Magnifying human fertility: microscopy and assisted reproductive technology

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Assisted reproductive technology (ART) has undergone rapid expansion over recent years due to the use of pioneering clinical techniques such as *in vitro* fertilisation (IVF), and intracytoplasmic sperm injection (ICSI). Microscopy represents an essential requirement for such techniques, allowing gametes and embryos to be visualised in sufficient detail, permitting the assessment of key clinical parameters and micromanipulation without compromising viability. Historically, microscopy has been essential to our understanding of the roles of sperm and oocytes before, during, and subsequent to gamete fusion. Indeed, microscopy continues to play a key role in furthering our understanding of the complex and intricate mechanisms which underlie sexual reproduction. Herein, we discuss the essential nature of microscopy, and its associated skills, within the many parameters of ART, as well as its continued fundamental role in research within reproductive sciences.

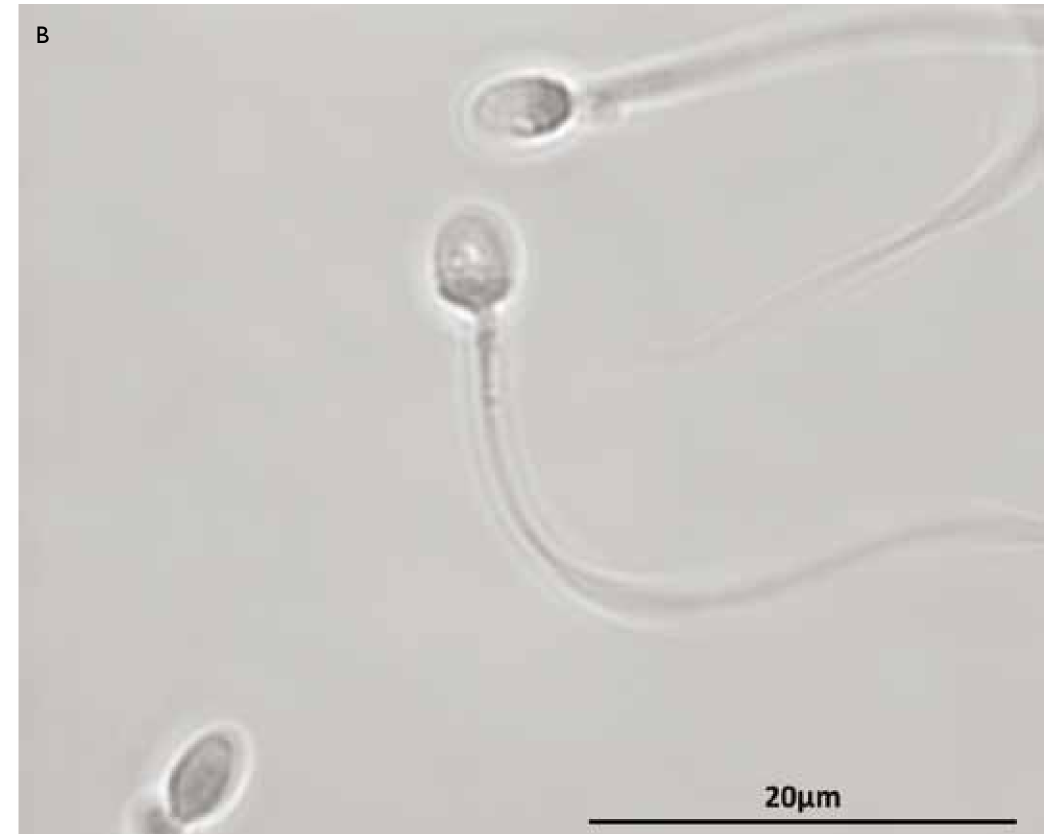


Figure 1: Images of motile human sperm taken during routine semen analysis using (A) conventional brightfield optics using a simple compound microscope, and (B) differential interference contrast DIC optics using a Zeiss LSM510 confocal microscope with an oil immersion objective. Copyright: Oxford Fertility Unit and Junaid Kashir, respectively. Whilst confocal analysis clearly provides superlative magnification and resolution for studying sperm and is widely utilised in scientific research, such systems are highly impractical for routine use in IVF units owing to the prohibitive costs involved.

Introduction

Sexual reproduction is a proliferative strategy employed by a multitude of species, and characterised by the transfer of genetic material from each parent to create a unique individual. In mammals, the male testes produce vast numbers of sperm (~53-55 µm in length). In females, the ovaries generate oocytes which are much larger in comparison (~100 µm in diameter). Oocytes develop within a protective follicle and are surrounded by a glycoprotein layer (the zona pellucida). Following maturation, oocytes are released and transported along the fallopian tube towards the uterus. It is along this pathway that the oocyte hopes to fuse with a sperm to create a genetically unique individual, a process known as fertilisation. Historically, microscopy has been essential to our understanding of the roles of sperm and oocytes before, during, and subsequent to gamete fusion. Indeed, sperm were one of the first cell types to be examined using the early microscopes of the 16th and 17th centuries. These early observations gave rise to the now famous homunculus and animalcule theories, which stated that sperm simply contained miniature versions of the human or animal to which they belonged, and were destined to be 'implanted' in the mother's womb. Of course, we now realise that such inspired observations were far from the truth, and advances in microscopy have served to illuminate the complex and multi-factorial mechanisms which are inherent within the process of sexual reproduction. In order to create a new life, both the sperm and oocyte need to overcome a plethora of obstacles, all of which must occur in a concerted manner for a successful outcome.

While in the testis, mammalian sperm are immature and immotile, and need to undergo a variety of modifications in order to gain fertilisation competency. The first step of maturation occurs within the epididymis, where sperm acquire vital proteins and cholesterols (Yoshida *et al*., 2008). During sexual intercourse, motile sperm begin to make their way up the female reproductive tract towards the oocyte, directed by a process termed chemotaxis, thought to be mediated by progesterone released by cumulus cells surrounding the oocyte in the oviduct (Lishko *et al*., 2011; Strünker *et al*., 2011). Assuming the two gametes meet successfully, a process of fusion commences, in which a number of biochemical modifications and processes ultimately lead to the plasma membranes of both gametes being transformed from two separate barriers into a single lipid bilayer. This represents a complex pathway but is thought to involve a variety of receptor/ligand interactions, on both the sperm and the oocyte.

