Light Microscopy in Clinical Embryology: Lighting the Way for Infertility Treatment

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INTRODUCTION
The World Health Organisation (WHO) estimates that approximately 50-80 million individuals (8% of couples) are now infertile [1]. Consequently, assisted reproductive technology (ART) has experienced a rapid expansion over recent years. Over one million treatments are performed globally each year, accounting for up to 7% of all births in some developed countries [2].

Clinical embryologists play a vital role in ART, not only by carrying out sophisticated diagnostic, analytical and manipulative techniques within the laboratory, but also by liaising with patients, senior clinical staff, and nursing teams. One key aspect is the close relationship between embryologists and scientific researchers which forms a key link in translational research and the continued development of technology within the ART laboratory. In the UK, embryologists carry out their duties in a highly standardised manner regulated by the Human Fertilisation and Embryology Act (1990, amended 2008) and the European Cells and Tissues Directive [3]. Their main remit is to analyse, prepare and process gametes for laboratory intervention, perform in-vitro fertilisation (IVF), microinject sperm into eggs (intracytoplasmic sperm injection, ICSI), select and process gametes or embryos for cryopreservation and/or implantation, and perform delicate biopsy procedures on live embryos for pre-implantation genetic diagnosis (PGD). Techniques are carried out in accordance with a standard operating procedure (SOP).

An essential technological concept in ART is the ability to examine, monitor, and assess live gametes and embryos for clinical use, and to visualise gametes and embryos in sufficient detail to allow delicate micromanipulative procedures to be performed without compromising viability. ART laboratories are thus equipped with a wide range of microscope equipment which invariably includes dissecting, compound, and inverted systems equipped with brightfield, phase contrast, and Hoffman modulation contrast (HMC) optical technology. Competency and consistency in microscopy is thus an essential aspect of an embryologist’s training, as is the ability to maintain and troubleshoot problems swiftly and effectively.

Clinical Embryology Training
As the number of patients seeking ART expands, demand upon ART clinics is mounting. This has led to increasing worldwide concern over the availability of appropriately trained embryologists to meet clinical demand. In an attempt to address this worrying situation, several institutions have devised specialist postgraduate courses for those wishing to pursue a career in ART.

One such course is the MSc in clinical embryology at the University of Oxford, UK, which was specifically designed to provide intensive training in theoretical and laboratory skills pertaining to ART [3]. Based in the new Institute of Reproductive Sciences alongside the Oxford Fertility Unit (OFU), and with scientific researchers from the Nuffield Department of Obstetrics & Gynaecology at the University of Oxford, this course aims to train, motivate, and inspire future leaders in reproductive science and ART by combining academic excellence with significant hands-on training in laboratory skills. A key feature of the course is training in the use of light microscopy equipment within the ART environment, provided by senior OFU embryologists (Figure 1a, b). Stu-
Students reading for this degree receive individual hands-on training in a variety of microscopy techniques using state-of-the-art optical systems and benefit from the combined teaching expertise of clinical and research staff from OFU and the University of Oxford. The acquisition of appropriate laboratory skills such as light microscopy is fundamental in providing students with the knowledge and motivation to consistently provide the best levels of patient care.

In this review, we briefly introduce the role of microscopy in the ART laboratory by discussing a range of clinical techniques, the importance of providing dedicated training facilities for those aspiring to become clinical embryologists, and by contemplating how advances in microscopy may enhance the future success of ART.

LABORATORY ENVIRONMENT

A vital aspect of ART is to control the laboratory environment within strict limits such that the viability of gametes or embryos remains uncompromised. The air supply is often filtered and separate to other areas of the building. To protect gametes and embryos from environmentally induced DNA damage, many laboratories use lighting systems that are devoid of ultraviolet radiation. Staff adhere to strict protocols in terms of attire to promote an aseptic environment, often extending to limited use of cosmetics and other toiletry products. Gametes and embryos are maintained within micro-environments in which temperature, humidity, pH, CO₂, O₂ and osmotic are monitored, generally and in vitro, are monitored constantly, and that equipment is calibrated and maintained regularly.

