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PII: S1472-6483(18)30639-4
DOI: https://doi.org/10.1016/j.rbmo.2018.12.011
Reference: RBMO 2072

To appear in: *Reproductive BioMedicine Online*

Received date: 17 August 2018
Revised date: 1 November 2018
Accepted date: 11 December 2018

Please cite this article as: Lien M. Davidson , Yaqiong Liu , Tracey Griffiths , Celine Jones , Kevin Coward , Laser technology in the assisted reproductive technology laboratory: a narrative review, *Reproductive BioMedicine Online* (2018), doi: https://doi.org/10.1016/j.rbmo.2018.12.011

Laser technology in the assisted reproductive technology laboratory: a narrative review

Lien M. Davidson[a,1], Yaqiong Liu[a,1], Tracey Griffiths[b], Celine Jones[a] and Kevin Coward[a*]

[a] Nuffield Department of Women's and Reproductive Health, University of Oxford, Level 3, Women's Centre, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, UK. [b]Oxford Fertility, Institute of Reproductive Science, Oxford, OX4 2HW.

[1] These authors contributed equally

*Corresponding author
Email: kevin.coward@wrh.ox.ac.uk; Telephone: +44 [0] 1865 618900; Fax: +44 [0] 1865 769141

ABSTRACT

To improve success rates, assisted reproductive technology (ART) procedures continually undergo optimization and enhancement such that we can identify and manipulate the best quality gametes and embryos, and thus improve clinical outcomes. Laser technology is now being applied across ART to reduce procedure times and increase the consistency and reproducibility of traditional ART techniques such as intracytoplasmic sperm injection (ICSI), embryo biopsy, assisted-hatching, cryopreservation, and sperm immobilization /selection. Herein, we examine the current status of cutting-edge laser-assisted reproductive technologies, investigate experimental techniques that are increasingly being applied clinically, and explore the future use of lasers as a research tool in the reproductive sciences. We highlight the benefits of lasers as a powerful technology at the forefront of both diagnostic and therapeutic treatments for general sub-fertility and male-factor infertility. However, it is important to note that while lasers are becoming increasingly commonplace in the ART unit, there is comparatively little information in the existing literature pertaining to the potential negative effects that laser application might impart upon the developing human embryo, thus creating the need for further investigative research.

KEY MESSAGE

This review discusses the current status of laser-assisted reproductive technologies in human infertility treatment. We highlight the benefits of laser technology in diagnostic and therapeutic treatments for sub-fertility and male-factor infertility, and the benefits of their application in cutting-edge techniques such as nuclear and spindle transfer and the genomic editing of embryos.

Keywords: Infertility; ART (assisted reproductive technology); Infra-red lasers; Zona pellucida; Assisted-hatching; Biopsy

INTRODUCTION

Infertility has become common with a 9 to 18% prevalence in the general population experiencing difficulty conceiving naturally (Aghajanova et al., 2017), and thus require medical intervention in order to achieve pregnancy. A combination of factors arising from

the male, female, or both sexes can contribute to infertility, such as the lack of oocytes or sperm, reduced oocyte quality, the inability of sperm to meet and fertilize the oocyte, or the inability of fertilized embryos to implant or grow in the uterus. In around 15-30% of cases, however, a clear cause is never established, leading to the diagnosis of 'unexplained' or 'idiopathic' infertility (Gelbaya et al., 2014).

Assisted reproductive technology (ART) has expanded rapidly over the past decades, largely owing to revolutionary techniques such as intracytoplasmic sperm injection (ICSI), preimplantation genetic diagnosis (PGD) and screening (PGS) and cryopreservation. During ART, oocytes are fertilized by sperm in vitro and then cultured to create embryos, which are then monitored and transferred to the uterus 3 to 6 days after fertilization, depending upon local protocols and patients' individual requirement. The ability of an embryologist to select high quality gametes and embryos is critical in optimizing procedural interventions, and thus outcomes. The use of lasers to manipulate gametes and embryos, beginning in the late 1980's and early 1990's (Tadir et al., 1989, 1990), represents a powerful advancement in ART, and is used to enhance and optimize procedural efficiency, thus reducing the exposure time of gametes and embryos to sub-optimal culture conditions outside of the incubator (Montag et al., 2009).

This review examines the advancements and improvements that laser technology imparts upon traditional ART procedures such as ICSI, assisted-hatching, embryo biopsy, cryopreservation and sperm immobilisation/selection. In addition, we highlight the potential negative effects that lasers might impart upon embryo viability and the relative lack of appropriate research in this area, particularly with regards to human embryos.

The development of lasers for assisted reproductive technology (ART)

The first solid state laser to be used for ART was used to trap sperm and was a neodymium:yttrium- aluminium garnet (Nd:YAG) laser operating at a wavelength of 1064nm (Tadir et al., 1989, 1990). Later, Tadir et al. (1991) coupled a Nd:YAG laser with a potassium titanyl phosphate crystal to double the working wavelength to 532nm and drill the zona pellucida; well defined craters were observed in the zona pellucida when the laser was

applied tangential to the oocyte sphere (Tadir et al., 1991). Over the next five years, multiple groups explored the ability of different lasers to drill preimplantation embryos (**Table 1**).

The aim of laser application at such a delicate stage of human development has, however, evoked considerable concerns. Most of these concerns focus on the potential risk of DNA damage, failed embryo development and possible congenital disorders (Montag et al., 2009). While early lasers were effective for drilling the zona pellucida in animal models (Blanchet et al., 1992; El-Danasouri et al., 1993; Palanker et al., 1991), these forms of lasers had limited value for clinical application. This was because such lasers operated within the DNA-damaging range of UV light, thus increasing the risk of cytotoxic and mutagenic effects (Kochevar, 1989). In 1992, the erbium-yttrium-aluminium-garnet (Er:YAG) laser was introduced, which operated at a wavelength of 2900nm and therefore avoided potential damage to the genetic structure of embryos (Feichtinger et al., 1992). The Er:YAG laser worked in the near infra-red range but required direct contact between the laser fibre and gametes/ embryos, thus raising further concerns relating to damage and contamination (Obruca et al., 1994). Over the next few years, attention turned to holmium:yttrium-scandian-gallium-garnet (Ho:YSGG) laser, which operated at 2100nm and exhibited a different absorption behavior in water than earlier lasers, thus making application possible without the need for additional micromanipulation (Neev et al., 1995; Schiewe et al., 1995). However, in order to use this laser, embryos had to be moved to quartz slides, which caused excessive stress, thus limiting its clinical application (Ebner et al., 2005).

The following years saw the emergence of non-contact lasers working in the infra-red or near infra-red range (800-1500nm), further away from the DNA absorption peak of 260nm. The first laser to have a major impact upon the advancement of ART was the indium-gallium-arsenic-phosphorous (InGaAsP) semiconductor diode laser, operating at 1480nm (infra-red) (1996; Rink et al., 1994), and is still commonly used today. (Rink et al., 1994) were the first to use the InGaAsP laser to drill mouse oocytes without the need for direct contact or micromanipulation to stabilize the oocytes. A beam emitted by the 1480nm laser was coupled with a visible aiming pilot red-light laser beam (670nm), and the collinear beams were directed through an inverted microscope objective (45X), along its optical axis, and then focused in 2-3μm diameter spots on the zona pellucida. Laser pulses (10-20ms), at a power of 60-70mW, were used to drill openings in the zona pellucida which were 5-7 μm in diameter. Using this method, (Rink et al., 1994) found that 70% of the drilled oocytes

successfully developed into blastocysts. Overall, this laser avoided the mutagenicity and contamination that was previously associated with UV contact lasers, thus rendering this new form of laser a valuable tool for ART. Its unique absorption characteristics in water and culture media allowed for non-contact application in standard culture dishes, thus enabling routine application without special equipment or additional steps (Montag et al., 2009). The first live birth reported using 1.48μm laser assistance was in 1995 in Lausanne, Switzerland (Germond et al., 1995).