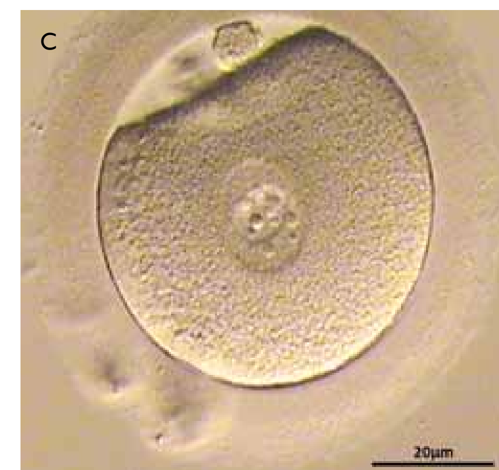
Mammalian oocytes remain arrested for most of their existence at the dictyate stage of prophase I (Jones, 2007), which then undergo first meiotic division within the ovary. Immediately after, and just before ovulation, oocytes become arrested at the metaphase stage of the second meiotic division (MII) following exclusion of the first polar body (Jones, 2005; 2007). Alleviation of MII arrest must be eliminated for cell division and subsequent embryogenesis to proceed. The process involved occurs following fertilisation via a series of concurrent events that are collectively referred to as oocyte activation. Only then, can the highly complex and multi-factorial process of embryogenesis begin.

Figure 2: Various stages of human oocytes and embryos as visualised using an inverted microscope and Hoffman Modulation Contrast. (A) germinal vesicle stage oocyte, (B) MII oocyte showing a polar body at the 12 O' clock position, and (C) fertilised oocyte showing two centralised pronuclei, and two peripheral polar bodies. Copyright: Mrs Tracey Griffiths, Oxford Fertility Unit.

As our understanding of the intricate mechanisms involved in gamete production, fertilisation, and embryogenesis develops, we are able to better understand the reasons behind their dysfunction. Enhanced understanding is vital in the development of new prognostic, diagnostic, and therapeutic protocols for the treatment of human infertility. Underlying this thirst for new knowledge is the worrying fact that the World Health Organisation (WHO) now estimates that approximately 50-80 million individuals (8% of couples) worldwide are now infertile (Ledger, 2009).

Microscopy and assisted reproductive technology (ART)

Assisted reproductive technology (ART) has undergone rapid expansion over recent years. One of the major reasons for the success of ART is the use of pioneering clinical techniques such as *in vitro* fertilisation (IVF), or intracytoplasmic sperm injection (ICSI) whereby a single sperm is microinjected directly into the oocyte. As a result, over one million treatments are performed globally each year, accounting for up to 7% of all births in some developed countries (Guner *et al*., 2009; Kashir *et al*., 2010; Shivhare *et al*., 2011). Microscopy represents an essential requirement for such techniques. Gametes and embryos need to be visualised in sufficient detail to allow for the assessment of key clinical parameters such as morphology and motility, allowing micromanipulation without compromising viability (Shivhare *et al*., 2011). ART laboratories are thus equipped with a wide range of microscopy equipment which invariably includes dissecting, compound, and inverted systems equipped with



brightfield, phase contrast, and Hoffman modulation contrast (HMC) optical technology (Shivhare *et al*., 2011). Competency and consistency in microscopy is thus an essential aspect of an embryologist's training, and thus represents a key requirement for educational courses aiming to train competent embryologists of the future, such as the MSc in Clinical Embryology at the University of Oxford.

Clinical ART laboratories utilise a range of microscopes with which to perform gamete selection and clinical procedures. Upright microscopes are generally used to visualise and assess sperm morphology, motility and viability, while stereo microscopes are used to assess oocyte and embryo morphology and viability. However, while both types of microscope are utilised within clinical and research settings, the most versatile form for ART are those with an inverted design. One critical adaption of this type of microscope is the fact that the specimen stage can be heated, thus allowing cells and embryos to be maintained

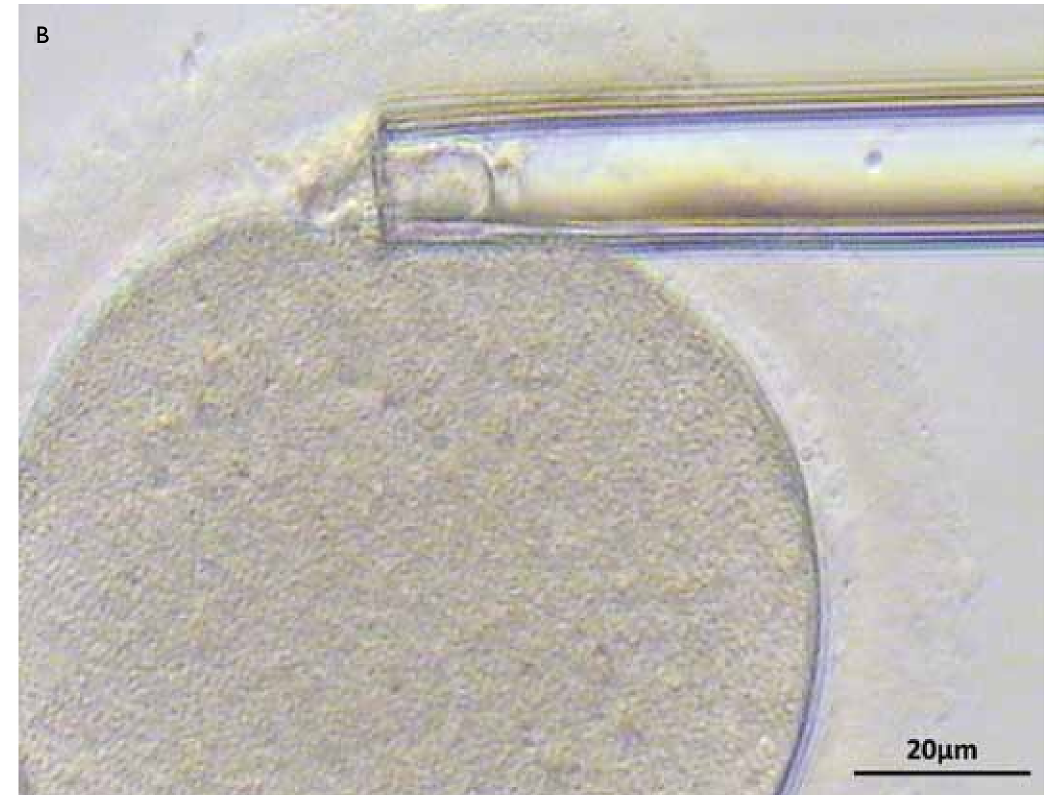


Figure 3: Images of an oocyte (A) before and (B) undergoing polar body biopsy, using an inverted microscope system. The oocyte is held in place with a holding micro-tool to the left. An incision is made in the zona pellucida using an infra-red laser and the polar body (blue arrow) isolated using a biopsy micro-tool. Copyright: Mrs Tracey Griffiths, Oxford Fertility Unit.

at a constant physiological temperature of 37°C. Whilst permitting the use of a wide range of magnification objectives, inverted microscopes also allow precise modifications to the stage to allow the incorporation and coordinated control of precision micro-tools. Such tools are fundamental requirements for the micromanipulation of sperm and oocytes during ICSI. Furthermore, infra-red laser systems can be added to inverted microscopes to allow the finely-controlled ablation of the outer embryo membrane, thus permitting single cells to be biopsied for genetic screening.

