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SYMPOSIUM: QUALITY MANAGEMENT IN ASSISTED REPRODUCTIVE TECHNOLOGY

Micromanipulation in assisted reproductive technology

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Dr Malter earned his PhD degree in Cell/Developmental Biology at Emory University. Over a 30-year career beginning in the large animal laboratory of Dr Ben Brackett and transitioning to human clinical work with Dr Jacques Cohen, Dr Malter has worked as a senior scientist at the New York Hospital and St Barnabas Hospital assisted reproduction programs, with the Reproductive Genetics Institute and the McGill Reproductive Center. He is currently the Director of Laboratories for the Fertility Center of the Carolinas in Greenville, SC and Fertility Solutions in Dedham, MA. His interests involve clinical and research aspects of human assisted reproduction and genetics.

Abstract Micromanipulation describes a set of tools and techniques for cellular microsurgery and manipulation. Micromanipulation techniques have played an important role in basic research and the development of clinical techniques in assisted reproductive technology. This work provides a review of the development and current practices involving micromanipulation in the human clinical assisted reproduction laboratory.

KEYWORDS: embryo biopsy, embryos, ICSI, micromanipulation, nuclear transfer, oocytes

Perhaps micromanipulation is not a worthwhile subject for isolated discussion? Micromanipulation simply describes a set of tools and techniques. However, these tools and techniques have played a key role in advancing knowledge in reproductive biology and expanding the repertoire of clinical methodology and options. They have essentially solved the dilemma of male factor infertility, they allow for the diagnosis and circumvention of inherited genetic conditions and they hold great promise for further advancement in the future. Therefore, I appreciate the opportunity to embark on this review of a subject I have had the great pleasure to have been intimately involved in for over 30 years. I hope to provide an analysis that puts the tools and techniques of micromanipulation in perspective with the science and clinical achievements that have resulted. We will begin with a historical perspective and then move into a categorical description of the various uses of micromanipulation in assisted reproductive technology (ART).

Historical perspective

No doubt from the first moments scientists looked through their microscopes into a new world, they wished to reach in and poke things about. Primitive simple manipulation devices – such as microscope-mounted needles – date back to the 18th

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century. Some of the earliest manipulative embryology experiments involved using such needles, already made from heat-pulled glass, to poke (and essentially destroy) individual cells of the early stages of simple aquatic organisms. Such destruction, in the case of these highly determinant embryos, resulted in satisfying downstream effects and manipulative embryology was born. Within 100 years, micromanipulation became a mature field with a substantial base of standard methodology and equipment, including the early versions of most current systems. Micromanipulation began to be used in a variety of reproductive developmental biology and animal husbandry settings. When the maturing science of ART was brought to bear in human clinical reproduction by Edwards and co-workers in the late 1970s, micromanipulation was not far behind. In the mid 1980s, several laboratories around the world began considering the application of micromanipulative techniques to human clinical material. The first efforts involved methodology to promote sperm–egg fusion and fertilization by circumventing the zona pellucida. After a few fits and starts, our group reported in 1989 on the first substantial series of pregnancies and healthy human births resulting from micromanipulated eggs in which the zona had been opened to supposedly facilitate fertilization in male factor cases (Cohen et al., 1988; Malter and Cohen, 1989a). Around the same time, other groups pursued simply injecting spermatozoa into the perivitelline space (Lacham et al., 1989; Ng et al., 1989). Within a few years, these techniques were quickly made obsolete by direct sperm injection, but human micromanipulation was established and other techniques addressing other clinical issues in human reproduction quickly followed. I will now review the historical development of micromanipulation techniques in greater detail as related to the stage of development.

Micromanipulation of eggs and spermatozoa

The interaction between egg and spermatozoa represents a primary mystery in developmental biology and is obviously of great clinical importance in both animal husbandry and human clinical reproduction. While we have still not solved this mystery, we have made great strides, and micromanipulation has played a key role in the associated detective work. Furthermore, using micromanipulation, obstacles to sperm–egg fusion and fertilization hampering reproductive success have been considerably ameliorated. The idea of simply sticking a sperm cell into an egg has had great appeal and perhaps represents the pinnacle of developmental biological "poking" (Markert, 1983). It seems quite amazing that this technique, which bypasses a great deal of natural sperm–zona–egg membrane interactions, can actually work as well as it does.