During ART manipulations, the embryo's culture environment must remain constant in order to optimise potential for a viable pregnancy. Therefore, to provide consistent, reliable, and reproducible results, ART lasers must be integrated into the equipment required for routine laboratory procedures in an efficient and non-hazardous manner. Modern lasers are constructed such that the beam travels through a specialized microscope objective of 40X or 20X magnification. These objectives are then fitted onto inverted microscopes with bright field, phase contrast, and Hoffman modulation optics, which are used routinely in ART. This set up facilitates the application of laser pulses during normal manipulations. Lasers that are currently available on the market for ART are described in **Table 2.** Among them, there are four types used in clinic, of which the wavelengths are 1480nm or 1460nm and the power is of 300 or 400mW.

THE APPLICATION OF LASERS IN ART

Assisted embryo hatching

For successful implantation into the uterus, the embryo needs to escape from the zona pellucida, referred to as 'hatching'. While the exact causes of failed implantation are unknown; increased maternal age, poor oocyte and embryo quality, and reduced endometrial receptivity are all recognized as contributing factors (Das and Holzer, 2012; Simon and Laufer, 2012). Furthermore, it has been hypothesized that the exposure of oocytes and embryos to the artificial conditions of *in vitro* culture may have a direct impact upon the embryo quality, thereby influencing the capacity for implantation and pregnancy (Bungum et al., 2002; Cooke et al., 2002; Friedler et al., 2007). Moreover, cryopreservation procedures imparted upon oocytes and embryos, including cryoprotectant solutions and temperature changes, may affect the physicochemical characteristics resulting in thickening or hardening of the zona pellucida, leading to embryonic hatching difficulties (Petersen et

al., 2006). Consequently, a procedure known as assisted-hatching is commonly offered to patients with a poor prognosis to improve the success rate of implantation (Kissin et al., 2014b; Martins et al., 2011; Technology and Medicine, 2006). This procedure can be offered to those experiencing recurrent IVF failure in an attempt to increase implantation potential (Sallam et al., 2003), patients of advanced maternal age to help overcome zona pellucida hardening due to age-related endocrine disruption and/or the absence of lysis (Tucker et al., 1996), or cyopresenrved-thawed procedures (Balaban et al., 2006).

Although the exact mechanism of how assisted hatching can improve implantation remains unclear, there are several hypotheses. Firstly, assisted-hatching provides a route for the transfer of nutrients from the culture medium, thus enhancing embryo development (Hershlag and Feng, 2005). Secondly, assisted hatching may promote the initiation of implantation since embryos with an artificial gap created in the zona pellucida can make contact with the endometrium earlier than embryos with an intact zona pelluzida (Malter and Cohen, 1989). Thirdly, the artificial gap formed via assisted hatching may serve as a channel for the exchange of metabolites and growth factors between the embryo and endometrium prior to hatching, which subsequently facilitates implantation (Hammadeh et al., 2011; Wan et al., 2014).

There are several methods of assisted-hatching: mechanical hatching, chemical drilling with Tyrode's acid and laser-assisted hatching (LAH). Assisted Hatching techniques have been introduced over years. Mechanical AH by partial zona dissection was followed by zona thinning with Tyrode's acid or pronase and finally by laser. Chemical opening of the zona pellucida has been performed particularly widely over mechanical method due to technical ease and relative cost-effectiveness. The use of acid, however, presents many technical challenges, including exposing the embryo to abnormal pH levels, and the need to change culture medium afterwards to remove excess acid. The efficacy of LAH compared to acid Tyrode's solution may be simply related to ease of use, efficiency, and reproducibility, which renders the laser approach to be a more favourable option (Lanzendorf et al., 2007; Wong et al., 2003). In one early study, LAH showed a significantly higher implantation rate, clinical pregnancy rate, and live birth rate compared to acid Tyrode's hatching (Hsieh et al., 2002). However in the same year, a retrospective study conducted Balaban and colleagues compared four AH methods, including partial zona dissection, acid Tyrode's degradation, diode laser pulses, and pronase ZP thinning, and showed that that the four different AH

methods yielded similar implantation and pregnancy rates when employed in a poor prognosis patient population (Balaban et al., 2002). In 2007, a randomized prospective study compared LAH with acidified medium, but no benefit of clinical outcomes was found when using LAH (Lanzendorf et al., 2007). Nonetheless, as ART laboratories strive for consistency and reduced variability between technicians to optimize pregnancy outcomes, LAH has become a preferred method, especially in conjunction with embryo biopsy, largely because it represents a more consistent approach. However, debate as to the clinical efficacy of LAH is still rife and is discussed in more detail in the following section.

Laser-assisted hatching techniques

There are three different methods for LAH methods with differing degrees of invasiveness; the optimal method is still under debate. First, the laser can be used to create a hole completely penetrating through the whole zona pellucida layer. Secondly, a small hole through most of the zona pellucida can be created which allows an embryo to partially hatch. Thirdly, the laser can be used to thin a large inner or outer area of the zona pellucida around the embryo thus facilitating natural hatching, which is referred to as quarter laser assisted hatching (QLAH) (**Figure 1**).

The precise effects of these different techniques remain controversial. Indeed, one retrospective study compared laser-assisted zona thinning, partial hatching and full hatching methods and recommended that zona thinning should be performed rather than total dissection for a higher pregnancy rate (Mantoudis et al., 2001). One hypothesis as to why zona thinning of large areas may show increased success rates is that a more extensive area of ablation increases the probability that the resultant artificially-induced opening corresponds to the naturally predisposed hatching site (Sathananthan et al., 2003). Conversely, it has been reported using time lapse imaging in a mouse model, that zona thinning, unlike partial hatching, does not facilitate hatching and results in an increased incidence of multiple hatching sites and incomplete hatching (Schimmel et al., 2014).

Controversy regarding the benefits of laser-assisted hatching

Assisted hatching (AH) was first introduced in clinical IVF in 1989 (Cohen et al., 1990). The first meta-analysis of randomized controlled trials (RCTs) was performed in 2003 to specifically investigate the benefits of assisted hatching (including different hatching methods), showing the increased of clinical pregnancy rate (2.4-fold), implantation rate (2.2-

fold) as well as ongoing pregnancy rate (2.3-fold) in poor prognosis patients (Sallam et al., 2003). While a retrospective cohort study analysing all ART cycles conducted from 2000 to 2010 in US fertility centers showed that the assisted hatching technique became increasingly commonly used (from 25,724 to 35,518 cycles), the association of assisted hatching with pregnancy outcomes was not statistically significant. However, this study was lacking details regarding the types of assisted hatching utilized, which may have varied over time or from one clinic to another (Kissin et al., 2014a). The introduction of laser technology created an option for standardization and reproducible hatching for the first time, which is paramount for analysing results from multi-centre trials and comparing results from different studies.