As the number of couples seeking infertility treatment increases, there is a growing global call for the universal adoption of elective single embryo transfer (eSET) to reduce the risk of multiple pregnancies associated with multiple embryo transfer (McLernon *et al*., 2010). It is therefore vital that embryologists are able to successfully identify the healthiest gametes for procedural intervention and subsequently, the most viable embryos for re-implantation (Shivhare *et al*., 2011). Approximately 40% of all infertility cases may be attributed to irregularities in sperm production or function (Barrat *et al*., 2011). Semen analysis therefore represents a vital diagnostic approach, and critically evaluates qualitative characteristics such as appearance, pH, viscosity, volume, sperm count, morphology, and motility (Guner *et al*., 2009; Shivhare *et al*., 2011). Samples are investigated within one hour of ejaculation and compared against WHO guidelines to identify abnormalities and plan an appropriate course of treatment. The gold standard for sperm counting uses cell counting chambers (e.g. Neubauer, Burker, or Makler) and a light microscope equipped with phase-contrast objectives (Fig. 1).

Whilst some of these analytical procedures are carried out macroscopically, many (e.g. concentration, motility, and morphology) rely heavily upon microscopy. Oocyte morphology is considered a key contributor to fertilisation success. Microscopy is used not only to assess oocyte viability using the maturity of the cumulus-coronal complex or the position of the germinal vesicle as primary markers, but also by considering polar body presence/position, cytoplasmic clarity, and the relative thicknesses of the zona pellucida and the perivitelline space (Fig. 2; Guner *et al*., 2009; Shivhare *et al*., 2011). Specific criteria used to identify and select the healthiest embryo for implantation include: pronuclear morphology and size, the number and location of nucleolar precursor bodies, blastomere shape, thickness of the zona pellucida, cytoplasmic fragmentation, indicators of early cleavage, and the rate of progression to the blastocyst stage (Shivhare *et al*., 2011).

Techniques such as breaking the tail of an individual sperm with a fine-tipped micro-tool are critical in effective sperm capture for ICSI but also represent highly challenging skills to acquire. Embryologists must be able to manipulate and transfer gametes from one drop of medium to another, and to be able to present oocytes and embryos for manipulation in a specific orientation to avoid damage (Shivhare *et al*., 2011). Another aspect of clinical embryology requiring the acquisition of absolute dedication and skill is the practice of embryo biopsy, a technique in which polar bodies or single blastomeres (Fig. 3, 4) can be safely removed from developing embryos for genetic analysis (e.g. fluorescent *in situ* hybridisation, single cell polymerase chain reaction, or comparative genomic hybridisation). Many ART laboratories also utilise specialised digital cameras with which to visualise or witness gametes or embryos undergoing micromanipulation. Similar digital imaging techniques are often applied to the

Figure 4: Images of an oocyte (A) before and (B) during zona drilling for biopsy as visualised using an inverted microscope and Hoffman Modulation Contrast. Copyright: Mrs Tracey Griffiths, Oxford Fertility Unit.

mounting body of scientific research underpinning the development of ART, although many research laboratories utilise imaging equipment of a much greater specification, often adapted specifically for a given research directive.

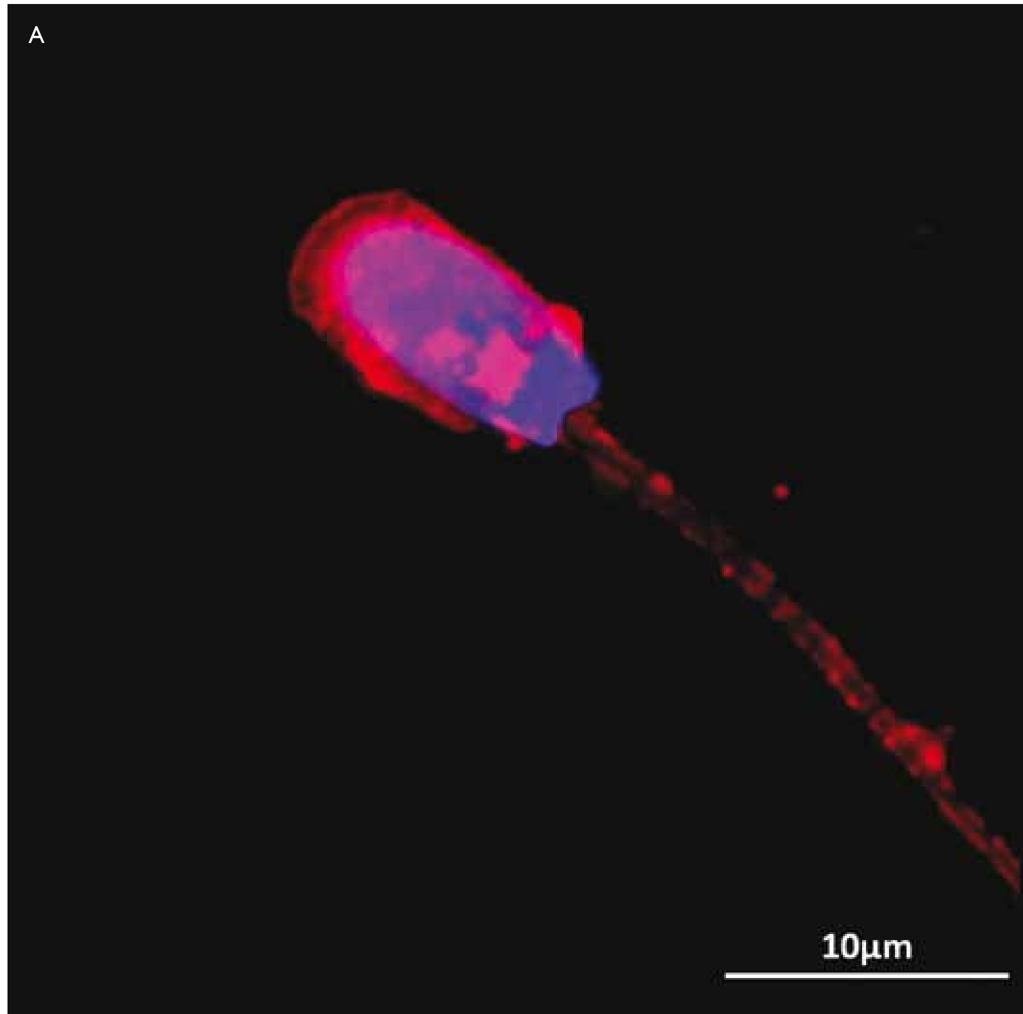
Microscopy in fertility research

Embryologists are constantly trained to follow and repeat clinical procedures in a consistent manner (Guner *et al*., 2009). This way, success rates are maintained and risk is minimalised. However, despite the rapidly advancing nature of ART, there are still many unknown factors which may be contributing towards male or female infertility and the prevention, or premature termination, of pregnancy. The elucidation of the reasons behind these factors is what intrigues and attracts scientists and clinicians alike towards research in this field.

