TECH-2-TECH

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Ultrasound to ultrasee – the use of ultrasound to determine pregnancy in mice

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Abstract

Several MRC Laboratory of Molecular Biology (LMB) groups require early-stage mouse embryos for their scientific research programmes. Traditionally, Animal Technicians have relied on visual checks or palpation of the abdomen to determine pregnancy in mice. In 2017 an ultrasound machine was purchased as the equipment would enable plugged mice to be scanned to confirm pregnancy prior to sacrificing, therefore, providing an increased accuracy to overcome the limitations of the existing methods. Therefore allowing females which do not appear pregnant to be mated again; a refinement to the current practice also reducing the number of animals used for experiments.

Initially, use of the ultrasound equipment allowed a 36% increase in determining pregnancy when compared with traditional methods between E.7.5 to E12.5. Given time and further practice the procedure was refined allowing successful detection of pregnancies at the earlier gestation periods of E6.5 and E5.5.

A successful in-house ultrasound training programme has been organised and there are now several competent technicians.

Introduction

Mice used by LMB researchers are housed in the MRC Ares breeding and experimental mouse barrier facility. All mice are bred in-house at the Ares animal facility under specific pathogen free conditions, at a temperature range of 20 to 24°C, humidity of between 45 to 65% and a 12 hour light-dark cycle. Animals are placed on Rettenmaier SDS RM1 diet with access to an automated watering system and are housed in

Tecniplast GM500 Green Line individually ventilated cages (IVCs)[1] with woodchip bedding, sizzlenest nesting, wood blocks, diamond twist and fun tunnel for enrichment.[2]

One of the research groups needed early time point embryos at E8.5. The females required for these experiments could have rare multi-allele genotypes i.e. only 1 in 25 pups could be the desired genotype. For these time points the traditional methods of pregnancy checking could not be applied, as visual checks are not possible until E12.5 and palpation until E9.5. Both methods are not accurate and can be subjective depending on who performs them. Palpating can also be quite stressful for females and can lead to further complications if not carried out correctly. Due to these limitations any timed mated mice for experiments requiring embryos at earlier time points were being sacrificed without the certainty of a successful pregnancy. This is undesirable as it led to unnecessary animal wastage. In 2017, an ultrasound scanner was purchased to refine the detection of pregnancies. Following a period of training and practice, successful pregnancy scanning was being routinely achieved down to E7.5 with great success. In 2022, new research needs required lower time points of E5.5 and retraining was undertaken to cover these time points.

The ultrasound device uses high frequency sound waves to create images of the inside of the body. The ultrasound travels through soft tissue and fluids then bounces back off denser surfaces. The denser the object the ultrasound hits, the more of the ultrasound bounces back. The bouncing back, or echo, is what gives the ultrasound image its features. Varying shades of grey reflect different densities i.e. bone appears whiter where tissues are greyer.

Some of the uses for the ultrasound in other facilities include blood flow measurements[3], tumour checks[4], organ imaging and embryo injections[5] however at Ares, ultrasound scanning is applied to confirm pregnancy or not. Mice are then either sent to the researcher for samples or experiments if pregnant and if not, they are reused for another mating as well as for checking the success of pregnancy using rotating males to speed up production.

Method

Often animals are required at a specific time point of gestation to meet the scientific needs. The most accurate way to do this is to arrange a timed mating. When a time point is established, the mating can then be arranged placing the two animals together until a copulation or vaginal plug (Figure 1) is found in the female and then the female will be separated from the male.[6] The time point when the plug is found is referred to as E0.5 (half a day) due to the nature of mice generally mating overnight. Despite the presence of a plug this does not confirm pregnancy.

Figure 1. An albino mouse with copulation plug.

There are ways of increasing the chance of a mating occurring over a required time frame and this can be done by either seeding the cage or oestrus checking the female.

Seeding the cage requires taking a small amount of male bedding and placing it in the female's cage a day or two before the mating. Smelling the scent of the male will trigger the female to come into oestrus.