The use of LAH first appeared in the scientific literature in the early 1990s (Tadir et al., 1993). Since then, different laser techniques and LAH methods have been developed to improve implantation. Non-contact infrared diode laser assisted hatching has become the most widely used due to its efficiency, safety and controllability (Hammadeh et al., 2011). However, clinical relevance of LAH remains controversial and elusive. In 2012, a meta-analysis by Carney et al. was conducted to analyse the effect of AH on pregnancy outcomes from 15 studies employing laser assisted hatching (Carney et al., 2012). However, no significant difference was found in live birth rates (5 RCT, OR=1.01, 95% CI 0.81 to 1.26), clinical pregnancy rates (15 RCT, OR=1.04, 95% CI 0.9 to 1.19), and miscarriage rates (8 RCT, OR=0.98, 95% CI 0.59 to 1.63) in the LAH group when compared with the control group. All studies included in this meta-analysis performed day-2 or day-3 embryo transfer. Among those, a prospective randomized study by Petersen et al. concluded that quarter-laser zona thinning AH improved the implantation rate (10.9% and 2.6%, P=0.02, respectively) in 150 patients with repeated implantation failures (Petersen et al., 2005). Another prospective randomized study assessed LAH on cryopreserved embryos, showing that LAH could increase the implantation rate (9.9% and 20.1%), clinical pregnancy rate (27.3% and 40.9%), as well as multiple pregnancy rate (16% and 40.3%) (Balaban et al., 2006). Conversely, other studies indicated that LAH showed no significant improvements in patients with good prognosis (Balakier et al., 2009; Baruffi et al., 2000; Carter et al., 2003; Sagoskin et al., 2007), poor prognosis (Kutlu et al., 2010; Petersen et al., 2005; Primi et al., 2004), or in patients with endometriosis (Nadir Çıray et al., 2005). A randomized controlled trial investigating LAH compared to non-assisted hatching initially found no significant differences in clinical pregnancy rates and implantation rates. However, when the results

were categorised based on patient age and frozen-thawed embryos, there was a significant improvement in clinical pregnancy rates following LAH in patients 35 years or older (70.0% versus 25.0%), and in frozen-thawed embryos transfer cycles (62.5% versus 29.5%) (Elhussieny et al., 2013). Due to the insufficient evidence regarding the hypothesis that AH improves birth rates (Carney et al., 2012), in 2014 the ASRM committee recommended that AH should not be recommended routinely for all patients undergoing IVF (Medicine and Technology, 2014). In 2016, an updated meta-analysis was published based on 36 randomized control studies, showing a significant increase in clinical pregnancy (OR=1.16, 95% CI 1.0-1.36) in AH groups compared with control, however the author suggested selecting AH patient more scrupulously (Li et al., 2016).

More studies focused on the effect of LAH on vitrified-warmed embryo transfer cycle due to the potential embryonic damage caused during freezing/vitrifying and zona hardening during thawing/warming, which may contribute to implantation failure. A prospective randomized controlled trial found QLAH did not improve the implantation rate in vitrified/frozen-thawed/warmed embryo transfer cycles when compared with the control group, reporting a similar implantation rate (13.3% and 15.6%), ongoing implantation rate (10.5% and 13.5%), and live birth rate (10.5% and 13.3%) per embryos transferred (Debrock et al., 2011). Conversely, Wan et al, found that QLAH could improve the clinical outcomes of vitrified-warmed blastocysts, especially for day 6 blastocysts that developed from low-grade cleavage-stage embryos (Wan et al., 2014). In addition, when compared with 335 control cycles, a higher implantation rate, clinical pregnancy rate and live birth rate were observed in 480 cryopreserved embryo transfer cycles where LAH was carried out on day-3 cleavage-stage embryos (Zhou et al., 2014). Recently, a meta-analysis including 12 randomized controlled trials and over 2574 participants found that for cryopreserved-thawed embryos, LAH is related to a higher clinical pregnancy rate, implantation rate, and multiple pregnancy rate; however, it is unlikely to improve the live birth rate (Zeng et al., 2017).

The conflicting nature of the results described in existing literature further defines the present debate of whether LAH is a beneficial, neutral or detrimental technique. Only a few follow-up reports have been published with regards to the health of children born following LAH. Kanyo and Konc followed the health of babies up to 1 year post-delivery and reported that there was no increase in the rate of major or minor congenital malformation, or the rate of chromosomal aberrations in 134 children born following LAH (Kanyo and Konc, 2003).

Similar results as for pregnancy and perinatal outcomes were found by Primi and Hagemann, and reported no increase in assisted groups compared to control group (Hagemann et al., 2010; Primi et al., 2004). However, three meta-analyses showed that multiple pregnancy rates were significantly increased when assisted hatching or LAH was conducted (Carney et al., 2012; Li et al., 2016; Zeng et al., 2017). Although the majority of ART-related multiple pregnancies are dizygotic, there are some trials reporting monozygotic (MZ) twinning (Hagemann et al., 2010; Hurst et al., 1998; Schieve et al., 2000)). So far, data from 6 randomized control trials has not shown an increase in the rate of MZ twin pregnancy with AH (Carney et al., 2012). While there is good evidence that AH or LAH is associated with multiple pregnancies, there remains insufficient evidence for an association with MZ twin pregnancy. One study reviewed 8 years of data to identify risk factors for MZ twinning with no association found in relation to assisted hatching (Knopman et al., 2010). The trend towards single blastocyst transfer may help to decrease the multiple pregnancy rates as well as make it more feasible to investigate the effect of new technology on embryo implantation. With regards foetal malformations and anomalies, 23 malformations and 2 anomalies have been reported thus far. Of these malformaltions, 9 were major, such as umbilical deformity, spina bifida and congenital heart disease, and 14 were minor, such as atrial septal defect, Torticollis, and congenital naevus; however, there is no increasing trend compared to those without assisted hatching pregnancies (Hagemann et al., 2010; Kanyo and Konc, 2003; Zhou et al., 2014). Overall, there is insufficient data at present to allow us to definitively describe the impact of AH upon offspring. Although, LAH has been investigated previously in animal models, we still do not understand the precise mechanisms and true value of LAH and potential harmful effects on embryo development cannot be excluded. Further, large-scale studies as well as mechanism researches are now required to fully investigate the health of embryos subjected to laser treatment as well as the health of children born following laser treatment.

Embryo biopsies

The genetic testing and diagnosis of embryos, known as preimplantation genetic screening (PGS) and preimplantation genetic diagnosis (PGD), is becoming increasingly applied to test for chromosomal disorders and hereditary disorders, respectively. To perform such tests, embryonic cells must first be biopsied from a viable preimplantation stage embryo.

Historically, embryos were biopsied using acid Tyrode's solution, which was used to create a hole in the zona pellucida. However, because of the potential danger caused by the embryo being exposed to abnormal pH levels, and also the need to change culture media (Geber et al., 2011), a mechanical method was developed as an alternative which used sharp glass pipettes to breach the zona pellucida (Tarín and Handyside, 1993). However, both acid and mechanical techniques are highly variable, lead to inconsistent hole sizes, and could easily result in damage or the loss of cells. In current clinical practice, laser-assisted embryo biopsy is the preferred method of choice, since it simplifies the process and reduces damage to the oocyte or embryo while resulting in equivalent, or even increased success rates. Laser pulses are directed at the zona pellucida, creating a uniform hole through which a blunt glass capillary can be inserted to extract target cells. The ability to use a blunt capillary, rather than a sharp glass needle, to puncture the embryo avoids damage such as mechanical strain and deformation of the embryo (Rienzi et al., 2001). Moreover, laser equipment enables multiple embryos to be kept in the same dish as they do not need to be washed from the acid afterwards. Studies comparing the use of acid Tyrode's with laser zona drilling for embryo biopsy have shown that laser drilling is an easier and faster procedure, resulting in a greater number of intact blastomere cells, minimizing the time the embryo spends outside of the incubator, and often increased pregnancy success rates (Chatzimeletiou et al., 2005; Geber et al., 2011; Joris et al., 2003). Prior to laser-assisted procedures, the biopsy technique was limited by both operator skill and a non-standardized tunnel size.