As sperm–egg fusion and fertilization began to be dissected by developmental biology and animal science researchers, early experimental attempts sought to first simply bypass the zona pellucida. Beth Talansky in Jon Gordon's laboratory performed the pioneering mammalian experiment in which an ingenious "hydraulic drilling" technique was used to burn a tiny hole in the zona of mouse eggs using a flow of acidified solution from a micropipette (Gordon and Talansky, 1986). This "zona drilling" resulted in substantially increased fertilization when the population of spermatozoa was compromised in various ways. The technique seemed a likely candidate for improving human clinical IVF. However, human eggs unfortunately were considerably more sensitive to the acidified media, and success was not obtained (Garrisi et al., 1990; Gordon et al., 1988). Our group, under the direction of Jacques Cohen, developed a simple mechanical alternative to dissect a gap in the human zona, which quickly resulted in facilitating healthy births in many mild male-factor couples (Cohen et al., 1988; Malter and Cohen, 1989a). This consistent clinical success demonstrated that micromanipulation could be integrated into the human ART laboratory setting and established a set of basic aspects, such as appropriately sized microtools for human eggs/embryos and strict temperature control to maintain the integrity of human eggs during the procedures. One lesson from these early manipulative developments was the failure of rodent eggs and embryos as a model for the human. As mentioned, the reaction of human eggs to acidified zona drilling was not commensurate with the prior rodent experiments. Another major discrepancy was the level of polyspermy observed when the zona barrier was compromised. Partial zona dissection of mouse eggs resulted in essentially no increase in polyspermy, whereas in the human it was considerable and basically rendered the technique unusable (Malter et al., 1989). This was of course a basic developmental biology finding in identifying a distinct difference between the two species in the basis of the polyspermy block. However, it was a frustrating cautionary tale in the attempt to model and develop human techniques in the mouse, and other developmental discrepancies between the species would continue to be identified.

The injection of a spermatozoon under the zona was another "bypass" technique that was successfully pursued, but early failures at direct cytoplasmic injection of spermatozoa in the human clinical setting put a damper on the pursuit of that idea (Lanzendorf et al., 1988; Sakkas et al., 1992). My co-worker Carol Keefer in Ben Brackett's large animal research laboratory had already produced the first mammals (Dutch belted rabbits) from direct cytoplasmic sperm injection in 1989 (Keefer, 1989). However, her success was hard fought and required a rather complex manipulative protocol and considerable skill, and had a restricted survival and success rate. Fortunately, Gianpiero Palermo and co-workers in Brussels observed random success when human eggs were fully pierced during clinical subzonal injection attempts. They pursued and refined the technique to create a working direct cytoplasmic injection technique somewhat ponderously termed intracytoplasmic sperm injection (ICSI) to delineate it from the subzonal procedure (Palermo et al., 1992). With ICSI, excellent fertilization and development was achieved with single sperm injection, providing a truly revolutionary functional clinical solution to even the most severe level of male factor infertility. It is worth noting that, again, human eggs and embryos fortunately have considerable developmental distinctions from other model systems. Direct sperm injection "works" much better in the human than in basically all other species in which the technique has been pursued, including other primates (Hewitson et al., 2000).

Another technique involving the egg is the manipulation of ooplasm. A variety of research and large animal experiments have used either membrane-bound cytoplast transfer or direct injection to modify the ooplasm. Some

experiments have manipulated the ooplasmic mitochondiral make-up, whereas in others cytoplasmic developmental determinants were transferred between strains (Muggleton-Harris et al., 1982; Smith and Alcivar, 1993; Smith et al., 2000). Based on evidence of positive downstream effects from ooplasmic manipulation, the direct injection of human donor egg ooplasm was attempted using a modification of the ICSI technique (Cohen et al., 1997). When this was applied in couples experiencing a history of cryptic prior ART failure and developmental compromise, a considerable effect was observed ameliorating their prior ART failure (Barritt et al., 2001; Cohen et al., 1998). Since the technique resulted in the transfer of mitochondrial DNA between distinct individuals in some instances, it was determined to fall under regulatory supervision by the US Food and Drug Administration and was quelled. Further research in this area continues with both supportive and cautionary findings (Sansinena et al., 2011; Sharpley et al., 2012; Yao et al., 2014). A version of the technique has been resurrected in pursuing a new protocol in which a mitochondrial preparation of the patient's own ooplasmic material would be injected into potentially compromised eggs to improve outcome (Woods and Tilly, 2012).