Oestrus checking involves visually checking the female to determine at what point she will be in oestrus (Figure 2), or this can be determined by producing vaginal smears of cells under a microscope.[7] Although in this case visual checks are used as they are less invasive and this is the preferred method used within Ares.

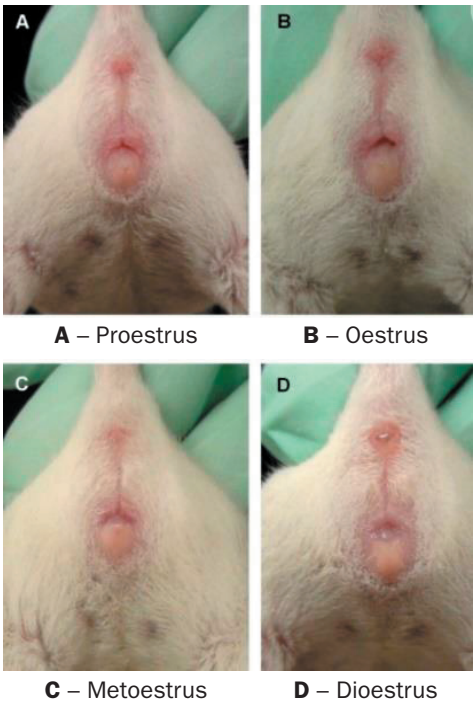

Figure 2. The four stages of oestrus.

Once a female at the correct stage of oestrus is identified she can be placed with the male where mating will usually occur overnight.

Oestrus checking is normally the quickest form of time mating but usually is most suited to wildtype lines. In most cases, a pool of wildtype females can be assessed to collect the animals required. When this involves transgenic animals, where a particular genotype is required, this can be harder to do making it a longer process.

The equipment used to carry out the scans is as follows: (Figure 3 and 4)

- mice – transgenic and wildtype *(Hsd:ICR-CD1)* lines
- 70% ethanol
- ultrasound gel
- disinfecting hand spray
- scanning probe suitable to species
- LOGIQ e ultrasound monitor[8]
- GE L8-18i-D hockey stick probe[9]

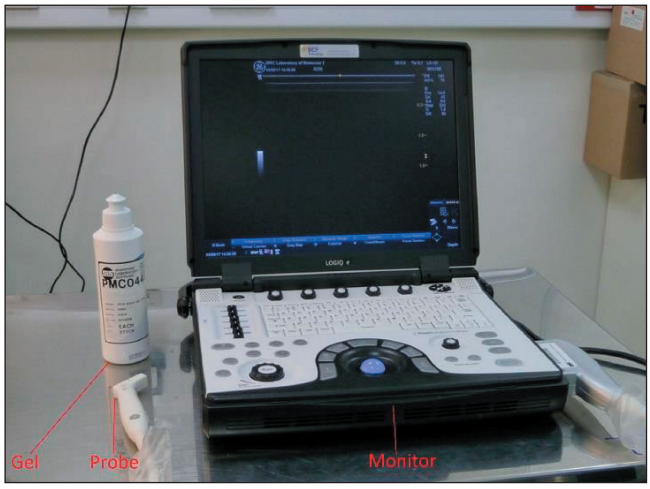

Figure 3. LOGIO e device.

Ares is a non-tail handling unit and using this method of handling further reduces the stress that could be caused to the female during the scanning process. The aim is to produce a clear scan that will confirm the presence of pups without keeping the female away from their cage too long. A researcher at the LMB with previous experience of scanning mice said that it was necessary to anaesthetise and shave the mouse abdomen. We have refined this by carrying out a normal scruff to restrain the mouse and then applying 70% ethanol to the stomach and smoothing down the fur to remove any air bubbles that may distort the image. Other solutions were tested to see if the percentage of ethanol could be reduced or eliminated, however it was found these were not as effective. Ultrasound gel (the same type as used on humans) is then applied to the abdomen and this is what provides the connection between the body and probe to allow the sound waves to pass through.

Figure 4. Set up required to perform a scan.