Whilst manipulative techniques such as ICSI and embryo biopsy underlie the current success of ART, it is important to note that these techniques rely unconditionally upon the quality of the manipulator system employed and the optical equipment used to visualise specimens. Indeed, although ART has played a critical role in reducing infertility, groups of infertile couples for which ART has not yet proven successful still persist. Furthermore, whilst advances in ART have revolutionised infertility treatment, success rates vary, with pregnancy and delivery rates for IVF and ICSI rarely exceeding 30% and 23% respectively (ICMART, 2009; de Mouzon *et al*., 2010). Consequently, it is critical that ART protocols are constantly modified in line with scientific advances so that clinics can consistently provide patients with the best chances of conception.

The evolution of clinical ART techniques may lead to improvements in ART efficiency and pregnancy success rates. Indeed, advancements such as *in vitro* maturation (IVM), involving the maturation of germinal vesicle (GV)-stage oocytes from a diverse range of follicular stages (Smitz *et al*., 2011), may allow ART to become available to more patients, as well as reducing the need for hormonal stimulation, and thereby reducing safety concerns for the patient. Another possible advance for semen analysis is the use of computer-assisted sperm analysis (CASA) for sperm counting, motility analysis, and, in some cases, morphology. Relying heavily upon mathematical algorithms, CASA is based upon a combination of light microscopy and sophisticated computer software, often using negative phase-contrast objectives (Tomlinson *et al*., 2010). However, whilst popular amongst research scientists, CASA has yet to become fully established within IVF clinics.

Figure 5: Image of a human sperm acquired via transmission electron microscopy (TEM), courtesy of Professor David Ferguson (Nuffield Department of Clinical Laboratory Sciences, University of Oxford). Copyright: Professor David Ferguson. Whilst the image clearly provides a superior level of magnification and resolution for studying sperm ultra-structure/morphology, and is widely utilised in scientific research, such systems are highly impractical for routine use in IVF units owing to the prohibitive costs of such equipment and the highly skilled procedures involved.



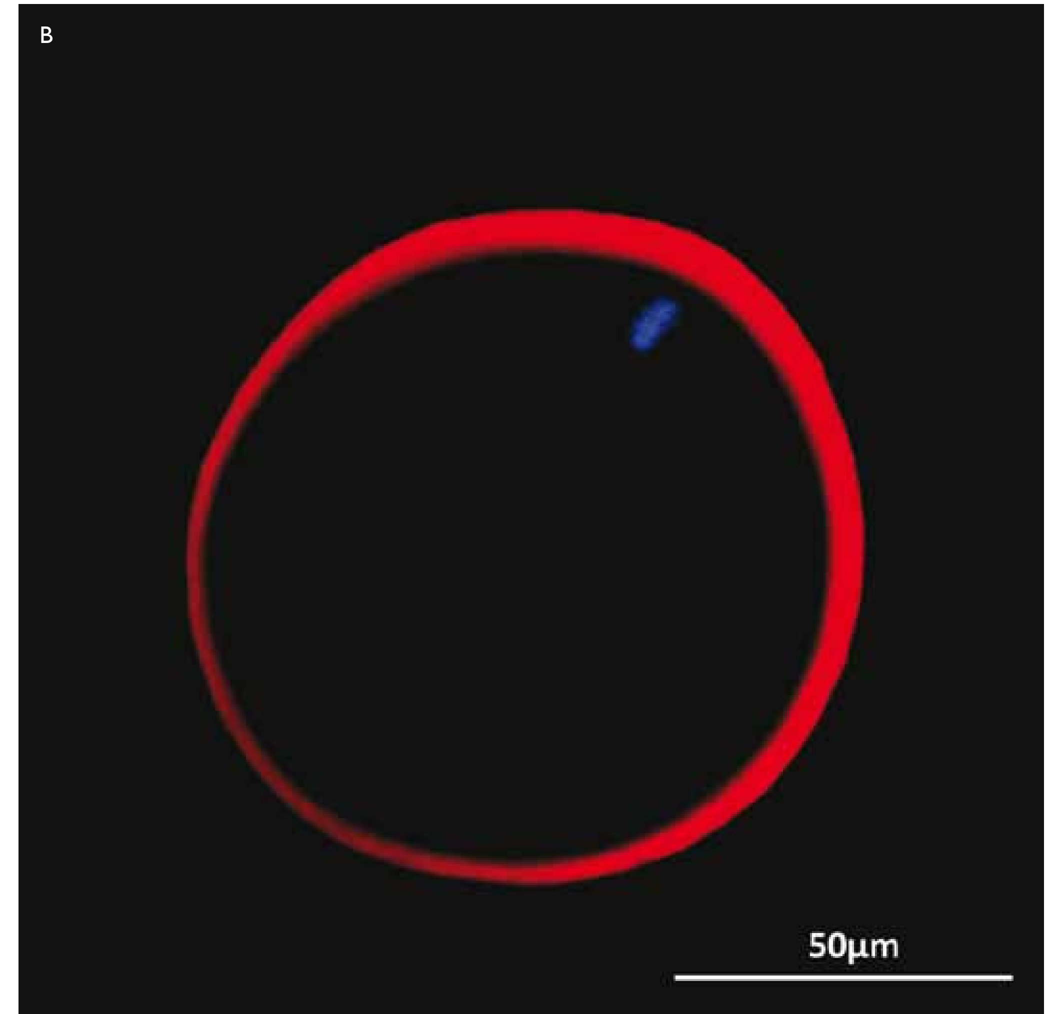


Figure 6: Confocal microscopy images of plasma membrane staining (red) of (A) boar sperm obtained via a Z-stack reconstruction of multiple images taken along different planes of view, and (B) an optical slice of an MII mouse oocyte. Blue staining indicates DNA and therefore represents the nucleus and chromosomes in the mouse oocyte. Images were taken using a Zeiss LSM510 confocal microscope with an oil immersion objective. Copyright: Mr Junaid Kashir.

The realm of reproductive science provides scientists with the opportunity to use much more sophisticated microscopy techniques, such as scanning or transmission electron microscopy (SEM and TEM respectively). SEM uses a high-energy beam of electrons which interact with atoms within the sample being imaged to produce signals representing the surface topography of the target sample. In this way, scientists can acquire detailed and highly resolved images of a target specimen's surface features. TEM operates on a similar principle but uses ultra-thin sections prepared from the sample to provide intricate and detailed images. While such techniques allow for highly detailed information pertaining to ultra-structure and ultra-morphology (Fig. 5), such protocols involve highly skilled and time-intensive procedures which are unlikely to be applicable in a clinical setting. However, a modified version of ICSI has been gaining momentum over recent years, and while yielding interesting results, it currently remains unclear as to how popular this technique will become in IVF units of the future. Referred to as intracytoplasmic morphologically selected sperm injection (IMSI), this technique specifically selects the best quality sperm for injection at a much higher magnification than ICSI and is based upon morphological criteria (Berkovitz *et al*., 2006), such as the avoidance of sperm exhibiting vacuoles. Indeed, IMSI-selected sperm have resulted in successful pregnancy in cases of aberrant sperm morphology where pregnancy and implantation success would otherwise be very low (Souza Setti *et al*., 2010; Sermondade *et al*., 2011).