There are three types of biopsies performed to obtain genetic material for screening. First, polar body biopsy, which involves screening genetic material from the unfertilized maternal oocyte. Secondly, blastomere biopsy, where a single cell is extracted from a day-3 embryo for genetic examination. Thirdly, trophectoderm biopsy, where a cluster of cells from a day-5 embryo are genetically tested.

Laser-assisted polar body biopsy

Polar bodies are extruded from the oocyte, and while considered as genetic discards, these structures can contain valuable information which can help determine the health of a given embryo (Hou et al., 2013). In some countries, such as Switzerland, Austria, Germany and Italy, polar body biopsy is the only legally acceptable method for genetic investigation as it only screens unfertilised oocytes (Kuliev et al., 2006). Laser-assisted polar body biopsy was first explored in 1997 by Montag and colleagues; of 921 human oocytes which were laser-

treated to open the zona pellucida to retrieve a polar body, 443 oocytes were identified as being euploid using fluorescence in situ hybridisation (FISH), 293 were fertilised, 214 were transferred to the uterus, and 27 pregnancies resulted. The take home baby rate was 18.3% per transfer cycle, which was comparable to the mechanical drilling method (Montag et al., 1998). Hammoud et al., further investigated the effects of using lasers for polar body biopsies and concluded that there were no deleterious effects of laser drilling for polar body biopsy in in vitro matured oocytes in terms of oocyte lysis, oocyte activation, or chromosome breakage in embryos (Hammoud et al., 2010). In another study, laser-treated groups showed significantly higher chemical pregnancy rates, clinical pregnancy rates, and live birth rates when compared to controls (Levin et al., 2012). Figure 4 shows a typical polar body biopsy.

Laser-assisted blastomere biopsy

Blastomere biopsy for PGD/PGS is a well-accepted method of choice due to its diagnostic ability to detect both maternal and paternal contributions to the embryo. At day-3 post-fertilization, the cleavage stage consists of identical cells called blastomeres. Boada et al. (1998) published the first case report of laser-assisted blastomere biopsy for PGD in two patients with haemophilia, which resulted in a single successful pregnancy. Two to four laser pulses were applied to 13 embryos, three normal female embryos were replaced in the uterus on day 3, and one pregnancy resulted (Boada et al., 1998). Since then, numerous studies have reported the use of laser-assisted blastomere embryo biopsy in successful PGD/PGS analysis (Jones et al., 2006; Joris et al., 2003; Kahraman et al., 2004). Joris and colleagues report that using a laser to open the zona pellucida for blastomere biopsy is an easier procedure which results in significantly more intact blastomeres compared to acid Tyrode's but did not influence pregnancy and implantation rates (Joris et al., 2003). In another study, Jones et al. reported that laser biopsy does not impair embryonic development to the blastocyst stage in terms of expansion and cell numbers in the inner cell mass and trophectoderm (Jones et al., 2006). Figure 5 shows a typical blastomere biopsy. While the impaired human embryonic implantation potential after blastomere biopsy (Adler et al., 2014), and a potential risk of impaired development by blastomere biopsy during both pre- and postnatal life shown on mouse model draw clinician and scientists' attention to the potential long-term risk of this technique (Zacchini et al., 2017). Further study on the effect

of blastomere biopsy on the born children in their adulthood and/ or their offspring are necessary.

Laser-assisted trophectoderm biopsy

Between days 5 to 6 post-fertilisation, the embryo becomes a blastocyst which involves differentiation into the trophectoderm (TE), which forms the placenta, and an inner cell mass (ICM) forming the embryo. The advantage of blastocyst biopsy is that multiple trophectoderm cells can be collected for analysis, thus allowing for more reliable results, and also that no cells that form the embryo proper are compromised. Historically, the zona pellucida was breached and trophectoderm cell samples were obtained by mechanical excision. Conversely, the zona pellucida can be breached and herniated cells separated from the blastocyst using laser pulses. The laser procedure is much faster, requires less mechanical skill, and imposes less mechanical stress on the blastocyst (Montag et al., 2009). Figure 6 shows a typical example of trophectoderm biopsy.

Although blastomere biopsy has been used in the majority of PGD cycles for the past decades, trophectoderm biopsy has replaced blastomere biopsy in clinical practice over the last ten years (Zacchini et al., 2017). The use of lasers has now become the accepted convention for embryo biopsy and has been used to investigate differences between blastocyst trophectoderm biopsy and cleavage stage blastomere biopsy. For example, Kokkali et al., compared laser blastomere biopsy versus blastocyst biopsy for beta-thalassaemia PGD and found that blastocyst biopsies resulted in significantly higher diagnosis rates and implantation rates compared to blastomere biopsies (Kokkali et al., 2007). In support of this finding, many other studies have shown laser trophectoderm biopsy to be more reliable and successful than blastomere biopsy for PGD (Adler et al., 2014; Forman et al., 2011; Scott et al., 2013). Internationally, clinics are moving towards selective blastocyst stage biopsy to allow for more accurate tests and thus improved success rates. One study used H1 human embryonic stem cell models to quantify laser impact, showing that laser usage in the range commonly used to perform trophectoderm biopsy had no impact to mosaic anomalies (Kelk et al., 2017). Practically, in some IVF clinics LAH and laser assisted trophectoderm biopsy were combined for PGD/PGS, whereby LAH is conducted on cleavage-stage embryos, followed by trophectoderm biopsy after 1-2 days culture. LAH could help embryo hatching so that hatching/hatched embryo can usually be observed on day-5, which helps embryologists to arrange a working routine (Personal Communication,

Miss Jenny Spencer, IVI, London, UK). However, research regarding the effects of trophectoderm biopsy on the further development of offspring, epidemiological changes and other changes at the molecular level remains sparse (Zacchini et al., 2017).

Embryo cryopreservation

Removing necrotic blastomeres

Cryopreservation procedures at the day 3 cleavage stage can impart damage to embryos via several mechanisms including solution effects, osmotic effects, or physical damage by growing ice crystals (Hendriks et al., 2015). Frozen-thawed day 3 embryos may experience partial blastomere damage, and are considered to have survived if at least half of the blastomeres are intact. Such partially-damaged embryos have lower implantation rates than fully-intact embryos (El-Toukhy et al., 2003; Guerif et al., 2002). The poor implantation rates associated with partially-damaged embryos may be due to the release of toxic metabolites from necrotic blastomeres, and the action of such metabolites upon the remaining healthy blastomeres. If the healthy blastomeres survive and resist the harmful actions arising from degradation products or metabolites, they may still have to engage in detoxification and defence mechanisms, which could deplete energy and resources needed for further development and differentiation, thus weakening the embryo (Rienzi et al., 2002).

However, laser techniques can facilitate the removal of necrotic blastomeres from the embryo by aspiration via a small hole created by a laser through the zona pellucida. Removal of necrotic blastomeres from partially-damaged embryos has been shown to significantly increase the developmental potential of partially-damaged frozen-thawed embryos; indeed, clinical pregnancy rates and implantation rates more than double compared to when necrotic blastomeres are not removed (Liu et al., 2006; Liu et al., 2005; Rienzi et al., 2002). Rienzi et al. showed that the developmental potential of partially-damaged frozen-thawed embryos can be equivalent to normally healthy embryos if necrotic blastomeres are removed with the aid of laser technology, even if multiple blastomeres need to be removed (Rienzi et al., 2005).