Two main types of scans are carried out which are the pelvis and spinal scans. The pelvis scan involves placing the probe horizontally to give a clear view of the pelvis and then the probe is turned vertically to give a spinal view. Once these are done, the female is cleaned as much as possible before being placed gently back into the cage. Due to the mice not being anaesthetised and the short time the process takes, the mice show no signs of stress or exhibit behavioural changes.

On average the whole process from start to finish takes around 50 seconds. This can vary depending on the size of the mouse and gestational time point.

The pelvic scan (Figure 5) is a scan showing the method used to produce a pelvic image.[9] Gentle pressure is applied across the top of the hips.

Figure 5. Pelvic scan using hockey stick probe.

The image in Figure 6 is produced when using this method. In the centre of the screen, the pelvis can be seen with left and right femurs branching off at each side. When searching for pregnancy, the areas above the pelvis are looked at usually to the left and right.

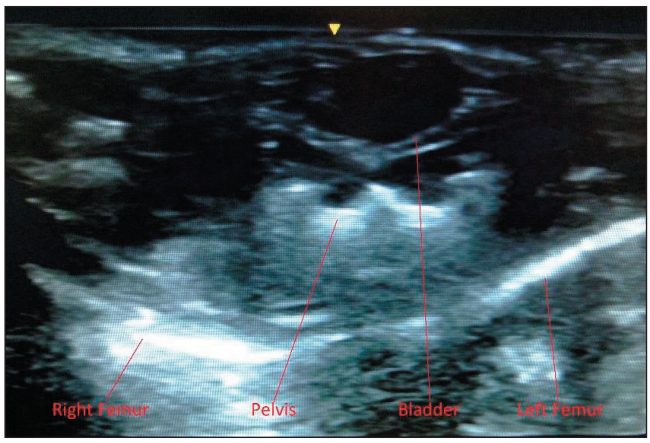

Figure 6. Ultrasound image of pelvic scan.

The next angle is the spinal view and to achieve this the probe is held vertically which allows the side profile of the spinal column to be seen (Figure 7).

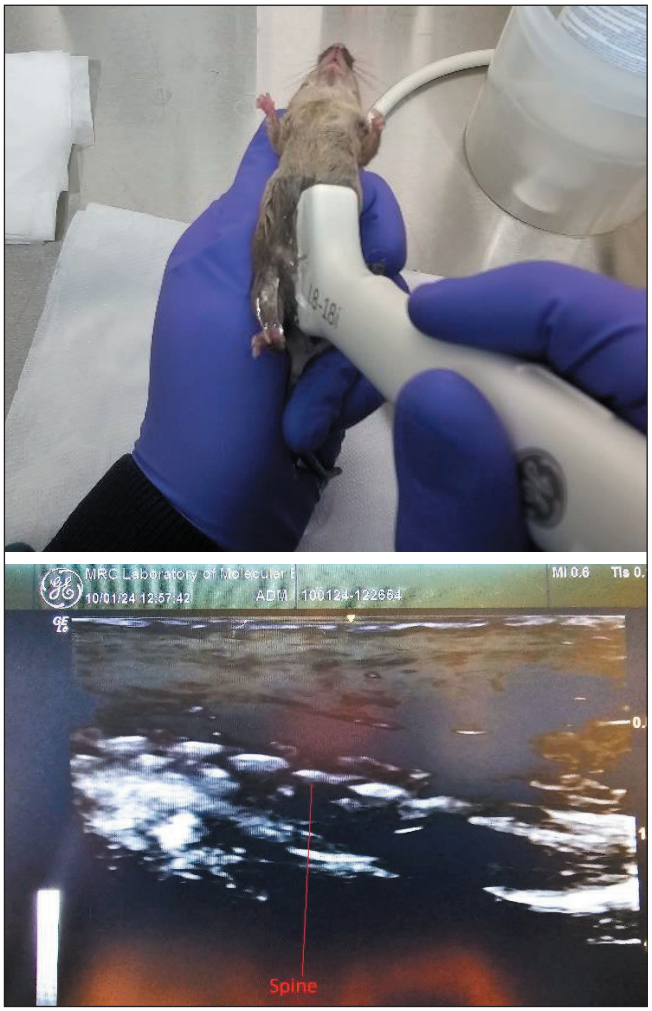

Figure 7. Spinal scan position and ultrasound image of a spinal scan.