Collectively, these protocols protect the viability of gametes and embryos at all times. Even a minor change in environmental conditions can lead to compromised embryo implantation and/or pregnancy. Many laboratories also use a computerised tracking/witnessing system, such as ‘VF Witness’ from Research Instruments (UK), in order to reduce the risk of human error.

ART laboratories are routinely equipped with a range of microscopy equipment, thereby providing capability for many optical techniques. The Oxford teaching laboratory mimics such facilities by providing a sufficient number and range of optical platforms (supplied by Nikon Instruments) such that graduate students can gain significant hands-on training tutored by senior scientific and clinical staff, supported by Nikon specialists. It is of paramount importance that clinical embryologists understand how different types of microscopes operate, how systems can be optimised and calibrated, and how different optical techniques are utilised in differing ART procedures.

Table 1 provides a summary of the light microscopes and micromanipulators currently held by the Oxford Clinical Embryology MSc laboratory along with indicators of the equipment’s general application within the training curriculum.

<table>
<thead>
<tr>
<th>Platform [number of units]</th>
<th>Specialist Optical Technology</th>
<th>Role in Teaching Curriculum</th>
</tr>
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<tbody>
<tr>
<td>Eclipse E200 upright microscope [10]</td>
<td>Brightfield, darkfield and phase contrast CFI E Plan objectives delivering long working distances, high numerical apertures, and flat images with virtually no curvature. Objectives: E plan 4x NA 0.10, 40x NA 0.65, 100x NA 1.25, phase contrast LWD 20x NA 0.40</td>
<td>Reproductive histology, sperm and oocyte morphology, semen analysis (motility, counting)</td>
</tr>
<tr>
<td>SMZ 1500 stereomicroscope [1]</td>
<td>Digital imaging, image analysis/measurement. Brightfield/darkfield with diascopic stand and external cold light source. Zoom range of 0.75x to 11.25x allowing both macro- and micro-analysis.</td>
<td>Developmental biology of model organisms, IVF oocyte grading, embryo grading, mechanical removal of cumulus cells from oocytes, pre-preservation and transfer of gametes and embryos to/from media drops, analysis of embryos pre- and post-vitrification, assessment of micro tools prior to use.</td>
</tr>
<tr>
<td>SMZ 445 stereomicroscope [10]</td>
<td>0.8x – 3.5x zoom magnification with diascopic stand and external cold light source.</td>
<td></td>
</tr>
<tr>
<td>Eclipse 80i fluorescence microscope [1]</td>
<td>Objectives: CFI plan Fluor 4x 0.13, 10x 0.30, 20x 0.50, 40x 0.75, DLL100x 0.30, Plan Fluor DLL 10x 0.30, Phase contrast CFI plan Apo 10x 0.25 Fluorescent filters: UV-2A, FITC, TXred, G-2A Basic Research Software/NI Elements for digital imaging/image analysis</td>
<td>Computer-assisted sperm analysis (coupled with Hamilton Thorne CEROS system), reproductive histology, semen analysis.</td>
</tr>
<tr>
<td>Eclipse T1-S inverted microscope with Narashige micro-manipulators [4]</td>
<td>Nikon CFI80 optics. Objectives: brightfield (CFI) 4x 10x, 10x 0.25 Hoffman (CFI HMC) LWD 20x F 0.40, 40x 0.55 Narashige NT-88-V3 micromanipulators</td>
<td>Gamete micromanipulation, sperm capture and tail break, ICSI, embryo biopsy using acidified Tyrode’s solution and infrared laser ablation.</td>
</tr>
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RESULTS

As more couples seek infertility treatment, there is increasing call for the universal adoption of elective single embryo transfer (eSET) in order to reduce the risk of multiple pregnancy associated with multiple embryo transfer [5]. A vital skill of the embryologist, therefore, is to successfully identify the healthiest gametes for procedural intervention and subsequently, the most viable embryos for reimplantation. Light microscopy plays a crucial role in the laboratory assessments used in such procedures.

Analysis of Semen and Spermatozoa

Approximately 40% of all infertility cases are attributable to irregularities in sperm production or function, either alone or in combination with other factors [6]. Semen analysis therefore represents a vital diagnostic approach and evaluates qualitative characteristics such as appearance, pH, viscosity, volume, sperm count, morphology, and motility [7]. Samples are investigated within one hour of ejaculation and compared against WHO guidelines to identify abnormalities and plan corrective treatment. Whilst some of these analytical procedures are carried out macroscopically, many (e.g. concentration, motility, and morphology) rely heavily on the use of light microscopy (Table 1; Figure 2 a, b).