Blastocoele collapse for vitrification

Although it has not been shown that the application of laser-assisted manipulation to post-thaw blastocysts is necessary to optimize ability of implantation, it does appear to have a

benefit prior to cryopreservation. In the day 5 blastocyst stage, the embryo has a fluid-filled cavity called the blastocoele which is at risk of forming ice crystals during cooling and warming. Artificial shrinkage of the blastocoele cavity, in order to reduce fluid volume prior to vitrification, has been shown to increase embryo survival rates (Chen et al., 2005; Vanderzwalmen et al., 2002). Shrinkage of the blastocoele can be achieved by three methods: 1) mechanical puncture with a sharp glass needle; 2) manual puncture and aspiration of fluid from the blastocyst into an ICSI pipette (Hiraoka et al., 2004) and 3) by osmotic shock using non-permeating solution to induce the transport of water out of the embryo. The latter method is an approach which only requires a short laser pulse to induce embryo shrinkage without any other form of manipulation or mechanical strain to the embryo (Iwayama et al., 2011). Several studies have shown that human blastocysts vitrified after mechanical and laser collapse show fewer damaged cells, lower levels of DNA damage, higher rates of expansion, significantly better blastocyst hatching and implantation rate, higher pregnancy rates, and better gestational duration and birth weight compared to non-collapsed blastocysts (Desai et al., 2008; Hur et al., 2011; Min et al., 2014). In addition, laser collapse was shown to be a superior method with a significantly higher blastocyst hatching rate, longer average gestational duration and lower premature birth rate (Cao et al., 2014).

Nonetheless, it is important to note that the necessity to apply blastocoele collapse to vitrified warmed blastocysts may be a cryoprotectant-induced phenomenon. For example, previous studies all used DMSO-containing solutions during the vitrification. Nevertheless, Stachecki et al. showed that glycerol containing, DMSO-free, vitrification solutions did not require mechanical collapse of the blastocyst in order to attain high rates of post-warming survival (Stachecki et al., 2008). Furthermore, it was recently shown that high survival and pregnancy rates could be achieved without pre-collapse of the blastocyst, in conjunction with HA (sodium hyaluronate), a extracellular adhesion molecule, even after trophectoderm biopsy/PGS testing, vitrification, warming and subsequent transfer (Stachecki et al., 2014) .

Laser-assisted sperm manipulation and intra-cytoplasmic sperm injection (ICSI)

Although laser-assisted techniques have been widely used in a range of different ART procedures, sperm trapping was the first application of lasers in ART (Tadir et al., 1989). Tadir et al. (1990) described the potential use of a laser trap to control sperm and guide them

into the oolemma. Since then, several new applications for the use of lasers in sperm manipulation have emerged.

Immobilization of spermatozoa

Prior to ICSI, the sperm tail must be immobilised so that its beating does not cause damage inside the oocyte (Catt and Rhodes, 1995; Gerris et al., 1995; Van den Bergh et al., 1995; Vanderzwalmen et al., 1996). Traditionally, sperm tail immobilization is performed using a fine-tipped glass micro-tool, which is manually brought down and across the tail, thus causing the tail to break (Van den Bergh et al., 1995). In the year 2000, the non-contact 1.48μm diode laser was introduced as a new means of immobilizing sperm (Montag et al., 2000); in this technique, low level laser pulses are applied to the sperm tail to incapacitate its beating without affecting viability; this practice also significantly reduces manipulation time and reduces exposure to PVP (Ebner et al., 2001; Vizziello et al., 2005). Ebner et al (Ebner et al., 2001) compared the conventional mechanical stimuli method with the laser-assisted immobilization method with human sperm and found that the laser method requires less manipulation time and that there was no differences between the two techniques in terms of ICSI outcomes, fertilization rate, embryo quality and blastocyst formation (Ebner et al., 2001). Furthermore, a case report describing a successful birth clearly supported the safety of laser-assisted sperm immobilization (Ebner et al., 2002). A further investigation regarding external physical damage and distinct external phenotypic effects between mechanical and laser-assisted immobilized human spermatozoa was visualized by using scanning electronic microscopy (SEM). Chan et al, found evidence of external damage at the SEM level showing reduced membrane integrity on mechanically immobilized sperm, but not on laser-assisted immobilized sperm. This study revealed the impact of laser immobilization upon morphological changes when the laser was targeted to different sperm regions (Chan and Li, 2017). However, additional clinical evidence is still required in order to confirm the relative benefit of laser-assisted sperm immobilization.

Laser-assisted selection of immotile sperm

ICSI relies upon the selection of ‘good quality’ viable sperm, usually taking morphology and motility as the prime considerations. However, there are cases where patients exhibit reduced sperm motility (asthenozoospermia) or lack sperm completely (azoospermia). This is most often seen in cases where sperm has been surgically retrieved from the testes, but

can also be seen in ejaculated samples (Nordhoff, 2015). Immotile live sperm can still successfully fertilise oocytes, albeit with a lower fertilization rate, poorer quality embryos and a lower pregnancy rate compared to motile sperm (Esfandiari et al., 2005; Javed et al., 2010; Montjean et al., 2015). Some viability tests can help to identify viable and suitable sperm for ICSI from a population of immotile sperm, such as the hypo-osmotic swelling test, the chemical induction of tail movements, the sperm tail flexibility test, and laser-assisted sperm selection (LASS) (Montag and Morbeck, 2017). The main disadvantages of the first three techniques are that they are time consuming, require additional chemicals, and cannot be used on cryopreserved sperm (Nordhoff, 2015). Collectively, LASS is considered to be quick, easy, repeatable and a safe means of viability testing (Nordhoff et al., 2013) by identifying viable sperm by targeting a single pulse (129 μJ for approximately 1.2 msec) to the tip of the sperm tail; this causes the tail of live immotile sperm to curl has no effect upon non-viable sperm (Aktan et al., 2004). This technique is conveniently performed immediately prior to ICSI injection, does not require the use of additional incubation steps or chemicals which may cause side effects, and does not cause damage to sperm DNA (Montag and Morbeck, 2017). Aktan et al (2004) reported that LASS exhibits the same level of viable sperm detection (22.0%) as the hypo-osmotic swelling test (21.5%). Furthermore, compared with randomly-selected TESE (testicular sperm extraction) sperm, LASS created improvements to fertilization rate (45.5% and 20.4%) as well as take-home baby rate (19.0% and 5.9%). In 2017, a successful pregnancy case was reported using a completely immotile but viable frozen-thawed spermatozoa selected by laser, providing a new insight into male fertility preservation (Chen et al., 2017). Additionally, a live birth was achieved in a couple with male Kartagener's syndrome using pentoxifylline resistant ejaculated immotile spermatozoa selected by laser assisted viability assessment. This report indicated that the application of laser selection has the potential to allow clinics to avoid using testicular sperm from Kartagener's syndrome by selecting the immotile ejaculated spermatozoa (Alvarez et al., 2018).

