Recipient Mice

With methods developed and tested for the isolation, preparation, fluorescent labelling, and irradiation of the donor splenocytes, the next stage in the development of the *in vivo* method was the passive transfer of the donor cells into the recipient mice.

Administration

The most common route of administration for adoptive transfer of splenocytes/T lymphocytes into mice has long been intravenous injection via the lateral tail vein (Sprent and Miller, 1973; Butcher and Ford, 1986; Albright *et al.*, 1998; Miller *et al.*, 2002). Injecting into the tail allows direct access to the circulation without the need to shave the skin to expose the vein (c.f. Saphenous vein on hind limbs) and the lateral tail vein can be easily dilated by increasing the ambient temperature. Comparisons between routes of administration have shown similar tissue lodging kinetics between tail vein and retro-orbital injection of thymocytes in mice, with the main drawback of either intravenous method being the first-pass loss of cells to the lungs (Price *et al.*, 1984).

Injecting cells into the lateral tail vein can be performed without anaesthesia, with the aid of a physical restraining device (Marshall *et al.*, 1994). However, after the initial pilot experiments conducted using a physical restraint, it was decided to continue the tail vein injections using an anaesthetic as a chemical restraint to assist in the accurate delivery of the cells and to reduce stress on the recipient animals, known to induce profound changes in gene expression in mouse lymphocytes (Flint *et al.*, 2005), to alter lymphocyte homeostasis in the spleen (Wei *et al.*, 2003) and compromise immune function (Tournier *et al.*, 2001). The inhalant anaesthetic

isofluorane (*VCA I.S.O.*, Veterinary Companies of Australia Pty Ltd, NSW, Australia) was chosen to induce and maintain anaesthesia during the injection procedure (Loepke *et al.*, 2006). In human patients undergoing isofluorane anaesthesia during minor surgery, no changes in white blood cell counts or immune depression is seen (Durlu *et al.*, 2002) whilst isofluorane anaesthesia on normal healthy human subjects not undergoing surgical procedures has been reported to have only transient and minor effects on immune function (Procopio *et al.*, 2001).

Method:

Recipient mice were warmed to dilate the tail vein by placing the cage within 30 cm of a 175 W infrared heat lamp (Koninklijke Philips Electronics N.V., the Netherlands) for no more than 15 min (*Figure 4.14*). Anaesthesia was induced by briefly placing the mouse on a mesh plate above Isofluorane-soaked cotton wool contained inside a Bell jar. As soon as the mouse was still, it was transferred to an anaesthetic apparatus (*Figure 4.15*) delivering a continuous isofluorane/oxygen mix (isofluorane 2–3% in oxygen at 2 $L.min^{-1}$). Using a tuberculin syringe (29-gauge needle) 200 µL of donor cell suspension was injected into the lateral tail vein towards the distal end of the tail. Any bleeding was stopped by applying pressure with tissue paper to the injection site. The anaesthesia was then immediately withdrawn and the mouse was closely monitored until normal breathing and activity resumed. Recipient mice were returned to their original cages where they remained until the end of the experiment.