Another egg/zygote micromanipulative technique is the manipulation of early nuclei. Nuclear manipulation had been performed for many years in pursuing a variety of developmental biology research and for animal husbandry pioneered by Steen Willadsen (McGrath and Solter, 1983; Willadsen, 1986). Simply from a physical perspective, zygotes – with their large, clear pronuclei – present a tempting target. Observing our early attempts at developing practical manipulative techniques, my colleague Michele Mazzanti suggested that if I could remove one of the pronuclei from a mouse zygote intact, he could attempt to patch clamp the nuclear membrane and elucidate its electrical behaviour. With the exuberance of youth, we made the attempt and by combining our two micromanipulation systems within a Faraday cage and taking care to use an appropriate media that allowed excised pronuclei to remain relatively physiological, we were successful, resulting in a landmark publication demonstrating the first evidence of ion channels in the nuclear membrane (Mazzanti et al., 1990). In a more practical vein, the idea of simply removing "extra" pronuclei (arising from supernumerary spermatozoa) to "fix" polyspermic zygotes was also pursued with some physical success in the human ART laboratory (Malter and Cohen, 1989b; Rawlins et al., 1988). This concept remained pre-clincial apart from a single case report of chromosomally normal embryos and a live birth following enucleation by a slightly modified method (Kattera and Chen, 2003). Despite this apparent success, the basic idea would seem to be made highly questionable by the recognition that the associated extra sperm asters in polyspermic zygotes consistently result in an aberrant spindle and abnormal chromosome segregation. Therefore incomplete removal of the extra sperm aster spindle along with the pronucleus would compromise development. This is another critical mouse/human difference. (Feng and Gordon, 1996; Sutovsky and Schatten, 2000). In the post-ICSI era, the similar removal of supernumerary egg-derived pronuclei in single-sperm injected zygotes was also considered and has had some clinical use to my knowledge (J Cohen, personal communication, 2000).

Another use of pronuclear manipulation involves exchanging or altering the ooplasmic make-up. This idea occurred to me the first time I had heard a lecture on the ooplasmic inheritance of mitochondrial mutations. The oocyte represents the initial pool of mitochondria that will replicate and go on to populate the developing individual. In the case of mitochondria harbouring deleterious mutations, these result in severe developmental problems and dysfunction. The compromised oocyte population could potentially be "corrected" at this early stage by the replacement or augmentation with ooplasm containing genetically normal mitochondria. As previously discussed, basic animal research and clinical work in the human showed that mitochondria from donor cytoplasm could be retained during development (Smith et al., 2000). However, this was in fact a rare event with limited scope following simple ooplasmic injection and therefore probably unsuitable to achieve the necessary replacement or at least clinically relevant dilution of genetically dysfunctional mitochondria (Barritt et al., 2001; Ferreira et al., 2010; Roberts, 1999). Spindle transfer in metaphase II oocytes or nuclear transfer at the zygote stage, which would effect an almost complete cytoplasmic exchange, has in fact been proposed and developed for circumventing the inheritance of human mitochondrial genetic disease and is shortly to be attempted in the first human patients (Craven et al., 2010; Tachibana et al., 2013). Considerable ethical debate over the application of nuclear transfer for mitochondrial disease prevention was conducted in the UK, including both the Human Fertilization and Embryology Authority (HFA) and Parliament. On 3 February 2015, the UK House of Commons approved a statutory instrument allowing clinics to apply for licensing for clinical mitchondrial donation by nuclear transfer (Commons Digital Outreach Team, 2015). Another rationale for early nuclear manipulation would be to move about or exchange nuclei in creating artificial eggs/early embryos for the purpose of "rejuvenation" – creating theoretically more functional cytoplasmically "younger" eggs for women of advanced maternal age or where no eggs can be obtained (Heindryckx et al., 2007; Liu et al., 1999). Considerable effort has been made in this area but this type of difficult inefficient manipulation has probably been made moot by the ability to create eggs from patient-derived stem cells (Hayashi and Saitou, 2014; Irie et al., 2015; White et al., 2012). As mentioned, an alternate protocol for "rejuvenating" human oocytes has been proposed using homologous ooplasmic injection. Technical hurdles still remain and the ultimate utility of these concepts will await future judgement. An intriguing option would be to use such techniques to create appropriate gamete combinations or artificial embryos for same-gender procreative couples (Woods and Tilly, 2012).