While advances in microscopy will undoubtedly improve pregnancy rates within ART, one of the most exciting prospects of such advances are their implications within the reproductive sciences, which in turn may aid in the advancement of ART efficiency. Indeed, Ajduk *et al*. (2011) used live cell particle imaging of fertilising mouse embryos to identify the importance of the sperm in triggering dynamic oscillatory behaviour of the actomyosin cytoskeleton at fertilisation, proposing that the pattern and timing of the ensuing movements may represent a powerful method of assessing an embryos ability to achieve full developmental potential. Further to this, Wong *et al*. (2010) demonstrated that the rate of the first cell division within mouse zygotes may be a predictive indicator of embryogenesis, with zygotes that undergo rapid or delayed division arresting before reaching the blastocyst stage. Collectively, these studies indicate that observations of the minute dynamics involved during early embryogenesis may serve as indicators of zygote and embryo health, and may represent promising techniques for future clinical application.

Another key remit of microscopy within fertility

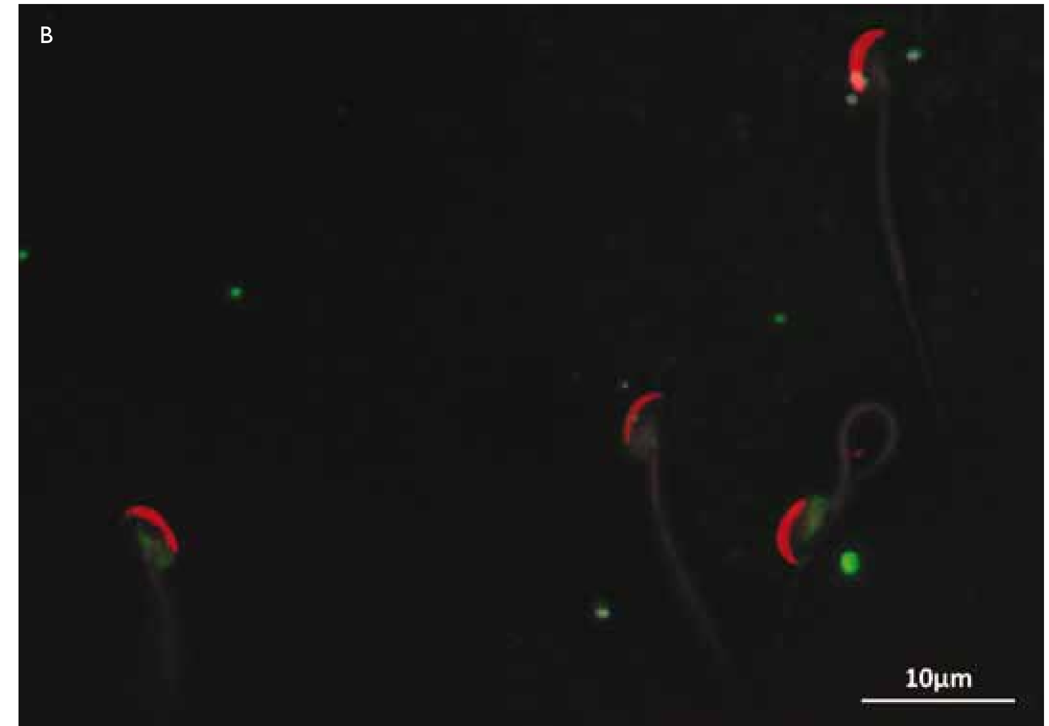


Figure 7: Images of mouse sperm. (A) Darkfield image illustrating diversity in sperm head morphology inherent within various mammalian species. (B) Composite of darkfield and fluorescence microscopy showing the oocyte activation factor PLCζ (green) and the acrosome (red) of mouse sperm. Images were taken using a Nikon Eclipse fluorescence microscope. Copyright: Miss Walaa Ramadan.

research is its use alongside fluorophores; molecules or proteins which fluoresce in a particular colour when exposed to a specific wavelength of light, a technique referred to as fluorescence microscopy. The utilisation of fluorophores conjugated to antibodies specific to particular markers of gamete function and viability may allow us to visualise and assess the complex and intricate mechanisms of not only gametogenesis, but also the biochemistry of gamete interaction and function. A further modification of fluorescence microscopy is confocal microscopy, which operates in a manner similar to fluorescence microscopy, but utilises much more powerful light sources with more specific wavelengths, allowing the dissemination of a greater level of detail, and resolving power, in captured images.

Fluorescence microscopy plays a key role in the identification and characterisation of factors involved in sperm/oocyte fusion (Fig. 6). Such examples include the ADAM (A Disintegrin and Metalloprotease) family of integral membrane proteins, or characteristic proteins such as ZP3 (Talbot *et al*., 2003; Muratori *et al*., 2009). Another such factor is *Izumo*, a testis-specific protein, only detectable on acrosome-reacted sperm and when knocked out, renders male mice sterile, despite otherwise normal mating behaviour (Rubinstein *et al*., 2006). Other markers also include proteins involved in capacitation (a series of physiological, biochemical, and biophysical modifications that occur within the female reproductive tract and bestow fertility; (Yoshida *et al*., 2008), such as soluble adenylate cyclase (sAC). Additionally, similar markers of gamete function have also been proposed for oocytes. Indeed, higher levels of activin A have been associated with increased oocyte quality (Lau *et al*., 1999), while higher levels of GDF-9, an oocyte secreted factor, is associated with improved oocyte quality within culture (Yeo *et al*., 2008; Gilchrist *et al*., 2008).

A further application of the diagnostic potential of fluorescence and confocal microscopy is the visualisation of the endogenous sperm factor; a testis-specific phospholipase C (PLC), PLCzeta (PLCζ), strongly believed to be responsible for the controlled release of intracellular calcium within the oocyte at fertilisation in mammals (Fig. 7). The localisation patterns of PLCζ are thought to be highly dynamic within both mouse and human sperm (Grasa *et al*., 2008; Young *et al*., 2009), depending upon the stage of sperm maturity. Morever, reduced levels or absence of PLCζ has been implicated as a causative factor for infertility in sperm from men who routinely fail ICSI (Yoon *et al*., 2008; Heytens *et al*., 2009; Kashir *et al*., 2010). Indeed, PLCζ is thought to represent a future prognostic and diagnostic marker of a sperm's oocyte activation capability (Fig. 8; Kashir *et al*., 2010; 2011a; 2011b).

