Masui, 1983). First, they avoided conventional homogenisation, but instead they lysed eggs by centrifugation of packed eggs. Secondly, the extracts contained very little exogenous buffer and so maintained cellular components at near physiological concentrations. Thirdly, they took steps to exclude calcium from homogenisation buffers and by including EGTA they were also able to counteract the en-

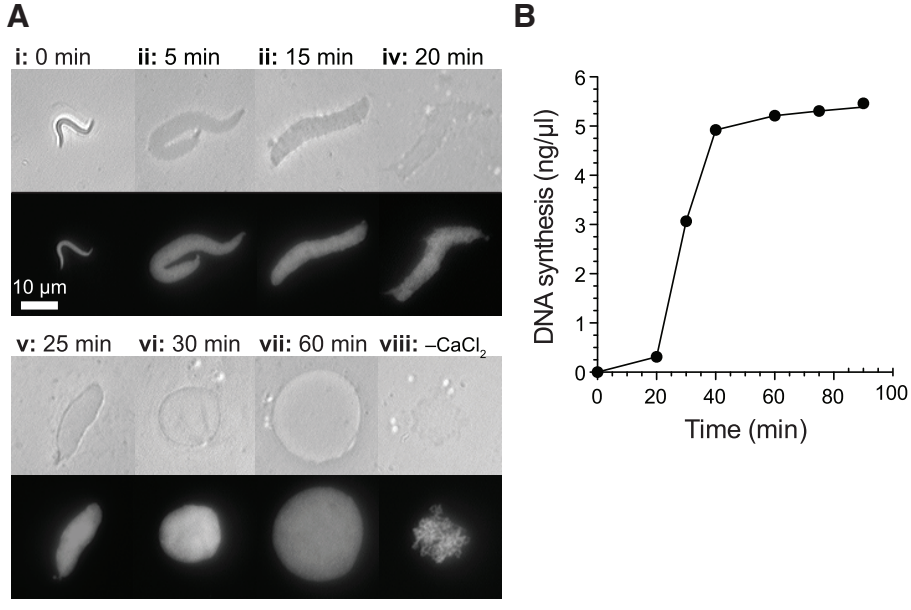

Fig. 3. Nuclear assembly and DNA replication in *Xenopus* egg extracts. *Sperm nuclei were incubated in metaphase arrested* Xenopus *egg extract released into interphase by addition of 0.3 mM CaCl2.* **(A)** *Nuclear formation was followed over the course of 60 minutes by phase contrast (upper panels) and UV (lower panels) microscopy (i-vii). Sperm nuclei incubated in extract in the absence of CaCl2 were visualized after 60 minutes (viii). Bar, 10 µm.* **(B)** *DNA synthesis was followed over the course of the incubation by measuring incorporation of [α^{32}P]-dATP. Reproduced from (Gillespie* et al.*, 2012).*

dogenous calcium in the extract, thereby preventing spontaneous release from cell cycle arrest in meiotic metaphase II (Lohka and Masui, 1984a). Furthermore they used low speed supernatants rather than taking only the soluble fraction. Indeed they showed that both the soluble fraction and a particulate membrane fraction were required for maximum activity in the systems (Lohka and Masui, 1983, 1984b). When these three procedures were followed they obtained extracts that were capable of decondensing sperm chromatin, assembling the chromatin into nuclei and then allowing DNA synthesis, which others subsequently showed was genuine semi-conservative replication (Fig. 3) (Blow and Laskey, 1986; Blow and Watson, 1987). The nuclei were subsequently disassembled and the chromatin condensed as the extracts entered mitosis in vitro. When protein synthesis was blocked so that extracts could not enter mitosis, these extracts supported exactly one round of DNA replication as evidenced by density substitution (Blow and Laskey, 1986) and flow cytometry (Blow and Watson, 1987) experiments, suggesting that the DNA replication was occurring under normal cell cycle control. Egg extracts prepared by the principles of Lohka and Masui appear to faithfully support all of the cytoplasmic activities that occur in the early embryo and which do not require the plasma membrane. This means that they essentially represent a 'cell in a test tube'. As a consequence, *Xenopus* egg extracts have been widely exploited for the study of many further processes including nuclear assembly, nuclear cytoplasmic transport, DNA repair, and chromosome structure. Perhaps the most important application of Lohka and Masui's egg extracts was the isolation by Lohka and Maller of maturation promoting factor or MPF (Lohka *et al.*, 1988; Gautier *et al.*, 1988). This elusive factor had been a 'holy grail' of cell cycle studies and its isolation allowed integration of the whole cell cycle field when it was shown that MPF consisted of a cyclin, complexed to a cyclin-dependent kinase (Gautier *et al.*, 1990). Both activities had been discovered previously but their relationship to one another, and their roles in the cell cycle, became very much clearer when they were identified as the two sub-units of MPF.