Embryo manipulation

Once fertilization and the initial developmental divisions have taken place, other micromanipulative utility remains prior to implantation. Jacques Cohen again suggested that opening the zona of cleaving embryos could facilitate the subsequent hatching process required before implantation (Cohen et al., 1990). Early experiments established the suitability of such manipulation, and clinical trials demonstrated that "assisted hatching" could improve implantation in some cases where the natural process might be compromised (Cohen

et al., 1992a, 1994; Malter and Cohen, 1989c). Assisted hatching has been controversial, with some trials failing to show any benefit, and no doubt there are patient-specific issues that determine if any benefit is to be had (Cohen et al., 1992b). In teaching assisted hatching for 20 years or more, I also felt that it was the most poorly/inaccurately performed procedure, particularly when acidified media were used, no doubt leading to negative effects in some cases. An ingenious extension of the hatching procedure developed by Mina Alikani involved a dual use for the hatching tool in removing excessive fragmentation debris at the time of hatching with some apparent benefit to subsequent blastulation and development (Alikani et al., 1999; Eftekhari-Yazdi et al., 2006).

Zona opening procedures have also been critical in the removal of cells for embryonic biopsies. Polar bodies, blastomeres and trophectoderm cells are all easily removed using appropriate aspiration pipettes once the zona is opened (De Boer et al., 2002; Handyside et al., 1990; Verlinsky et al., 1990). Each technique requires a unique zona gap, and as will be discussed below the beautifully controlled micromanipulative laser is the tool of choice. Trophectoderm biopsy is rapidly becoming the technique of choice when late-stage embryonic manipulation is allowed (Kokkali et al., 2007; McArthur et al., 2005). In this case, the zona opening is usually made earlier in development, and limiting the size of the zona gap is critical in setting up the desired artificial hatching event that facilitates separating an appropriate trophectoderm sample. The laser, again, is a critical tool in separating the trophectoderm biopsy from the remaining embryo, perhaps making a microscope laser an absolutely required tool in all ART laboratories.

Laser microsurgery

The use of lasers at the cellular level followed mechanical manipulation rather quickly (Berns et al., 1981). Laser energy can be used in a highly focused and accurate fashion basically to destroy and cut. This focused destructive power has proved a perfect solution to ablating sections of the zona pellucida for various reasons and has also been used to immobilize spermatozoa prior to ICSI (Malter et al., 2001; Montag et al., 2000). Initially, the use of a laser required that a large, cumbersome optical bench be set up adjacent to the working microscope to allow for the generation and introduction of laser light energy. A variety of lasers were used, including Nd:YAG (used in the first true "zona drilling") and ultraviolet Excimer lasers from which the first animals born from laser-ablated embryos were produced (Laufer et al., 1993; Palanker et al., 1991). However, the complexity of these systems and potential negative irradiation issues precluded regular clinical application. Infrared lasers, for instance the Er:YAG, became the tool of choice and first found their way into a human infertility programme (Feichtinger et al., 1992). As laser development and clinical utility advanced, small microscope-mounted units were developed based on pulsed infrared diode lasers operating at a 1.48 μm wavelength (Rink et al., 1994). This basic system, either mounted into the optical path or inside of the objective itself, became the clinical standard and forms the basis of the three principal clinical laser ablation units currently in use. Infrared laser energy pulses basically instantly ablate cellular material such as the zona pellucida. Energy is highly focused and the area adjacent to the pulse target site is affected only by localized heating resulting from the ablation event. Micromanipulator laser cutting systems include a specific indication of this zone of heating – varying based on pulse energy – to avoid interaction with adjacent embryonic cells.