Analysis of aneuploid embryos, in which there are an abnormal number of chromosomes, demonstrated alterations in amino acid turnover *in vitro*, in comparison to genetically normal embryos, indicating a potential association between amino acid turnover and chromosome aneuploidy *in vitro* (Picton *et al*., 2010). Furthermore, modification of oocyte-secreted glycoproteins by oocyte-specific deletion of core 1-derived O-glycans is known to increase litter size in mice and thought to be mediated by increased follicle survival (Williams & Stanley, 2008). Cumulous cells, specialised cells surrounding the oocyte, are known to possess trans-zonal cytoplasmic projections which penetrate the zona pellucida and form gap junctions within the oocyte. These remain loosely attached to the oocyte following ovulation and continue to support the oocyte during fertilisation (Huang & Wells, 2010). Recent evidence suggests that cumulous cells may support oocyte maturation and development *in vivo,* as well as following IVM, by acting as a relay for endocrine and other environmental signals (Huang & Wells, 2010), which may allow for the future identification of further factors indicative of oocyte and embryo health and viability.

The potential diagnostic applications of both fluorescence and confocal microscopy in conjunction with this growing array of 'viability factors' may permit further improvement of

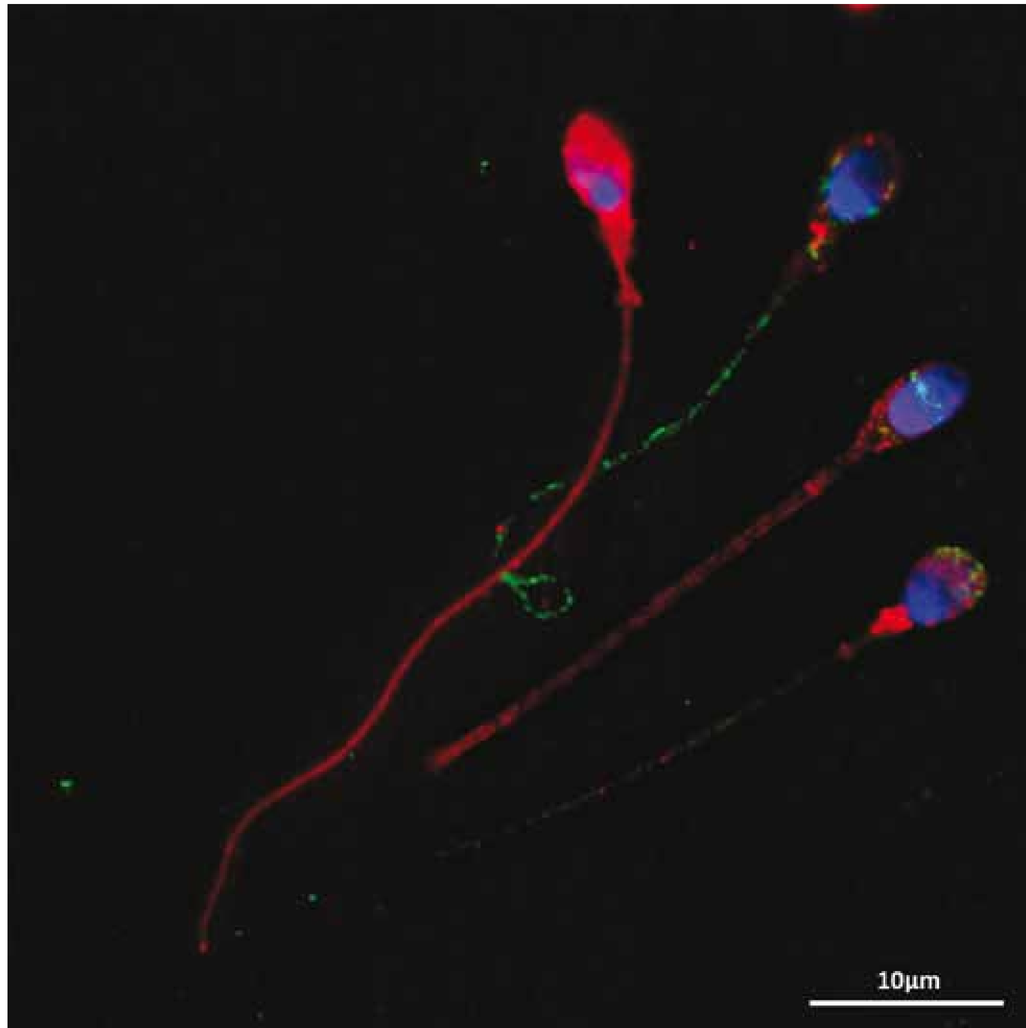


Figure 8: Optical slice of human sperm indicating plasma membrane (red), nucleus (blue), and PLCζ (green) staining (green staining in the sperm tail represents non-specific binding of the PLCζ antibody) as created via confocal microscopy. PLCζ staining indicates the potential held by PLCζ as a diagnostic indicator of male infertility, but also shows wide variability within the same sample. Images were taken using a Zeiss LSM510 confocal microscope with an oil immersion objective. Copyright: Mr Junaid Kashir.