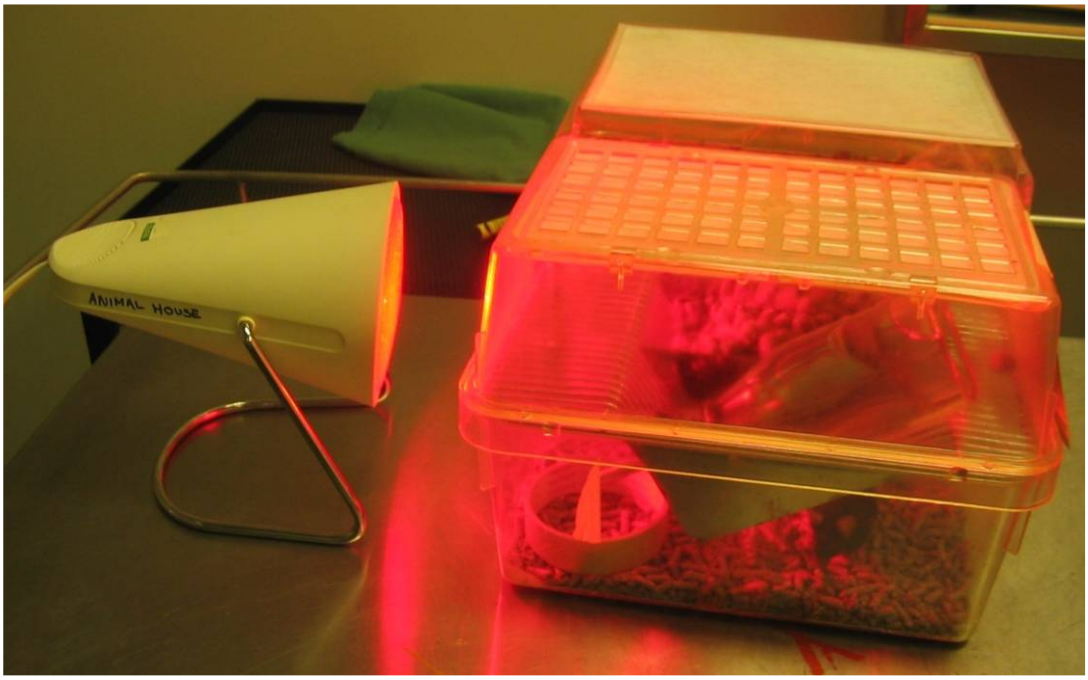

Figure 4.14: Pre-warming of cages to dilate mouse tail veins

Cages were placed within 30 cm of an infrared heat-lamp to warm the cages to assist in dilating the mice's tail veins. Mice had access to water *ad libitum*.

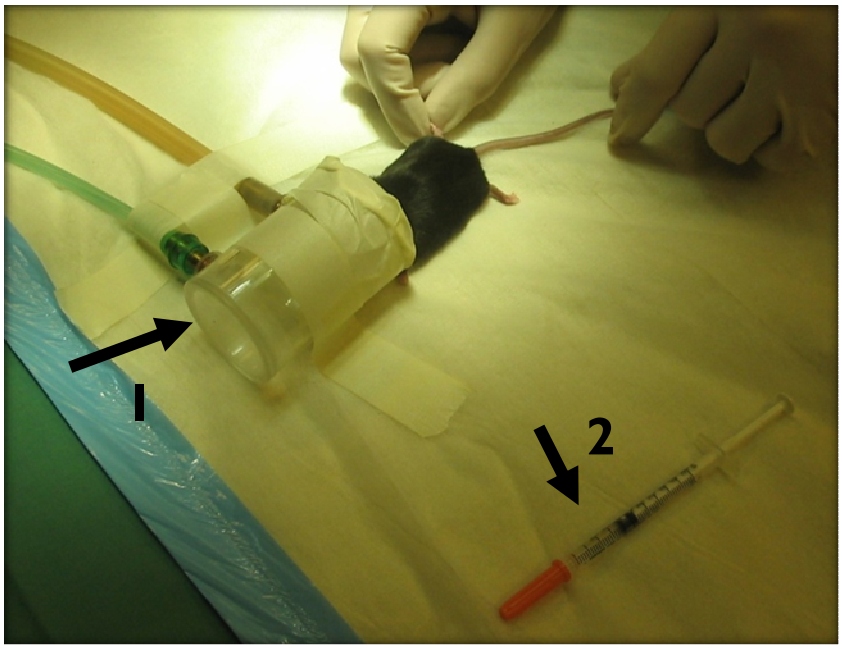

Figure 4.15: Recipient mouse tail vein injection setup

Recipient mice were anaesthetised with 3–4% isofluorane in oxygen using a custom-built anaesthetic delivery system (arrow 1) with vacuum scavenging. For each mouse, 200 µL of the donor cell suspension was injected using a 29-gauge needle (arrow 2).