Laser energy provides a seemingly perfect methodology for cutting or removing sections of the zona pellucida. This has provided a great improvement over the prior chemical or mechanical-based protocols (Boada et al., 1998), which has been particularly valuable in biopsy scenarios where the requirement for the secondary "cutting" tool can be alleviated (Montag et al., 1998). Clinical laser units can be adjusted very precisely to create a wide range of ablation outcomes well within the requirements of all clinical protocols. Another common use of the laser is in facilitating blastocoele collapse prior to cryopreservation via a single intercellular aimed pulse (Mukaida et al., 2006).

The current clinical micromanipulation laboratory

Clinical techniques – guidelines

We will now turn to a review of the current aspects of the human clinical micromanipulation laboratory, beginning with a discussion of the main techniques. Mature versions of several of the micromanipulation techniques described have been standard daily procedures for all human clinical embryology laboratories for many years now. A didactic exhaustive description of these is not required, but some comments are perhaps useful.

The most common current technique is certainly ICSI. Many clinics no longer inseminate oocytes and use ICSI on all cases. This can provide some benefit in ensuring fertilization is achieved (though by no means a guarantee) and streamlines and regiments the daily activity – particularly useful for large, busy operations. On the other hand, ICSI is certainly not required in a large percentage of cases and, for modest laboratories, avoiding the need to prepare and inject eggs has some value. ICSI is performed in a wide variety of individual fashions including variation to the control of the injection pipette (precise oil-filled microsyringe systems to mouthpieces), tool angle and method of membrane breakage. In surveys of ICSI, these variations would not seem to be associated with significant differences in success or outcome (Joris et al., 1998; Malter and Cohen, 2002). Critical factors that have been identified include the proper immobilization of the sperm cell and ensuring that oocyte-membrane breakage, and hence true ICSI, takes place. The specific nature of the ICSI technique will vary between individuals no matter how regimented their training or adherence to laboratory-specific rules. Therefore ICSI success by individual embryologists should be regularly monitored to ensure that reasonable fertilization rates (at least 75 – 80%), very low egg degeneration rates (at most ~3%), and appropriate embryonic development and outcome rates are achieved. Modifications of the ICSI technique have used laser assistance in both sperm immobilization and zona breaching. (Abdelmassih et al., 2002; Ebner et al., 2001; Moser et al., 2004; Rienzi et al., 2001).

Assisted hatching is another common clinical technique. As discussed, most clinics are now using infrared lasers as the cutting tool for zona opening, and this essentially "point-and-shoot" methodology has greatly simplified things. The use of acidified Tyrode's solution requires considerably more care and again is, in my experience, frequently an ill-performed technique. Proper acidified media zona drilling really requires the use of a mouthpiece to provide the immediate control that even the most carefully set up microsyringe cannot duplicate. The flow of media must be "turned on" and immediately and aggressively directed against the zona to produce efficient, quick dissolution and then instantly stopped when the desired effect is achieved. Immediate aspiration, so easily achieved by well-trained mouthpiece users, can remove excess acidified media from the area adjacent to the embryo and zona gap. Tool positioning/geometry including the critical "focus" position is extremely important in acidified media drilling and ill-positioned tools can spell disaster. The incorrectly "aimed" tool will simply direct the flow of acid media in the general area without the desired effect on the zona – and no doubt negative consequences on the embryo. Another important factor for both acid media drilling and laser ablation is the size of the gap created. Proper assisted hatching requires a relatively large gap in the zona to bring about the desired "assistance" when blastocyst expansion and hatching begins. As was well demonstrated during the development of the technique, very small gaps in the zona can in fact be detrimental, resulting in partial hatching (that may ultimately not be resolved), the trapping of blastocyst tissue in the gap and in some cases the complete separation of trophoblastic vesicles (Malter and Cohen, 1989c). This is one aspect where acidified media drilling may be more forgiving, as it results in "softer" edges to the gap itself where the chemical dissolution of the zona fades away to unaffected tissue. Laser ablation results in gaps that are delimited by stiffer heat-modified zona edges. This brings to mind the use of laser ablation to create very limited zona gaps of a few micrometres in setting up for aberrant early hatching and the formation of herniated trophectoderm for D5/6 embryo biopsy. In this case, the precise control of the laser as well as the "stiff" nature of laser-generated gaps is of value in achieving the desired effect, allowing for easy aspiration and separation of trophectoderm biopsies. There are definitely differences between the two zona-opening methodologies, but both can be used successfully (Chatzimeletiou et al., 2005).