The study of DNA replication in Xenopus *egg extracts*

Lohka and Masui's extracts also allowed rapid progress in analysis of the control of DNA replication in higher eukaryotes. Previously Mechali and Harland had shown that homogenates of *Xenopus* eggs were capable of synthesising a complete complementary DNA strand on single stranded DNA templates (Mechali and Harland, 1982). However, they were unable to obtain initiation of DNA replication de novo on double stranded templates. Adoption of Lohka and Masui's extract preparation procedures allowed clear demonstrations of the initiation of DNA replication *in vitro* (Blow and Laskey, 1986; Blow and Watson, 1987). Since then *Xenopus* egg extracts have played an important part in many fundamental discoveries about the control of DNA replication.

An early discovery was that the initiation of DNA replication depended on the template DNA being assembled into a functional interphase nucleus supporting active nuclear import (Blow and Watson, 1987; Newport, 1987; Sheehan *et al.*, 1988; Blow and Sleeman, 1990; Cox, 1992). Further work led to a model whereby re-replication of DNA was prevented by there being two non-overlapping phases that are required for DNA replication: prior to nuclear envelope formation, future origins of replication are 'licensed' for a single initiation event, but once the nucleus was assembled licensed origins could initiate but no further origin licensing could occur (Blow and Laskey, 1988). These principles were further used to identify double hexamers of Mcm2-7 as the proteins that are assembled onto origins to license them for replication (Chong *et al.*, 1995; Kubota *et al.*, 1995; Madine *et al.*, 1995; Kubota *et al.*, 1997; Gambus *et al.*, 2011). Subsequent work led to the identification of all the proteins necessary to license origins *in vitro* and a reconstitution of the licensing reaction with purified proteins (Carpenter *et al.*, 1996; Coleman *et al.*, 1996; Romanowski *et al.*, 1996; Rowles *et al.*, 1996; Maiorano *et al.*, 2000; Gillespie *et al.*, 2001). The role of the nuclear envelope in preventing re-replication of DNA was subsequently explained by nuclear import regulating the activity of geminin, a potent inhibitor of the licensing reaction (McGarry and Kirschner, 1998; Tada *et al.*, 2001; Hodgson *et al.*, 2002; Li and Blow, 2005).

Other work on the initiation of replication in *Xenopus* egg extracts has elucidated the role of S phase Cdks and the Cdc7 kinase in replication initiation (Blow and Nurse, 1990; Fang and Newport, 1991; Jares and Blow, 2000; Walter, 2000), has shown how otherwise dormant replication origins can be activated to allow completion of replication under conditions of replicative stress (Woodward *et al.*, 2006) and has led to the identification and analysis of key proteins involved in replication initiation and replisome function, including Treslin, RecQ4 and the GINS complex (Kubota *et al.*, 2003; Kumagai *et al.*, 2010; Sangrithi *et al.*, 2005). Technology for using *Xenopus* egg extracts to study DNA replication has also developed since the initial protocol of Lohka and Masui. Of note is the development of 'nucleoplasmic extract' (NPE) which provides an extract that can support the initiation of DNA replication without the DNA having to be assembled into a nucleus (Walter *et al.*, 1998). The preparation of NPE exploits the fact that intact nuclei float in *Xenopus* egg extract. When extracts containing nuclei are centrifuged, a layer of nuclei floats to the top and can be harvested and subsequently crushed to release an undiluted extract of nucleoplasmic protein

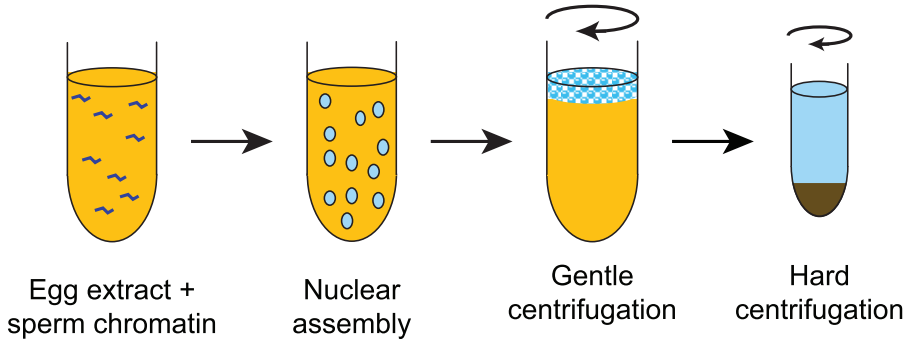

Fig. 4. Nucleoplasmic extract cartoon of the method of making 'Nucleoplasmic Extract' from *Xenopus* eggs according to the original protocol of Walter and Newport. *Sperm chromatin is incubated in standard 'low speed supernatants' (prepared as shown in Fig. 2) allowing the assembly of interphase nuclei. The extract is then gently centrifuged (~16,000* g*) to allow the nuclei to float to the top of the extract. Nuclei are then aspirated from the top, put into a new tube and centrifuged hard (~250,000* g*) to break the nuclei, releasing the soluble 'Nucleoplasmic Extract'.*