When completing a scan, both of these angles are used as this allows for confirmation of what has been seen with no further restraint to the animal.

We have completed scans over a range of different time points. Below is a time line of different gestational stages for a mouse, and shows the difference a day can make (Figures 8- 15).

Figure 8. Day 5.5 – The earliest time point successfully scanned.

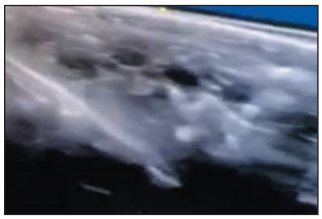

Figure 9. Day 6.5 – Cavities appear empty but located high.

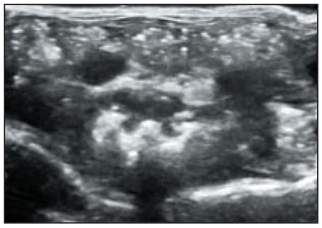

Figure 10. Day 7.5 – Multiple round empty cavities can be seen closer to the pelvis.

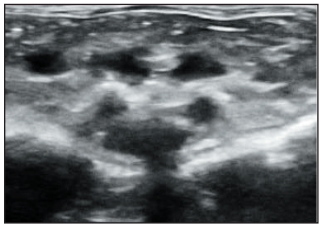

Figure 11. Day 8.5 – Cavities increase in size but are still empty.

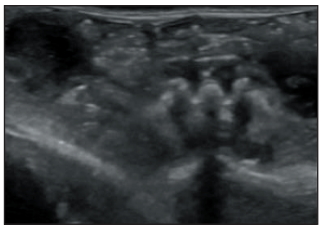

Figure 12. Day 9.5 – Cavities larger with some greying mass starting to appear.

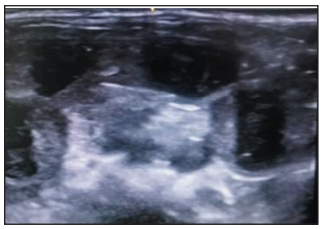

Figure 13. Day 10.5 – Bone mass has started to develop and can be seen in pale grey – heartbeats can be seen as a slight flicker.

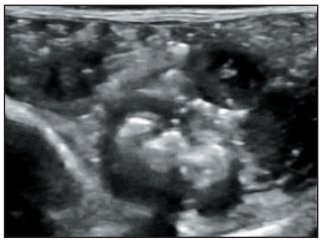

Figure 14. Day 11.5 – The pups' bones become denser and can be seen more clearly.

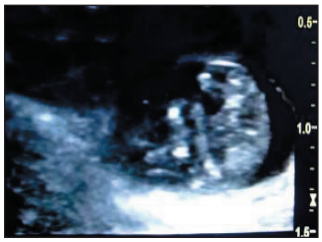

Figure 15. Day 15.5 – Pups appear fully formed. Eye sockets can be seen along with the rib cage and limbs. Pups can be seen moving at this point.

Results

During 2018, data was collected (Figure 16) and scans carried out between and including E7.5 to E12.5. From these scans 91% were correctly identified as pregnant or not pregnant. The animals that were not pregnant could be reused for future time matings, 2% were incorrectly scanned and kept as not pregnant but went on to have litters which were used for colony expansion with the females being reused for time matings when cleared. 7% were scanned incorrectly and determined as pregnant. Based on the time points of these animals before the ultrasound 45% would have been used regardless as there would have been no way to confirm pregnancy and only 55% could have been visually checked or palpated but as already mentioned these methods are not ideal and this means by scanning the animals, accuracy has increased by 36%.