A final zona opening technique still in clinical use is simple dissection with a rigid sharp microneedle. In the absence of a laser, this can be a very convenient and quick method with a single properly-positioned zona dissection event sufficient for facilitating easy polar body biopsy or perhaps setting up for D5/6 herniation. For creating a larger opening for blastomere biopsy a cruciform double-dissection can be performed by creating a single gap, positioning this gap at the '12 o'clock' position and then piercing the zona across this gap from side to side to create a second tear in the zona at approximately 90 degrees to the first gap (Cieslak et al., 1999). One aspect to consider in zona opening is the very existence of the opening. Eggs and embryos with opened zonae present a real and present danger in further handling. Obviously a holding pipette can quickly and efficiently aspirate the zona contents when an existing gap becomes inadvertently lined up with its suction orifice. Also the ICSI procedure, where the zona is compressed by the microneedle as injection begins, can cause expulsion of oocyte material for instance from a gap created for first polar body removal. Care needs to be taken in the positioning of these gaps and an awareness of gap position is critical in all subsequent handling and micromanipulation procedures.

Once the zona is opened, oocyte or embryo biopsies are easily accomplished using appropriately sized microtools. Polar bodies can actually be removed using a single-step method with a sharp bevelled tool of appropriate size, which can directly puncture the zona in the appropriate orientation for subsequent aspiration of the polar body. Cellular aspiration for biopsy has traditionally been performed using the handy mouthpiece control system. For those wishing to avoid mouthpiece use, slowing down the aspiration/expulsion speed of the fluid inside of the biopsy pipette can be critical in facilitating efficient successful biopsy. In this case, the biopsy tool can first be back-filled with a viscous fluid such as high viscosity (Dow 200 60,000 centistokes) silicone oil using a simple syringe filled with the fluid and connection via appropriately sized tubing to fit the typical ~1 mm outer diameter microtool. The filled tool is then placed in the holder and the flow of the viscous solution adjusted in a separate drop prior to actual manipulation. The flow rate of such fluid within the microtool is considerably reduced over media or oil and any well-maintained ICSI-type microsyringe can produce exquisitely slow flow control for pulling/expelling biopsied cells or trophectoderm tissue sections. Silicone fluid is toxic and at minimum will lyse cellular material on contact, but this is easily addressed by drawing up a column of handling media and/or oil in the terminal section of the biopsy pipette that the biopsy material will potentially contact. With care, expulsion of the viscous loading fluid into the actual biopsy drops is easily avoided.

Teaching micromanipulation techniques

Micromanipulation techniques present a unique challenge in the training of new staff members or the introduction of new clinical procedures. Immediately following the first successes at human clinical micromanipulation, outreach was attempted in extending these techniques to the human ART community through workshops, symposia and on-site consultation. It became quickly and gratifyingly evident that any reasonably skilled embryologist could quickly learn and perfect the required techniques. In our early attempts at workshop-based instruction, going back to 1990, it is interesting to note that the micromanipulation equipment companies wanted nothing to do with us and the human clinical milieu owing to fears about regulatory/liability issues, and plainly stated that their apparatus was not for clinical use. We were forced to drag our own early micromanipulation set-ups to the classroom in order to facilitate instruction. In a few short years, the clinical success of micromanipulative techniques led to a massive expansion of micromanipulators into every human clinical laboratory, changing this situation quite rapidly.