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. The number of sperm occupying a defined area/volume of the chamber is counted and extrapolated to the number of sperm per ml of semen. Motility characteristics are determined in duplicate samples at room temperature using phase contrast and classified into four categories: Grade A – rapid progressive motility >20µm s⁻¹

Figure 2: Light microscope images of male human sperm taken during routine semen analysis using (a) brightfield and (b) differential interference contrast optics using a Zeiss LSM10 confocal microscope with an oil immersion objective. Copyright: Oxford Fertility Unit and Junaid Kashir, respectively. Whilst confocal analysis clearly provides superb magnification and resolution for studying sperm and is widely utilized in scientific research, such systems are highly impractical for routine use in IVF units owing to the prohibitive costs involved.

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at 20°C; Grade B – slow progressive motility; Grade C – non-progressive motility; and Grade D – immotile. Samples in which at least 50% of sperm are motile are classified as being ‘normal’. Those with <40% motility are investigated further using vital staining and bright-field optics to identify immotile and dead spermatozoa [7]. Additional brightfield light microscope analyses determine the proportion of sperm exhibiting abnormal morphology of the head (e.g. the shape or presence of vacuoles), neck, mid-piece and tail. Sperm morphology is considered to be one of the main causes of male infertility [8] whilst vacuolated sperm is associated with reduced pregnancy rates [9]. Consequently, analysis of patient semen samples provides information central to the design of subsequent treatment options. For example, sperm exhibiting normal morphology and motility characteristics will normally be used in conventional IVF protocols whilst sperm exhibiting abnormal characteristics will be used for ICSI.

**Determination of Oocyte Viability**

Oocyte morphology is a key contributor towards fertilisation success. Consequently, screening and identifying the healthiest oocytes forms an essential aspect of ART (Figure 3 a,b). Hoffman modulation contrast (HMC) optics, or high magnification bright-field stereomicroscopy, are 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 [7].

In some cases, the induction of oocyte maturation by exogenous hormone preparations is not recommended. This is particularly dangerous in the case of women with polycystic ovarian syndrome (PCOS) who are at risk of ovarian hyper-stimulation syndrome (OHSS). In such cases, conventional IVF is replaced by in-vitro maturation (IVM), where immature oocytes are retrieved directly from the ovary and subsequently matured in vitro [10, 11]. Oocytes are cultured in an incubator for 24 to 48 hours and assessed microscopically (bright-field or HMC) at regular intervals to ascertain maturity and viability. Once maturity indicators reach an acceptable stage, oocytes can be fertilised in an appropriate manner.

**Selecting Healthy Embryos for Transfer**

Immense importance lies in the identification and selection of the healthiest embryo for implantation. Many parameters need to be considered, such as pronuclear morphology and size, the number and location of nuclear precursor bodies, blastomere shape, thickness of the zona pellucida, cytoplasmic fragmentation, indicators of early cleavage, and the rate of progression to the blastocyst stage. Embryologists commonly utilise brightfield, phase-contrast, and Hoffman modulation contrast light microscopy to visualise developing embryos and identify the healthiest embryos for transfer (Figure 4 a,b,c).

**Micromanipulation of Gametes and Embryos**

Micromanipulation of gametes and embryos involves the co-ordinated use of fine glass microtools with internal diameters of 5-35 µm and underlies the success of many clinical treatments including ICSI and embryo biopsy for PGD. Micromanipulation depends on several factors; a high quality inverted microscope with a magnification range of 40-400 X (normally using HMC optics), a precision-controlled micromanipulator system, and a bright-field stereomicroscope for egg and embryo preparation. Embryologists tend to favour HMC for micromanipulation as this provides excellent contrast whilst dealing with specimens with a 3-dimensional appearance. In this way, embryologists are more informed about the topography of oocytes and embryos.