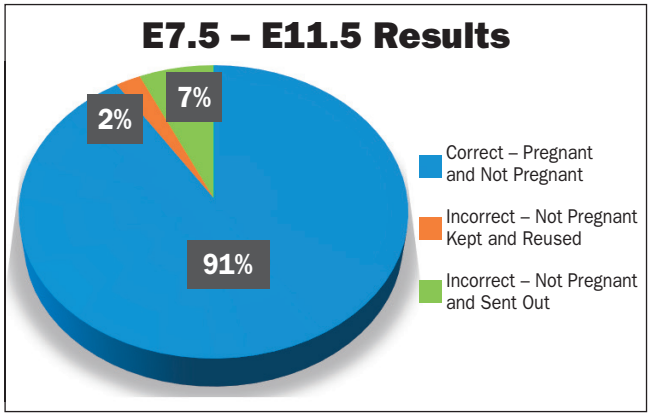

Figure 16. Data of scans completed between E7.5 and E11.5 gathered in 2018.

Moving on to 2023 when new research required embryos at E5.5 and E6.5, data was collected (figures 17 and 18) from 244 scans at these time points. 85% were scanned correctly as pregnant and not pregnant, again allowing the reuse of animals that were not pregnant. 12% were scanned incorrectly as pregnant and used unnecessarily. However before the use of ultrasound scanning was introduced all these early time point animals would have been used regardless with 41% of these were not pregnant. Ultrasound scanning has decreased unnecessary sacrificing of valuable animals by 29%. During this period scanning at these earlier time points was still being perfected, therefore moving into 2024 the aim is to see accuracy increasing further.

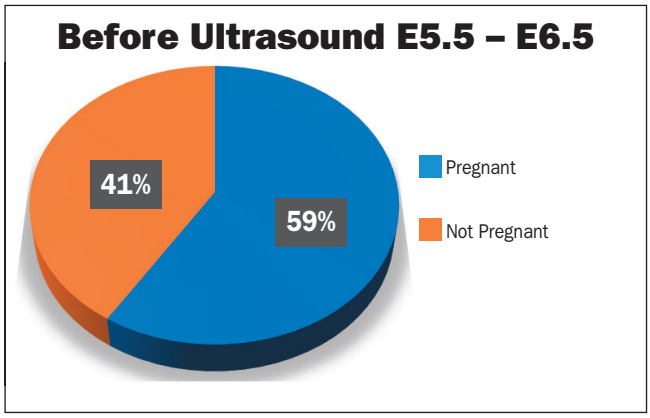

Figure 17. Percentage of animals pregnant and not pregnant that were used for experiments needing embryos at E5.5 and E6.5.

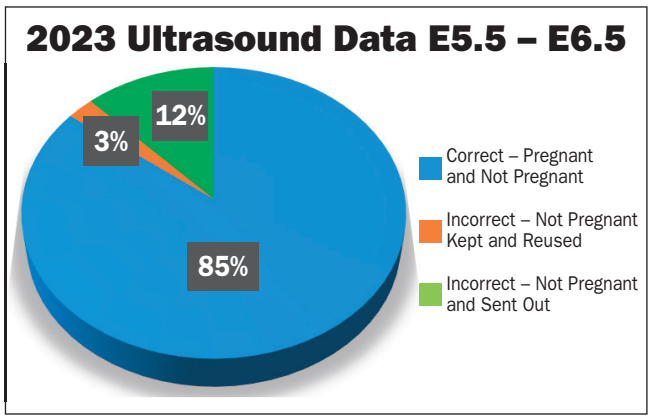

Figure 18. Data of scans completed between E5.5 and E6.5 gathered in 2023.

Discussion

When the ultrasound was first purchased, the basics of how to use the machine and how to find and use the pelvis as a focal point were learnt. After this there was no external training available, self-learning was required to identify what the embryos looked like on an ultrasound, starting with visibly pregnant time mated animals. Once each embryo time point was perfected and confidence gained, earlier days were worked on by decreasing one day at a time. This continued until E7.5 was reached and this initial training took around 6 months. When the need for earlier time points arose, the same process was followed until pups could be identified consistently at E5.5.