Tissue harvesting and storage

In the experiments presented here, only spleen tissues were examined from the recipient mice; however, liver and bone marrow were also routinely collected for future analysis. Spleen and liver tissues were stored frozen and unfixed to preserve antigen-reactivity and enzymatic activity, whilst the bone marrow cells were stored fixed to preserve morphology.

Method:

At the end of the *in vivo* lodging period, recipient mice were euthanised by CO_2 asphyxiation. An incision was made through the skin and peritoneal wall to expose the abdominal cavity and the spleen and left-anterior lobe of the liver were surgically removed. The tissues were embedded in cryoprotectant compound (Tissue-Tek® *O.C.T. compound*, Sakura Finetek Europe B.V., the Netherlands) within a labelled plastic mould. The moulds containing the embedded tissues were immediately snap-frozen on dry-ice and then stored at -70°C within an airtight bag (each mould individually wrapped in plastic film) or at -20°C prior to histological processing. The right hind-leg of each mouse was removed and the femur dissected out. The ends of the bone were removed with a scalpel blade to expose the marrow, which was flushed out with 2 mL of sterile PBS using a 21-gauge needle. A single-cell suspension was prepared by repeatedly flushing the dislodged bone marrow through the needle until no visible debris remained. The 2 mL of bone marrow cell suspension was then added to 2 mL of 4% formaldehyde (from paraformaldehyde in PBS) in a 10 mL plastic tube and stored at 4°C.

Pilot adoptive transfer experiments

The individual protocols developed for the adoptive transfer method were tested in a series of pilot experiments to ensure that the various steps: isolation, culture, radiolabelling, fluorescent labelling, administration and *in situ* identification of donor cells, worked together. Many of the parameters, such as: lodging kinetics, expected lodging frequencies and the required radioactivity of radiolabelling medium, did not need to be ascertained independently, since they could be derived from adoptive transfer/radiolabelling protocols in the literature. However, it was necessary to confirm that the parameters chosen were suitable in this system and that the system behaved as expected.

Following the protocols described above (chronic radiolabelling) donor T lymphocytes were prepared and sampled to confirm fluorescent and ^{3}H-thymidine labelling. The donor cells were brightly labelled with the CMRA fluorescent probe and the majority were successfully radiolabelled (*Figure 4.16*). When 5×10^{5} radiolabelled donor cells were injected into recipient mice, lodged donor cells were identified in the spleen by their CMRA fluorescence 22 h later (*Figure 4.17*). The majority of donor cells lodged in the spleen also showed ^{3}H-thymidine radiolabelling *in situ* by autoradiography (*Figure 4.17*). When the spleen sections from the recipient mice were examined by fluorescence microscopy, CMRA-positive donor cells were mainly observed loosely clustered around periarteriolar sheaths, although isolated cells were also visible (*Figure 4.18*).