Laser-assisted ICSI

Intracytoplasmic sperm injection (ICSI) is one of the most technologically impactful advancements applied to the treatment of human infertility, which has revolutionised the treatment of male factor infertility. Now, ICSI is used in over half of the treatment cycles carried out in the United Kingdom and in 67% of cycles carried out in the United States (Fertilisation and Authority, 2011; Gardner and Simón, 2017). However, conventional ICSI

is associated with potential mechanical damage to the oocyte caused by deformation of oolemma and exposure to negative pressure injection, which may lead to the degeneration of oocytes after ICSI. Especially, when the oolemma is very fragile and/ or the zona pellucida is very resistant to penetration of the ICSI injection needle, ICSI can result in unusual and extensive deformation of the oocytes (the oocyte has to be strongly compressed) **(Figure 2)** and subsequently compromise survival of embryos (Abdelmassih et al., 2002; Danfour and Elmahaishi, 2010; Jiaen et al., 1995; Nagy et al., 1995). In laser-assisted ICSI (LA-ICSI), a precise hole is drilled into the zona pellucida of oocytes, leaving the innermost layer of the zona intact, thus allowing easier access of the needle though the mechanical barrier with minimal membrane deformation. Previous studies have supported the notion that creating a micro-hole through the zona pellucida of the oocyte by a laser beam prior to ICSI results in less trauma following penetration of the injection needle (**Figure 3**), thereby resulting in reduced levels of oocyte and embryo degeneration, higher embryonic development (Abdelmassih et al., 2002), as well as higher rates of fertilization (Verza et al., 2013). In additon, better clinical pregnancy outcomes were also achieved in LA-ICSI compared to conventional ICSI for patients with fragile oolemas, with difficult zona pellucida penetration, repeat IVF failure, or even unselected patients (Choi et al., 2011; Rienzi et al., 2001; 2004). However, laser-assisted ICSI has not been widely-adopted by clinics, as there is some debate regarding the overall benefit of the procedure. For example, Richter et al found that there were no advantages of laser-assisted ICSI over conventional ICSI in unselected patients in terms of oocyte survival, embryo quality or clinical outcomes (Richter et al., 2006). In addition, some embryologists believe that it is the resistance and stretching ability of the membrane under the zona pellucida and around the cells, and not the zona pellucida itself, that relates to oocyte degeneration and embryo developmental competence following conventional ICSI, and that such damage can be reduced by simply using good techniques and good quality injection needles (Danfour and Elmahaishi, 2010).

Safety concerns associated with laser application

When the use of lasers was first induced into ART, there were understandable concerns about safety, largely due to its application at such a delicate stage of human development. Most concerns centered upon the risk of DNA damage, failed embryo development and possible congenital disorders (Hershlag et al., 1999; Makrakis et al., 2006; Montag et al., 2009).

Three characteristics of lasers that can exert impact upon embryos or gametes are wavelength, power, and pulse length. The ultra violet (UV) wavelength historically used in biomedical lasers posed significant concern, especially given the reputation of such instruments to be potentially mutagenic to DNA (Sinha and Häder, 2014). Consequently, laser technology deliberately adapted to operate in the infra-red (IR) wavelength for ART. The amount of power from a laser, while constant, can influence the diameter of the hole created and the amount of heat emitted; high power translates to a larger diameter and increased local heating (Taylor et al., 2010). Similarly, pulse length, which can vary from 20 ms down to the femtosecond level, affects hole diameter, with a longer pulse correlating to a larger hole and the delivery of more energy (Ebner et al., 2005). The laser uses high energy light, which comes into contact with the media surrounding the embryo. This heat is dissipated and transferred through the media, thus increasing the temperature of the environment surrounding an embryo. The risk of thermal damage was cause for significant concern, as this could potentially heat and damage the surrounding cells. However, initial studies failed to identify any significant harmful impacts upon blastocyst development following the laser treatment of thawed embryos (Wong et al., 2003). Furthermore, three different laser pulse lengths (0.604 mS, 0.708 mS and 1.010 mS) were found to have no influence upon the quality of day 5 embryos and lysed blastomeres when a laser was deployed during an embryo biopsy procedure (Taylor et al., 2010). Chailert et al. found that full thickness opening of the zona pellucida using LAH increased the number of completely hatched blastocysts compared to LAH thinning of the zona pellucida and controls, however, cell numbers (TE and ICM) were significantly decreased with both LAH and LAH thinning compared to controls (Chailert et al., 2013). In contrast, a significant perturbation in developmental potential was observed in mouse embryos after LAH at the 6-8 cell stage, and also that LAH treated cells at the 2-cell stage showed increased DNA fragmentation at the blastocyst stage (Honguntikar et al., 2015). In another study, the safety of IR lasers with respect to thermal damage was investigated using heat-shock protein response (hsp70i), and showed no elevation in terms of rescue mechanisms even at energy levels higher than those applied clinically, suggesting that the laser pulses used during ART do not cause heat stress to embryos (Hartshorn et al., 2005). More recently, Honguntikae (Honguntikar et al., 2017) used mouse embryos to investigate epigenetic changes in preimplantation embryos following LAH. These authors found that only the expression of two de novo methyl transferases (Dnmt3a and Dnmt3b) decreased after LAH at the 2-cell stage, whereas LAH at the 6-8 cell and blastocyst stage did not cause any effect on the expression of these genes.

On the other hand, long interspersed element (LINE-1) methylation, a maker for global genomic DNA methylation, did not change after LAH in any of the embryonic stages tested. In terms of embryo metabolism, one study indicated that LAH neither facilitates nor reduces the uptake of metabolites from the culture medium in human embryos, suggesting that laser manipulation may not cause any immediate metabolic and functional abnormalities in embryos for at least 24 hours post- laser treatment (Uppangala et al., 2016). Time-lapse monitoring provides us with an opportunity to observe embryo cleavage patterns (Meseguer et al., 2012). A morphokinetic study in mouse embryos showed that LAH thinning does not facilitate hatching compared to the control group (39.3% and 33.9%, respectively), furthermore, LAH thinning may disrupt the *in vitro* hatching process (60.7% did not complete hatching process). However, LAH opening did appear to improve the hatching process in that hatching occurring 14 hours earlier in laser-treated embryos compared to controls (Schimmel et al., 2014). Time lapse monitoring of human embryos for 24 hours after LAH did not reveal blastomere fragmentation adjacent to the point of laser treatment (Uppangala et al., 2016). Thus far, research involving both animal models and human subjects have provided some clues of how laser application may impact embryos at the molecular level, however, there is insufficient evidence to demonstrate the negative clinical impact of using laser manipulation on embryos. Further, more robust evidence of pregnancy outcomes and the health of offspring are now needed to support the long-term safety of laser techniques in ART.

CONCLUSIONS AND FUTURE DIRECTIONS

It is clear that the continued success of ART lies in the dedication and skill of clinical embryologists and to the continued development and improvement of laboratory technologies. This is imperative to providing patients with the highest level of care and greatest chance of pregnancy. The benefits of laser application upon embryos, which include minimizing mechanical strain, reducing manipulation times, and reducing the time required outside an incubator, place such technology at the vanguard of research developments. **Table 3** summarizes the common clinical applications of lasers in present-day ART. From available literature, LAH is likely to improve implantation and pregnancy rates, however uncertainty remains regarding the benefit on live birth rate. LAH is recommended for use in certain sub-populations, such as patients with repeated implantation failure or patient with poor embryo quality. It is recommended that trophectoderm biopsy for PGD/PGS is

performed with laser-assisted manipulation. There remains a lack of sufficient evidence supporting laser-assisted ICSI and embryo cryopreservation due to improvements of needle and cryoprotectant utilized. Large scale studies and meta-analyses are still needed to prove the clinical benefit and safety for laser-assisted sperm manipulations.