All training is held in-house and the same pathway that was originally used is followed with new trainees, starting at E11.5 and as confidence and competence is achieved, the time point is lowered. Once trainees can complete a scan at E8.5 competence is assessed as this is the lowest commonly used time point. Training plans need to be tailored to the individual as some trainees can analyse the screen image well whereas others are stronger at scanning and some take that bit longer to connect the two parts of the process. One thing that is the same for all trainees is that consistency is the key. Regular practice must take place to build up skill and confidence. Trainees could be ready for assessment after around two months.

When carrying out scanning, a mixture of transgenic and wildtype mice were used. More commonly in the wildtype CD1 line, it has been found that embryos from these

animals appear larger at the point of scan with more cavities present than those of the transgenic animals, although larger litters are more common for this line. As a consequence of these larger embryos, this can sometimes lead to incorrect assumptions that the embryos are at different time points from expected. Confirmation of the correct time points is made at the time of dissection when embryo development is assessed.

When handling animals for scanning, if often appears that those which are pregnant display behavioural differences from those that are not. Although all animals are handled using non-tail handling methods to reduce stress, these animals can appear more aggressive. Considering this behaviour combined with the observational evidence from the scan can help in pregnancy diagnosis. Behaviour varies from strain to strain but aggression does seem to be more prominent in transgenic lines compared to wildtypes.

The use of ultrasound scanning for pregnancy has also allowed the animals to be viewed for health checks. On occasions animals have been observed to show signs of a blocked bladder, observed as a very large dark cavity in the centre of the screen. Another heath concern which has been observed has been fluid filled kidneys. This again appears as a very large cavity but on one side of the animal. These animals have not presented any obvious signs of ill health at the time but have been able to provide an early warning to the research group before they make use of the animal.

Ares have been very involved with the development of this idea and rate its use but also understand the need to be mindful of the caveats.

- It is not possible to accurately count numbers of pups.
- It is not possible to accurately determine gestation without a timed mating but can estimate.
- Overweight animals are more difficult to scan.
- Cost of equipment.

But despite this, the 3Rs benefits far outweigh the limitations.

Pregnancy can now be confirmed at earlier time points and prevent animals from being unnecessarily used. This process itself is far less invasive and it has been possible with time and practice to make further refinements and it is no longer necessary to use anaesthetics or shave the animals.

In regard to pup numbers an estimation can be given which allows the opportunity to hold animals back if a litter appears small with the hope of a larger litter with the second pregnancy. This together with holding back non-pregnant females has enabled the reduction of the number of females unnecessarily used which in turn reduces numbers bred for use in these experiments.

Sometimes the use of animals of the correct genotype can be limited and selection of age-appropriate animals can be difficult. In these cases, animals are more commonly overweight causing the visibility of the pups to be difficult to see. However in these cases the settings can be adjusted on the ultrasound machine. The depth of the sound waves can be increased, also known as the gain and this can aid bringing pups into focus through the fat tissue of the mouse. Where possible the use of younger animals is more appropriate and steps have been taken to advise better colony management such as assisting with the selection of younger females where success rates are greatly improved.

This project has been expanded beyond the original expectations and is now used across the whole facility. The technique is a far more accurate way of highlighting pregnancy and a successful in-house training plan has been put together expanding our number of competent staff.

We have been extremely happy with the progress of our work using ultrasound scanning to increase the rate of successful pregnancy detection in timed mated mice and will strive to continue to develop this further and promote its use, especially as it is a great 3Rs success, incorporating a fantastic refinement of a traditional procedure, increasing our success rate of pregnancy determination and therefore enabling a considerable reduction in the numbers of mice sacrificed unnecessarily.

In the future, further work is planned to explore if the numbers within a litter impacts the visibility of embryos visualised using the ultrasound scanner. Data will be gathered of numbers of embryos collected for research studies and then compared to see how numbers impact visibility.

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