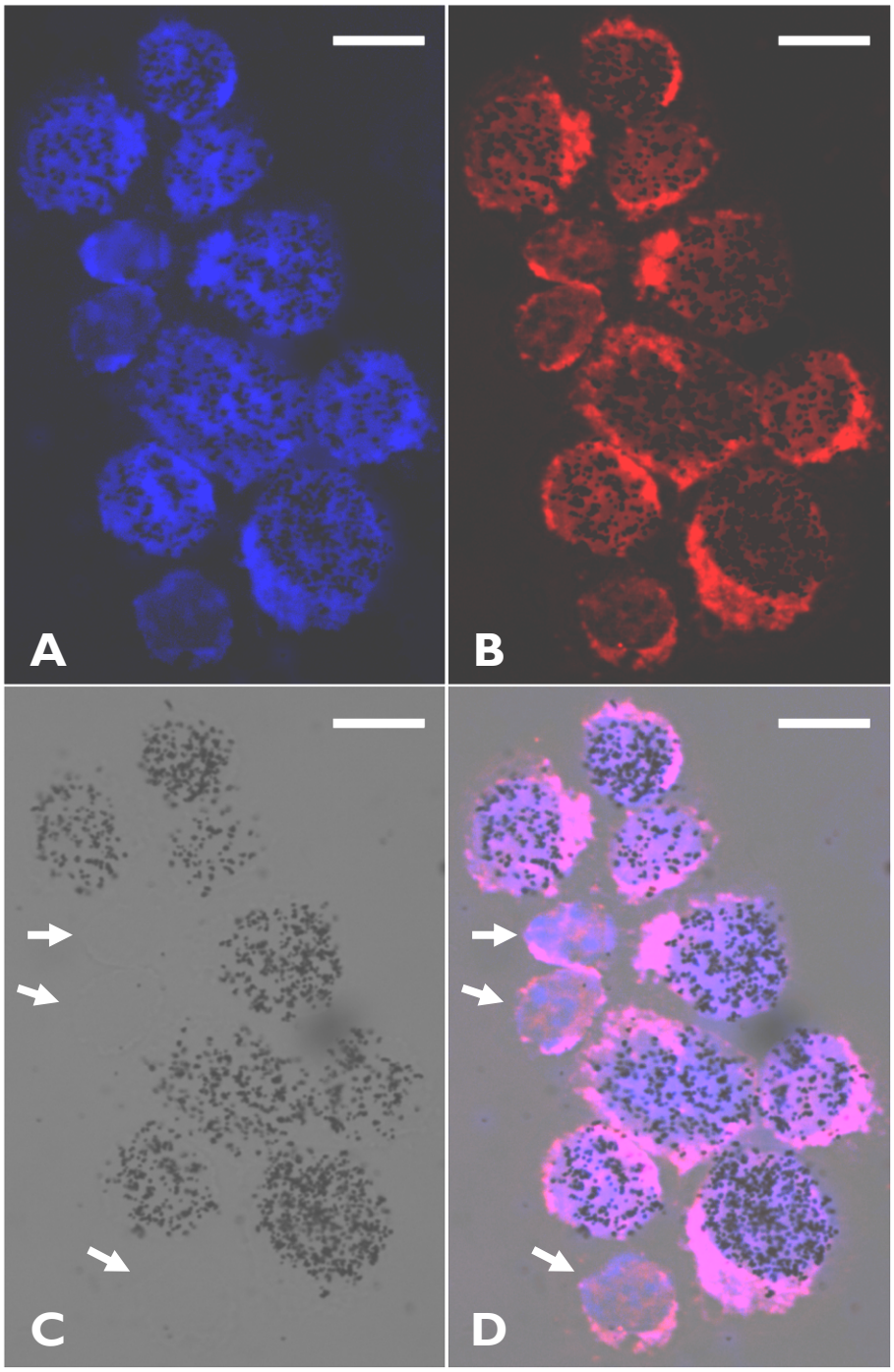

Figure 4.16: Radiolabelling and fluorescent labelling of donor cells prior to adoptive transfer

After 25 h in culture, donor T lymphocytes were incubated for 18 h with 1.48 kBq.mL^{-1} [^{3}H-methyl]-thymidine and 2.5 µM CMRA and then a sample of the cells were processed for autoradiography. (A) Donor cells viewed via DAPI nuclear counterstain under fluorescence microscopy (blue); (B) Same field viewing CMRA labelling under fluorescence microscopy (red); (C) Autoradiography grains viewed under brightfield microscopy; (D) Pseudo-coloured overlay of autoradiography grains (greyscale), CMRA fluorescence (red) and DAPI counterstain (blue). Non-radiolabelled donor cells are marked (arrows). Images were photographed with 100× objective lens. Scale bars each show 10 µm.