(Fig. 4). When DNA that has already been licensed is incubated in NPE (which contains active geminin and so does not support origin licensing), the initiation of DNA replication occurs very efficiently. The use of NPE provides a powerful extract system for studying replication where there is great control over the different stages of replication and which has allowed the detailed study of processes that are otherwise intractable, such as replication termination (Dewar *et al*., 2015).

DNA replication in cell-free systems from other species

In addition to *Xenopus* egg extracts, cell-free extracts from other systems have also been valuable for studying DNA replication. For example an *in vitro* system that supports DNA replication has recently been developed in budding yeast (Heller *et al*., 2011; Gros *et al*., 2014; On *et al*., 2014), and using this system replication initiation has recently been achieved using purified proteins (Yeeles *et al*., 2015). This opens opportunities to generate more mechanistic detail on the initiation process itself.

Efficient initiation of DNA replication in extracts of mammalian cells has been more difficult to achieve, though there has been significant progress in several areas. The first evidence of initiation in mammalian cell-free extracts was achieved using G1 nuclei in S-phase cytoplasm from HeLa cells resulting in cyclin/Cdk dependent initiation of replication in vitro, though efficiencies were not high and optimisation of the systems has been difficult (Krude *et al*., 1997). A variation of this system using mouse nuclei released from quiescence as the templates was able to discriminate distinct roles of Cyclin A/Cdk2 and Cyclin E/Cdk2 establishing a sequence of requirements for these two complexes and implicating Cdc6 as an important substrate for their activity (Coverley *et al*., 2002). A significant improvement was made by simplifying the synchronisation procedures by the use of mimosine and this has allowed exciting recent advances in mapping origins at which human DNA replication initiates. (T. Krude personal communication. Manuscript submitted).

The future of *Xenopus* egg extracts

There are still many complex processes that are not well understood and that can be addressed using *Xenopus* egg extracts. Important work continues to be done on the process of nuclear envelope and nuclear pore assembly, where a huge number of proteins need to be brought together to form a large macromolecular assembly. The *Xenopus* system has the disadvantage that it is not amenable to genetic analysis, which means that proteins need to be identified using antibodies rather than tags. However, a range of new technologies can be applied to their study. For example, mass spectrometry provides a powerful way to identify proteins in large ultrastructures, such as nuclear pores, replisomes, and chromosomes that can be assembled *in vitro* and then carefully separated for analysis of their contents (Gambus *et al*., 2011; Gillespie *et al*., 2012). There are now a large number of procedures that can be used to create and control cell cycle synchrony in egg extracts with unprecedented precision (Gillespie *et al*., 2016). *Xenopus* egg extracts can be used for live microscopy imaging, and in future this approach could be developed to deliver large amounts of data and 'high content' analysis of processes that can be followed by fluorescently marked components. As the acquisition of data becomes more and more powerful, a major challenge for 21st century biology is to understand all of the data that is produced. Cell-free systems, such as *Xenopus* egg extracts that support complex biological procedures should have a large potential in making sense of biological complexity by allowing scientists to disassemble, reassemble and manipulate highly controlled reactions that involve the interplay between many interacting components.

Translation of results from cell-free systems to produce diagnostic reagents for cancer screening and diagnosis

Proteins whose roles have been identified using cell-free extracts have emerged as powerful markers for cancer diagnosis and screening. Initial attempts focused on Cdc6 as a potential marker but its stability limited its value. In contrast MCM proteins of the MCM2-7 complex are robust and informative markers that have been tested extensively for both screening and differential diagnosis for a range of cancers (Baldwin *et al*., 2003; Coleman *et al*., 2006; Jackson *et al*., 2013). Clinical trials have been undertaken or are in progress to assess the value of antibodies against MCM2-7 in screening for cancer of the cervix, bladder, prostate, oesophagus and colon amongst other sites. One diagnostic product has already been launched and another is due to be launched later in 2016. These developments are direct consequences of studies using cell-free systems to study DNA replication in both frog and human systems.

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