Instruction must start by imparting a solid basic understanding of all components of the micromanipulation microscope and systems. Mastery of the necessary techniques is of course important, but this will be of little use to

embryologists unable to correctly address operational issues that prevent the proper use of the equipment and potentially endanger precious clinical material. Trainees must first understand exactly how the micromanipulators and peripheral equipment operate and the many ways in which the three-dimensional position and angle of the microtool is controlled. A frequent trainee error involves attempting to use manipulators that are at the limit of their fine control range and must be coarsely re-centred to allow function. A similar error involves using a microsyringe at the limits of its range where the proper degree of aspiration and expulsion is not possible. The next aspect to be mastered is setting up for particular procedures, including the handling and loading of the microtools and establishing their correct position for use. Again, a thorough understanding of all that is involved, including critical aspects of the microscope focus, positioning of tools for dish loading, tool angle and alignment, must be achieved rather than relying on some rigid, rote formula for setting the system up. In most laboratories, equipment is being used by multiple individuals and so changes to the microscope may occur that confound such rote set-up protocols. The aspects affecting tool focus in particular must be well understood to avoid lengthy adjustment scenarios and/or broken tools. Obviously, laser manipulation is another critical area where a thorough understanding of all operational aspects, including the optical path, video imaging and computer software control, is critical before actual technique training begins.

One issue in teaching manipulative technique faced in the training of new embryologists is the non-clinical training material to be used. Obviously rodent or other model system eggs/embryos are available and are certainly suitable for basic handling aspects. However, mouse eggs/embryos, perhaps the most available non-human mammalian material, exhibit considerable differences rendering them rather unsuitable for perfecting most human techniques. The mouse zona is less substantial and does not behave in a similar fashion to dissection and manipulation. The injection of mouse eggs with an ICSI pipette simply results in instantaneous death, although hamster eggs may be more suitable (Gvakharia et al., 2000). Mouse eggs can be used to teach basic holding pipette operation and are quite suitable for laser ablation training. Mouse eggs, cleaving embryos and blastocysts are also useful in learning the basics of polar body, blastomere and trophectoderm biopsy, although the latter is compromised by the relatively flimsy nature of the mouse zona pellucida. Fortunately, human ART clinics produce a variety of discarded human eggs and embryos (those exhibiting immaturity, abnormal fertilization or other serious abnormalities) that can be readily used for training with proper patient consent in place. This fresh discarded material is unfortunately often of compromised developmental quality. However, another obvious source is patient-discarded frozen material. This usually includes a range of good-quality embryos of various stages and is particularly useful in training in trophectoderm biopsy. Learning the very critical sperm handling techniques required for successful ICSI simply requires time and a sample of motile spermatozoa. As in the training of all clinical techniques, proper documentation of trainee participation and performance is essential and in most situations a regulatory mandate. Once sufficient skill has been demonstrated, trainees can begin to divide actual clinical treatment events such as ICSI, hatching or biopsy (i.e. perform the procedure on a percentage of the available material with an experienced embryologist supervising and performing the remainder) before moving on to full clinical proficiency. It should be noted that depending on local ethical oversight and regulations, for instance via the HFEA in the UK and National Health and Medical Research Council in Australia, specific patient consent, ethical approval and proper licensing is required for the use of any human gametes and embryos in such teaching scenarios.

Clinical micromanipulation equipment and tools

As discussed above, the basic clinical micromanipulation techniques are necessary components of human infertility laboratory treatment and so the required instruments and tools are very much standard equipment. In this area, not much advice can be provided. Several commercial instrument suppliers produce appropriate micromanipulators and microsyringes that can be mounted to all major inverted or fixed-stage microscopes and used for ICSI and embryo biopsy. Having used all commercial micromanipulator systems for human clinical work, I would suggest that all are equally suitable, perhaps with minor individual benefits and disadvantages. Those embarking on the purchase of a new system for the first time would certainly do well to at least try the various competing systems to determine if one provides a better "fit" to their own needs. Also, there is no reason why some system components, for instance microsyringe controllers, cannot be obtained from a different manufacturer to complete the necessary micromanipulation suite. I would suggest that particularly if trophectoderm biopsy is to be routinely performed that a micromanipulation laser system is also absolutely mandatory. Again, there are several competing systems, all of which offer excellent clinical utility in my experience. Further comments about the maintenance and quality control of clinical micromanipulation systems will be provided in a separate section below.