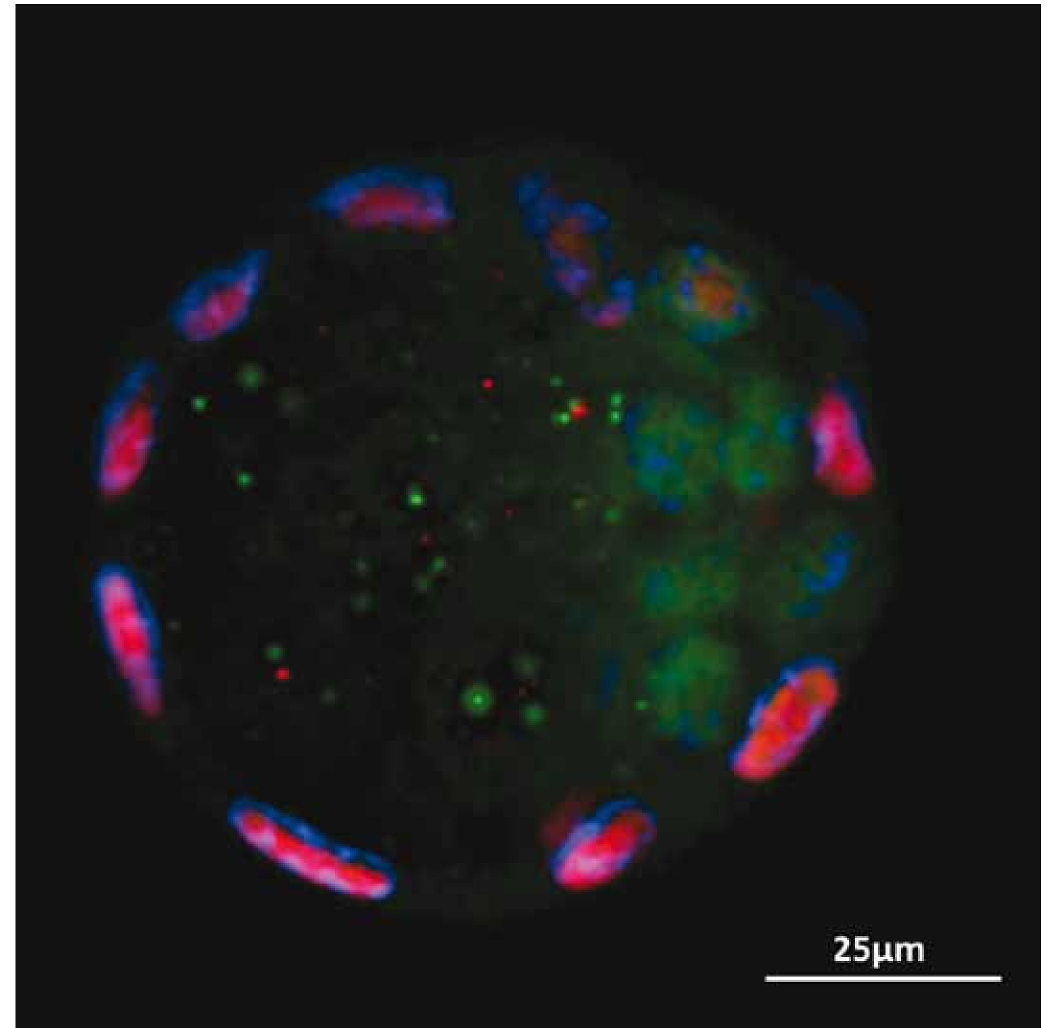


Figure 9: Optical slice through the centre of a day 4 free-floating mouse blastocyst, showing the inner cell mass stained for a pluripotency marker (green), the trophectoderm stained for a differentiation marker (red), and the nuclei of the embryo (blue). Images were acquired via fluorescence microscopy, and captured using Openlab software (Perkin Elmer) and processed using Volocity software (Perkin Elmer). Copyright: Miss Walaa Ramadan.

pregnancy success in ART by allowing the specific selection of the best quality gametes and embryos for downstream procedures. For example, there appears to be a large degree of variability with regards to the localisation pattern of PLCζ within human sperm. Within a semen sample, there are often sperm shown not to possess PLCζ at all and are thus theoretically incapable of activating an egg (Kashir *et al*., 2011a; 2011b). However, by using only brightfield optics, embryologists will not be able to differentiate sperm which contain PLCζ from those which do not, and may therefore, inadvertently select sperm which would not lead to successful fertilisation and pregnancy. Other studies aim to investigate the key potential of specific markers of oocyte and embryo health in the hope of understanding molecular and physiological processes underlying successful embryogenesis and implantation (Fig. 9-11). The study of such factors expressed within *in vitro* model systems may lead to increased understanding of the reproductive mechanisms in which they are involved. One such routinely used model system is the transformed human embryonic kidney (line 293; HEK293T) cells (Fig. 12). Indeed, Kashir *et al*. (2011b) utilised this system to express active recombinant human PLCζ protein (a potential therapeutic for male infertility) for the first time, as well as utilising such a system to investigate localisation patterns of fluorescently tagged isoforms of PLCζ.

One further avenue to explore for ART is the potential use of microscope platforms incorporating time-lapse imaging ability. These systems can capture minute changes in cellular movement with respect to the cellular micro-environment and may prove useful in predicting embryonic vitality. For example, novel systems are emerging that combine a powerful digital imaging system with a controlled and continually-monitored micro-environment, allowing operators to continually assess various aspects of cellular morphology and structure but without the need to remove the specimen from its protective micro-environment. These features are likely to appeal to IVF units and work is underway to assess the potential role for such systems.

It is clear that the continued success of ART lies heavily in the dedication and skill base of clinical embryologists and the multifaceted nature of microscopy. In order to provide future patients with the best possible level of care, it is vital that

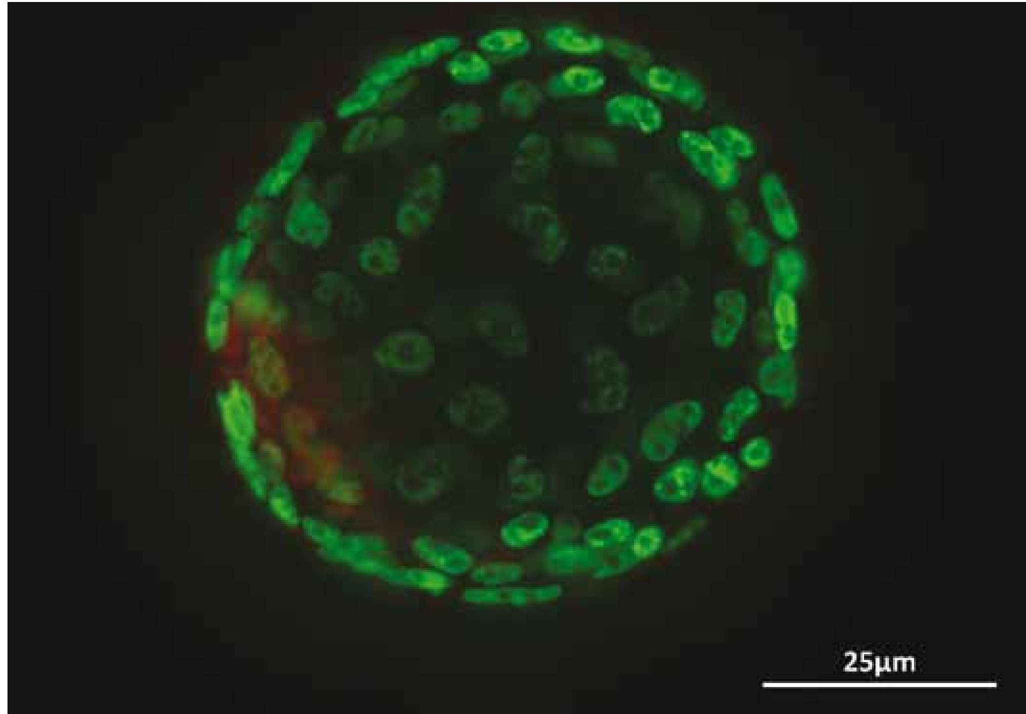


Figure 10: Extended focus image, formed by taking an image-stack of a day 5 free-floating mouse blastocyst. The inner cell mass was stained for a pluripotency marker (red), and the trophectoderm was labelled using a differentiation marker (green). Images were acquired via fluorescence microscopy, and captured using Openlab software (Perkin Elmer) and processed using Volocity software (Perkin Elmer). Copyright: Miss Walaa Ramadan.