Particular attention must be afforded to the environmental conditions surrounding the specimen(s) being manipulated and the time taken for the embryologist to perform the technique. Procedures should be performed at 37°C to prevent chromosome disassociation and subsequent aneuploidy (abnormal chromosome copy number). Microtubule depolarisation and damage to the meiotic spindle can occur during ICSI as a result of specimen cooling. This problem can be circumvented by fitting a temperature-controlled plate directly to the microscope stage, such as the Tokai Hit Thermo Plate, which provides specimens with a relatively stable thermal environment, thereby reducing the risk of chromosomal damage. Maintenance of environmental conditions is also crucial in minimising the production of reactive oxygen species (ROS) which can be harmful to gametes and cause DNA fragmentation.

Microscopes must possess a smooth-running mechanical stage to allow controlled movement of samples whilst manipulators must permit delicate control. For example, the Nikon-Narashige NT88-V3 micromanipulator system used by the Oxford MSc combines a 3D motor-driven manipulator and a 3D oil-hydraulic manipulator for increased accuracy, whilst the reduced distance from manipulator mount to microelectrode tip provides optimal stability. It is critical that embryologists can perform manipulative procedures swiftly and effectively in order to avoid compromising viability or integrity. Techniques such as breaking an individual sperm’s tail with a fine-tipped microtool are critical in effective sperm capture for ICSI but represent highly challenging skills to acquire. Embryologists must also be able to set up manipulator systems, optimise optics and HMC illumination, attach and align microtools, and be able to manipulate gametes from one drop of medium to another. Orientation of oocytes and embryos for manipulation represents another critical skill. During ICSI, oocytes must be orientated in such a way that an injection tool is not inserted into an area occupied by the meiotic spindle. By convention, most IVF units avoid this problem by injecting an oocyte at the 3 o’clock position whilst the polar body is situated at the 12 o’clock position (Figure 5 a, b, c).

Whilst the practice of ICSI has revolutionised the treatment of male factor infertility, primarily by circumventing problems associated with sperm motility, or sperm-egg binding,
one other area of micromanipulation experiencing significant expansion is embryo biopsy (Figure 6). Embryo biopsy represents a powerful addition to conventional screening techniques, as it allows single blastomeres or polar bodies to be removed from developing embryos for genetic analysis (e.g. fluorescent in-situ hybridisation, single cell polymerase chain reaction, and comparative genomic hybridisation). These techniques provide clinicians with critical information concerning the genetic composition of embryos and thus allow embryologists to select embryos for transfer that pose minimal risk for genetic abnormality or pre-disposition to deleterious genetic diseases. Biopsy must be carried out in a manner that causes the least invasive damage to the embryo during the delicate phases of early development. Initial methodology involved the application of acid Tyrode’s solution onto a selected region of the zona pellucida. More recently, IVF units have begun to use infrared laser systems in order to drill through the zona pellucida. Irrespective of the technique used, it is critical that the embryologist drills a hole in the zona pellucida of a specific size. Too small and the blastomere or polar body will be damaged upon retrieval. Too large, and the integrity of the embryo will be compromised and further blastomeres could be lost inadvertently subsequent to the procedure.

Whilst it is clear that 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. To attain the level of skill required to perform such techniques clinically on a daily basis, embryologists require significant levels of training, with ongoing assessment, not just in clinical application, but also in the ability to troubleshoot problem areas swiftly and effectively.

**DISCUSSION**

Whereas the techniques discussed herein have rigidly defined the way that ART is performed and underlie the current success of this rapidly expanding field, it is important to recognise that future advances in optical technology are highly likely to permit further enhancement and improvements in this crucial clinical area.

One future possibility 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 [12]. Whilst popular amongst research scientists, CASA has yet to become established in IVF clinics. The predominant reasons for this relate to the prohibitive cost of CASA software and concerns over the accuracy and reproducibility of such systems. One critical overriding factor, however, appears to be that by examining semen samples manually under a light microscope, the clinical embryologist can learn much more about a sample than by CASA alone, for example being able to distinguish cellular debris from dead or immotile cells, or by identifying signs of bacterial infection. With continued improvement in CASA software, it remains to be seen how widespread this technique will become in ART.