Currently, the laser energy applied for all ART procedures is at the discretion of each embryologist and is based on how much zona pellucida should be dissolved with each pulse applied. Further experiments into the more sensitive effects of laser pulses upon embryo health and negative clinical consequences could help further optimize laser settings and thus improve clinical outcome. Studies focusing at the molecular level, including protein and mRNA, of how laser techniques might influence embryo development and the processing of implantation are now needed to understand whether laser procedures could potentially change the physiology of the embryo, thus providing mechanical evidence for clinical application.

ACKNOWLEGEMENTS

Conflict of interest: None declared

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Figure Legends

Figure 1. The three methods used for laser-assisted hatching (LAH). **(A)** complete LAH, in which a single hole is drilled completely through the zona pellucida; (**B)** partial LAH, in which a single hole is created but without reaching the inner membrane; (**C)** quarter LAH, in which around a quarter of the zona pellucida perimeter is partially drilled. Images were created by the lead authors.

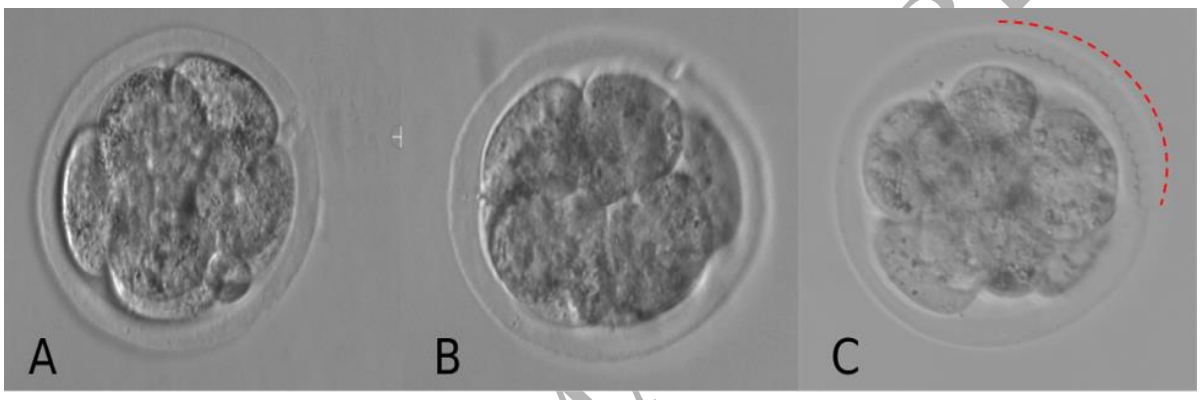

Figure 2. Resistance patterns of oolemma breakage during intracytoplasmic sperm injection (ICSI). (**A)** mild resistance from the oolemma (curved shape); (**B)** serious resistance from the oolemma (funnel shape). Modified from (Danfour and Elmahaishi, 2010) with permission.

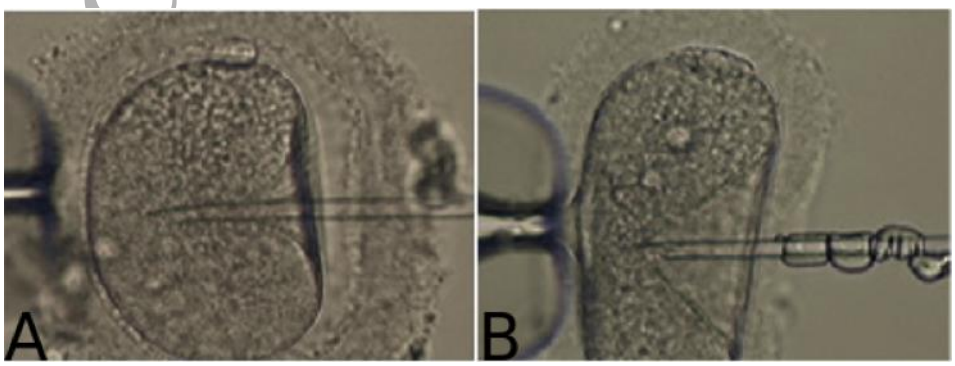

Figure 3. Laser-assisted intracytoplasmic sperm injection, in which holes are created in metaphase II oocytes by an infra-red laser, to permit the more efficient introduction of a micropipette for intracytoplasmic sperm injection. Modified from (Rienzi et al., 2001) with permission.

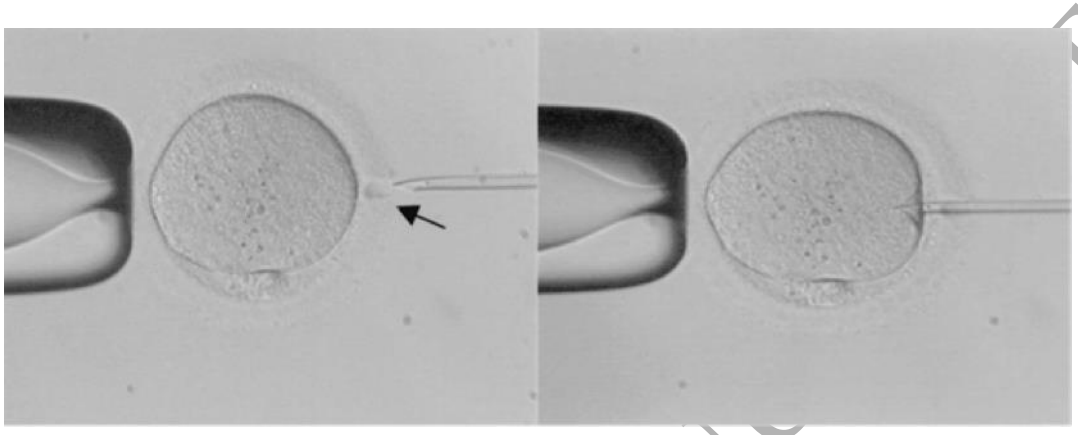

Figure 4. Laser-assisted polar body biopsy **(A)**, in which the zona pellucida is treated with laser pulses **(B)**, and the polar body is harvested for genetic analysis using a glass micropipette tube **(C).** Images were created by the authors.

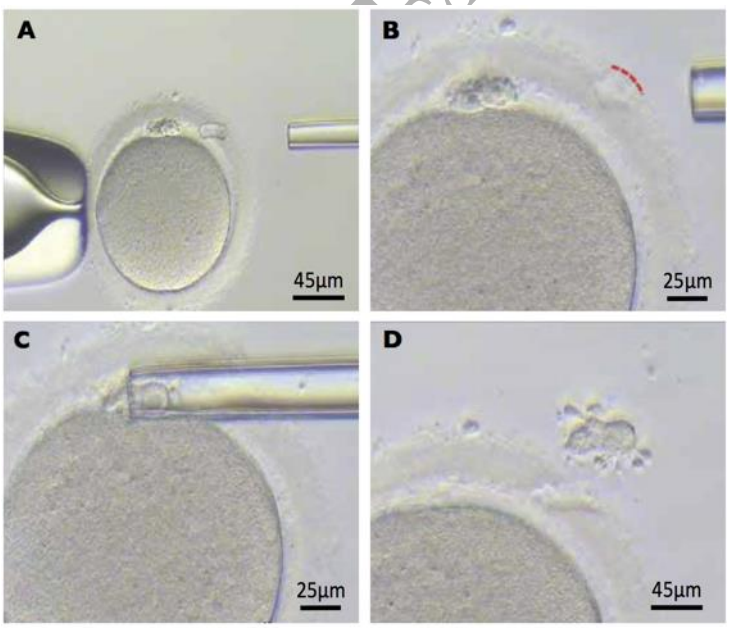

Figure 5. Laser-assisted blastomere biopsy. Images show the administration of laser pulses to the zona pellucida **(A, B),** and the insertion of a glass micropipette tube to select a blastomere close to the periphery of the embryo, followed by blastomere extraction **(C).** Images were created by the authors.