Clinical microtools are of course also readily available from several suppliers – again all providing good quality and utility in my experience. Commercial tools are sterile, tested and certified as tissue culture devices and have provided a huge advantage that has helped clinical micromanipulation to expand. In the past, a great percentage of micromanipulation workshop time was used in teaching the art of microtool fabrication and every clinical laboratory had to produce their own tools. This required considerable time besides the considerable expense of purchasing and maintaining the required toolmaking equipment (needle pullers, microforges and grinders). As with the micromanipulators themselves, commercial microtools from the various manufacturers have their own individual characteristics, and embryologists are encouraged to try out the various tools available to determine if individual types provide any advantage to their own work.

Maintenance and quality control

Like all other critical laboratory equipment, micromanipulation-related devices require regular maintenance and

monitoring for proper function. Most mechanical-based micromanipulators are usually very reliable and simply require monitoring for any loss of control or aberrant function. Mechanical components such as gears and drive knobs can become worn from repeated use and require replacement. Hydraulic force systems can develop leaks that reduce or abrogate function. Fortunately, these can also be individually replaced. Electronic devices (stepper-motor based manipulators, lasers and other such components) are also very reliable but do require regular function checks and should be checked periodically by an appropriate technical expert. Microsyringe systems (syringe, connectors, tubing and tool holder) particularly should be checked before every clinical use for proper fill level, excess bubbles, plunger position and the integrity of all seals. Some of the syringe/tool holder components, such as flexible plastic seals and o-rings, fail over time and require replacement. Spares for all of these components should be maintained on site and be available for replacement as needed.

Considering the great potential for destruction posed by laser devices, most laboratories require that proper laser function is confirmed before every clinical use. While new clinical laser systems include an active indication of laser targeting – a precise secondary coincident visible light source – older units lack this facility, and targeting is an essentially "blind" process based on the position of an arbitrary indicator such as an on-screen video-generated target. Unfortunately, this system can be subject to gross errors from slight changes in the positioning of the optical path components involved. For instance, slight rotation or movement of the associated video camera can greatly alter the apparent on-screen position of the laser target. For this reason, confirmation of laser targeting before any clinical use is highly recommended. This is easily accomplished using a glass coverslip covered with a film of dark-coloured ink, for instance from a "dry erase" type marker. A laser pulse will clearly ablate a small gap in the ink film and indicate the precise location of the target. As in all things, laboratory directors can best determine for their own operation which specific requirements and schedules for maintenance and quality control are needed. It is certainly prudent – and may be mandated in regulation – to also have redundant second or "back-up" units available for this critical equipment.

Future perspectives and conclusion

Every year brings slight changes in micromanipulation equipment and the repertoire of techniques. Some of these are simply marketing-based changes and the basic operation and principles of most equipment would seem to be essentially mature. However, no doubt further improvements are possible. For example, micromanipulation laser systems have seen several substantive improvements, including visible targeting indicators and the ability to automatically direct the laser pulses along defined paths. As the areas of computer control, robotics optics and micro-fluidics come together, it would seem that new "automated" methodologies could be developed. Recently a fully "automated" system for performing ICSI was developed that included automated sperm capture and immobilization, simultaneous positioning of multiple eggs and successful intracytoplasmic injection (Lu et al., 2011). One concept I feel could be pursued is the automated screening and selection of individual sperm cells based on fine morphology or other metrics and the delivery of "approved" cells to the ICSI system through micro-fluidics. This is a very exciting time for human clinical micromanipulation, over the next few years we will hopefully see both a human nuclear transfer technique (for circumventing mitochondrial disease) and an oocyte "rejuventation" technique via cytoplasmic injection become clinical realities (Russell and Turnbull, 2014; Woods and Tilly, 2012). As has been common throughout the history of micromanipulation in the human clinical laboratory, both techniques have been subjected to considerable criticism and controversy. Certainly there are critical issues of safety and efficacy that need to be addressed. It has been particularly gratifying to observe the situation in the UK in which the HFEA has acted to support prudent ART research by protecting both clinicians/researchers and the patient population they serve (Leese and Whittall, 2001). Human embryologists and clinicians have an obligation to develop the tools and techniques that will continue to solve biomedical challenges for our patients, and micromanipulation will continue to play an important role in this pursuit.

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