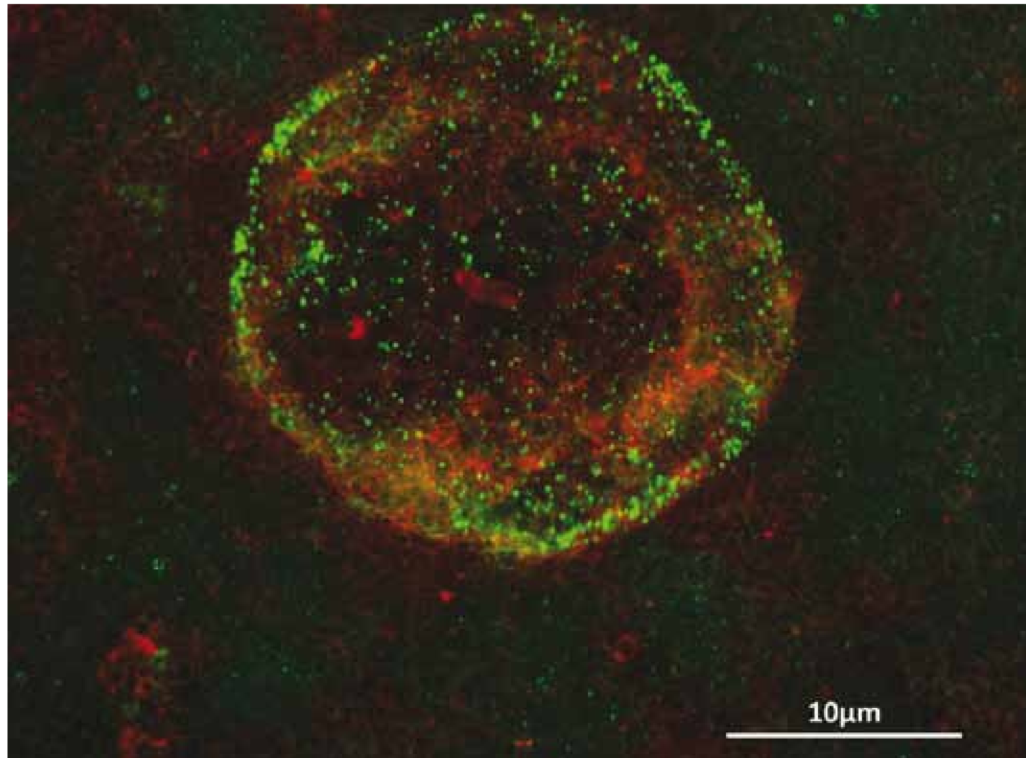


Figure 11: Optical slice image of a human embryo implanted on surrounding cells in vitro, indicating adhesion molecules (green) on the surface of the embryo and surrounding cells, along with staining for filaments (red) in the embryo and surrounding cells. Images were acquired via fluorescence microscopy, and captured and processed using Openlab software (Perkin Elmer). Copyright: Miss Youn-Jung Kang.

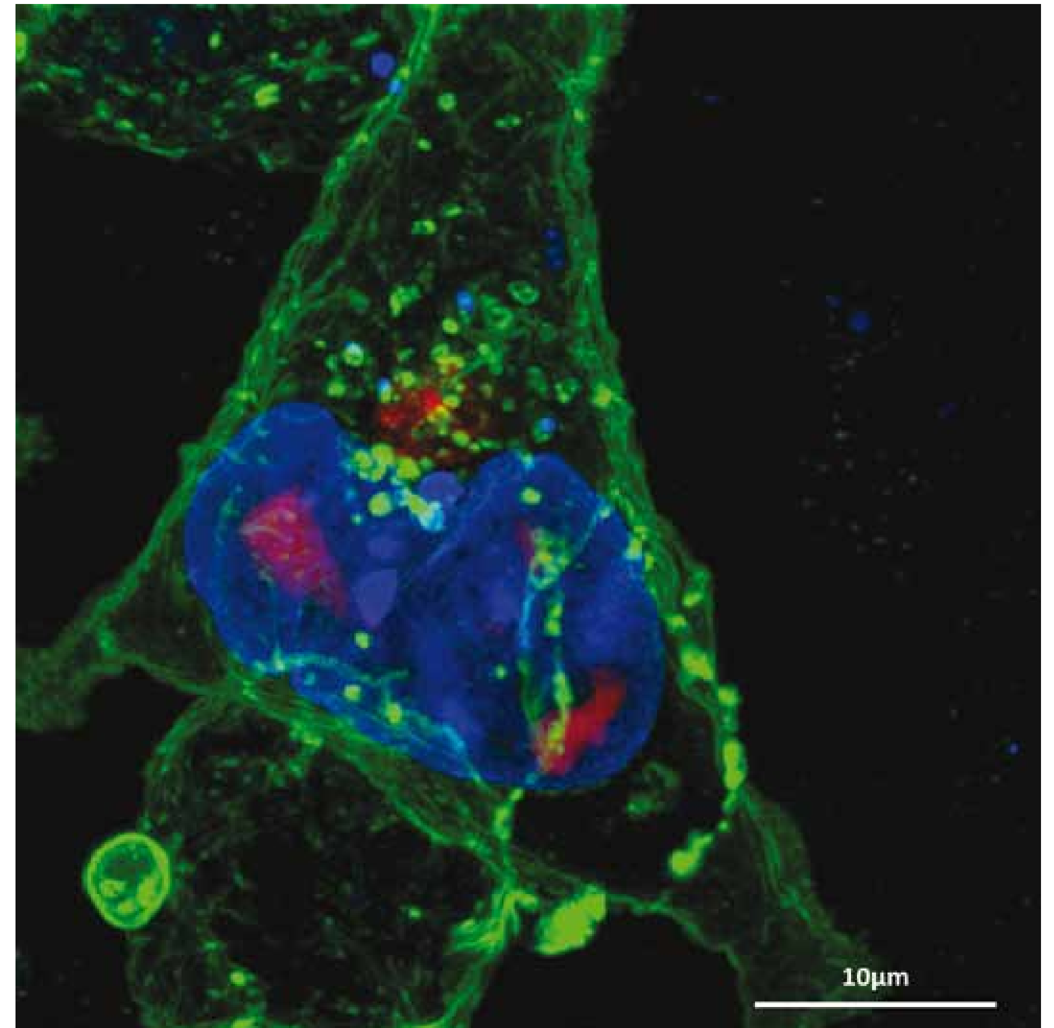


Figure 12: A Z-stack reconstruction of multiple images, obtained via confocal microscopy, taken along different planes of view of a HEK293T cell transfected in order to express a red fluorescent protein. Green staining indicates the plasma membrane, while blue staining represents the nucleus. HEK293T cells and similar systems may act as models for the study of localisation patterns and expression levels of key reproductive proteins. Images were taken using a Zeiss LSM510 confocal microscope with an oil immersion objective. Copyright: Mr Junaid Kashir.

IVF laboratories continue to adapt alongside technological and scientific advances with which to continue to provide the very best levels of patient care and the best chances of pregnancy success.

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