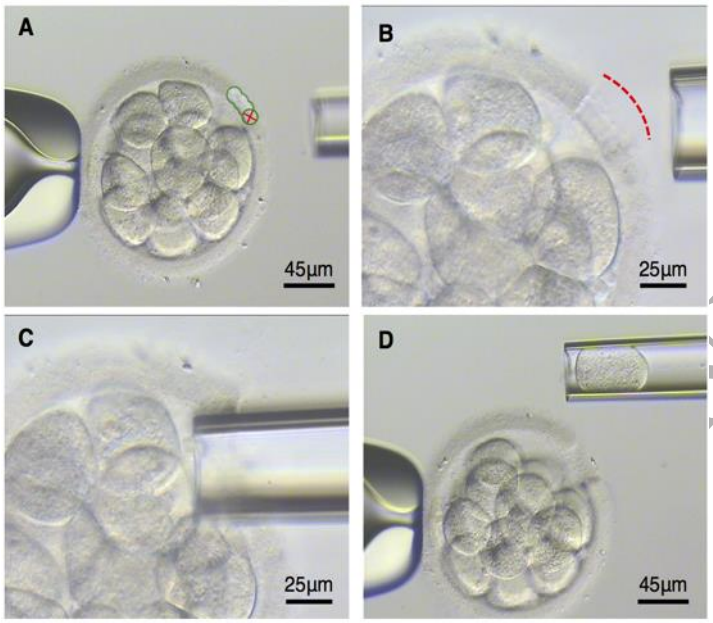

Figure 6. Laser-assisted blastocyst biopsy. These images show an example of a laser-assisted trophectoderm biopsy carried out on a blastocyst. The hole was created by laser pulses on day 3, thus allowing part of the trophectoderm to begin to hatch out of the zona pellucida through the hole created by the laser **(A).** An aspiration pipette was then used to hold the herniated cells and create tension between the cells **(B).** Laser pulses are normally applied at the cell junction in order to separate the trophectoderm cells from the embryo (C). Finally, a micropipette tube was used to collect the trophectoderm cells. Images were created by the authors.

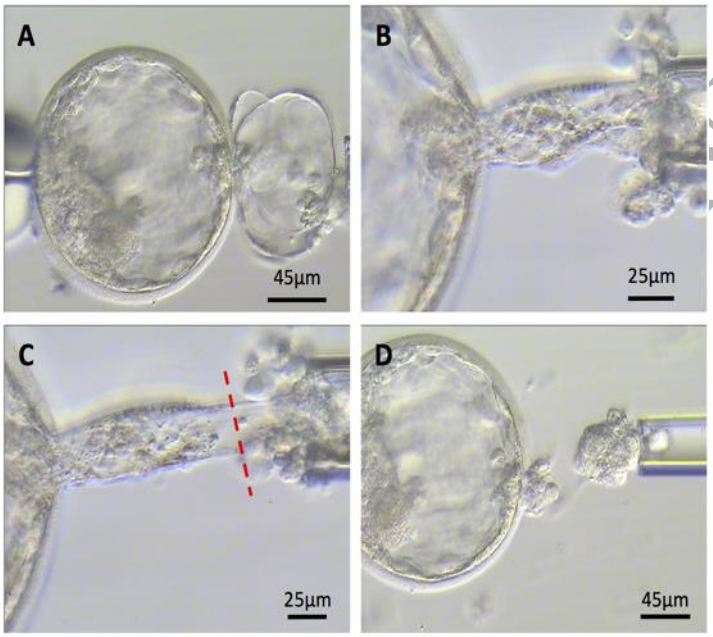

Table 1. History of laser development for ART.

Year	Laser	Wavelength	Application	Reference
1989	Nd:YAG	1064nm	Trap spermatozoa	Tadir et al.(1989)
1991	Nd:YAG	532nm	Drill zona pellucida	Tadir et al.(1991)
1991	ArF	193nm	Drill mouse zona pellucida, grow to blastocyst	Palanker et al. (1991)
1992	KrF	248nm	Drill zona pellucida of 2-cell mouse embryos, grow to blastocyst	Blanchet et al. (1992)
1992	Er:YAG	2900nm	Drill human oocyte zona pellucida prior to IVF	Feichtinger et al. (1992)
1993	XeCl	308nm	Drill mouse oocytes prior to improve fertilisation with vasectomised mice	Dana- souri et al. (1993)
1994	InGaAsP	1480nm	Non-contact drilling of mouse oocyte zona pellucida	Rink et al. (1994)

Table 2. Assisted reproductive technology lasers currently on the market

Name	Company	Wavelength	Power	Objective	Approved work
Saturn 5	Research Instruments Ltd.	1480nm	400mW	40X	Clinical
Saturn 5-Active	Research Instruments Ltd.	1480nm	400mW	20X, 40X	Clinical
LYKOS	Hamilton Thorne	1460nm	300mW	40X	Clinical
ZILOS-tk	Hamilton Thorne	1460nm	300mW	40X	Clinical
XYRCOS	Hamilton Thorne	1460nm	300mW	20X, 40X	Research
XYClone	Hamilton Thorne	1460nm	300mW	40X	Research

Table 3. Clinical applications utilizing lasers in the ART laboratory

Application	Procedure	Recommendation
Assisted hatching	LAH can be conducted on fresh or cryopreserved-thawed embryos. Different LAH methods: complete LAH; partial LAH; quarter LAH thinning	Although LAH has been reported with higher implantation and pregnancy rates, LAH is not recommended routinely for all patients due to the insufficient evidence regarding live birth rates; however it may be beneficial for certain subpopulations such as those with poor prognosis.
Embryo biopsy	A laser is used to create an opening in the zona pellucida for polar body, blastomere, or trophectoderm biopsy. Laser pulses are applied at cell junctions to separate trophectoderm cells for trophectoderm biopsy.	Laser-assisted trophectoderm shows higher diagnosis rates in PGD and implantation rates compared with polar body and blastomere biopsy.
Cryopreserved embryos		
Removing necrotic blastomeres	Laser pulses are applied to create an opening in the zona pellucida for aspiration of necrotic blastomeres from cleavage stage cryopreserved-thawed embryo.	Laser-assisted necrotic blastomere removal and blastocoele collapse may increase the embryonic developmental potential and pregnancy rate. However, with developments in the protocols used for cryopreservation or the cryoprotectants used in these protocola, it is possible that laser-manipulations may not be needed.
Blastocoele collapse	A laser pulse is applied to shrink the blastocoele cavity.	
Sperm manipulation		
Immobilization of spermatozoa	Laser pluses are applied to the sperm tail for immobilization prior to ICSI.	Laser assisted sperm manipulation is quick, easy and repeatable. More clinical evidence is needed to confirm the safety and benefit of the procedure.
Selection of immotile sperm	Laser-assisted sperm selection is processed by targeting a single laser pulse to the tip of the sperm tail for viability testing	
Laser-assisted ICSI	An opening into the zona pellucida is produced by laser pulses for easier access of the ICSI needle.	Laser-assisted ICSI shows limited advantage over conventional ICSI when good quality injection needles are utilized with proper technique.

Yaqiong is a DPhil candidate at the University of Oxford. She obtained a medical degree from Guangzhou Medical University, followed by a Masters in Reproductive Engineering from Central South University, China. She also holds an Master's degree in Reproductive Medicine and Heath from the University of Nottingham, UK.