One further aspect of semen analysis experiencing growing interest from the IVF community is the diagnostic analysis of DNA fragmentation. Whilst it has been established for some time that sperm DNA fragmentation can underlie human infertility [13], IVF units have generally avoided routine fragmentation assessments in their semen analysis protocols. However, the ‘Halosperm’ technique has generated much interest in providing a simple and cost-effective system for determining sperm DNA fragmentation, and only requires minor laboratory intervention along with a basic light microscope [14].

Over recent years, a modified version of ICSI has been developed, although, as yet, it remains unclear as to how popular this technique will become in IVF units. Intracytoplasmic morphologically selected sperm injection (IMSI) specifically selects the best quality sperm for injection at a much higher magnification than ICSI, based upon morphological criteria [15]. This technique requires microscopes with significantly enhanced resolution. Further enhancements to the ICSI technique may also be derived from recent developments in the visualisation of meiotic spindles. Maintaining this structure is vital if chromosomal damage is to be avoided. During ICSI, embryologists exert extreme care in orientating the oocyte into a position such that the injection microtool does not cause damage to the meiotic spindle. Currently, embryologists accomplish this visually by selecting the injection site in relation to the position of the polar body. By doing this, the embryologist can avoid the approximate location of the meiotic spindle but this, however, does not account for individual differences in oocyte architecture. One way of rectifying this situation is to use a system in which polarised light is used to visualise subcellular structures such as spindle microtubules. Modern polarised light microscopes are digital, orientation-independent, microscopes with exquisite sensitivity to birefringence exhibited by mammalian spindles [16]. Such technology has already proved to be beneficial in the IVF environment as demonstrated by enhanced fertilization and cleavage rates [17] and improved
embryo quality [18]. However, this technology has yet to become widespread amongst the IVF community, largely due to the high costs involved in running such systems. Perhaps the most significant area of likely advancement is in the use of microscopy to determine embryo quality. For example, work is underway to investigate whether lipid droplets, organelles heavily implicated in cellular metabolism, play a critical role in embryogenesis. These organelles are difficult to visualise without the use of chemical stains but research has identified adaptive harmonic generation microscopy as a means of visualising and quantifying lipid droplets in mammalian embryos [19, 20]. Whilst this technology may identify useful predictors of embryonic health, the equipment required to visualise lipid droplets is unlikely to be practical for IVF use in its present form due to its bulky nature and high cost.

Microscope systems incorporating time-lapse imaging ability can be used to capture minute changes in cellular movement with respect to the cellular micro-environment, and may prove useful in predicting embryonic vitality. For example, systems are emerging that combine a powerful digital imaging system with a controlled and continually monitored micro-environment (e.g. the Biostation IM from Nikon Instruments). Such systems are especially suited to live cell imaging since operators are able to continually monitor 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 be appealing to IVF units, and work is underway to assess the potential role for such systems.

It is clear that the success of ART lies heavily in the dedication and skill base of clinical embryologists and the multifaceted nature of light microscopy. In order to provide future patients with the best possible level of care, it is vital that IVF laboratories continue to adapt alongside technological and scientific advances. As the demand for ART increases, there is mounting support for training programmes that adopt a holistic approach to human infertility, such as the Oxford MSc in clinical embryology.

Critically, the course is based within a motivational and inspiring environment in which scientific research is used to derive new technologies or enhance diagnostic procedures. Investing such powerful resources in new generations of clinical embryologists is crucial in sculpting laboratory personnel who are not only academically informed and highly skilled, but are also aware of the need to continually adapt and revise techniques and equipment in line with emerging research.

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ACKNOWLEDGEMENTS
Sourimaa Biswas Shivhare and Ioannis Ladas are graduate students reading for the Oxford MSc in Clinical Embryology. Junaid Kashir is a PhD student and laboratory demonstrator for the MSc in Clinical Embryology. Celine Jones is the Laboratory Manager for the MSc in Clinical Embryology. Tracey Griffiths and Karen Turner are Senior Embryologist and Consultant Embryologist, respectively, for the Oxford Fertility Unit. Kevin Coward is the Director of the MSc in Clinical Embryology, a Principal Investigator for the Nuffield Department of Obstetrics and Gynaecology, and a Lecturer in Medicine for Worcester College, Oxford.

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WEBSITES
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