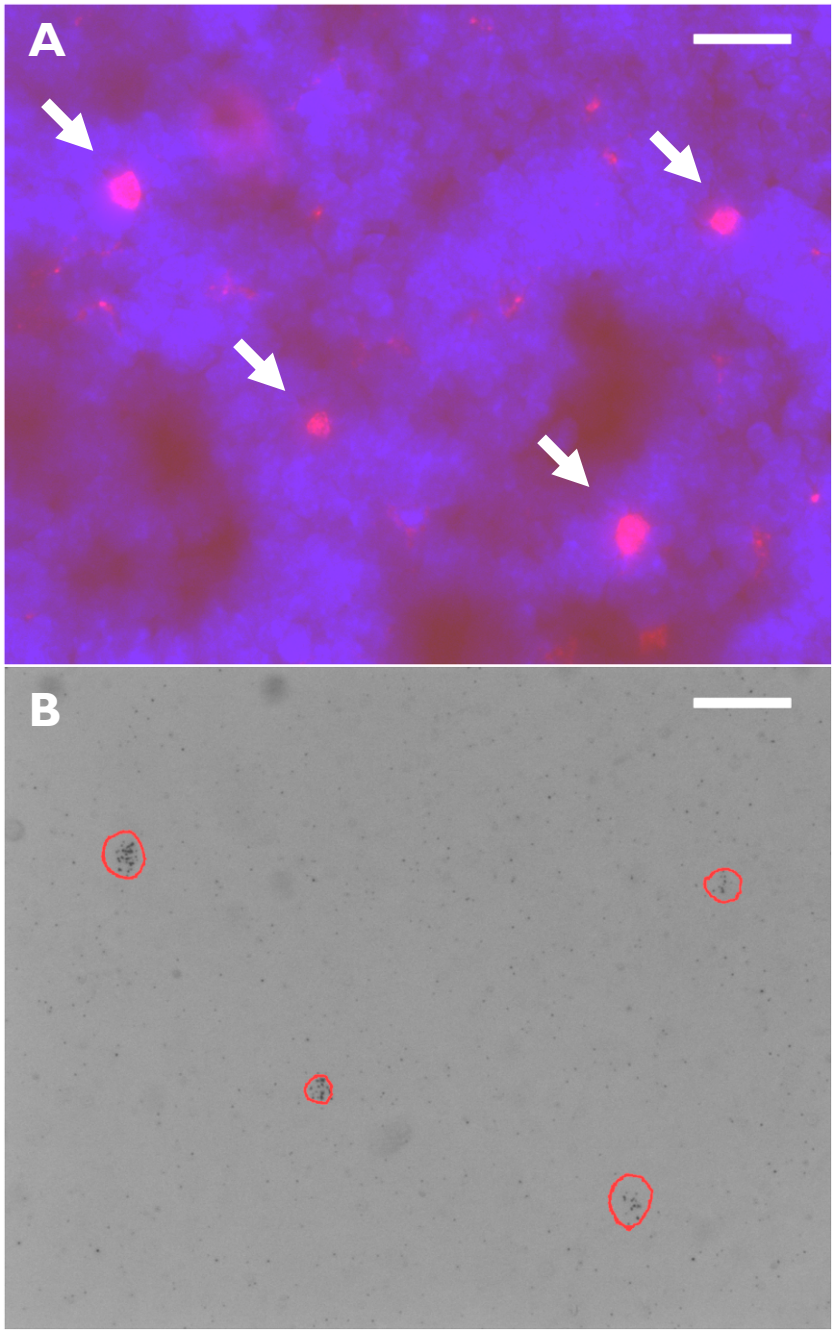

Figure 4.17: ^{3}H-thymidine radiolabelled donor cells identified lodged in recipient mouse spleen

A recipient mouse was injected with 5 × 10^5 radiolabelled donor cells and the spleen was collected and frozen 22 h later. 5 µm sections were cut, fixed and processed for autoradiography. Under fluorescence microscopy (A), DAPI-stained cell nuclei (blue) and four lodged CMRA-positive donor cells (arrowed) can be seen (red). Other smaller objects visible using the CMRA fluorescence filterset were autofluorescent particles that did not align with DAPI-stained nuclei. Under brightfield illumination (B) the same field shows autoradiography grains (black) in each of the four donor cells (locations outlined in red). The images were photographed with the 40× objective lens, scale bars show 25 µm.

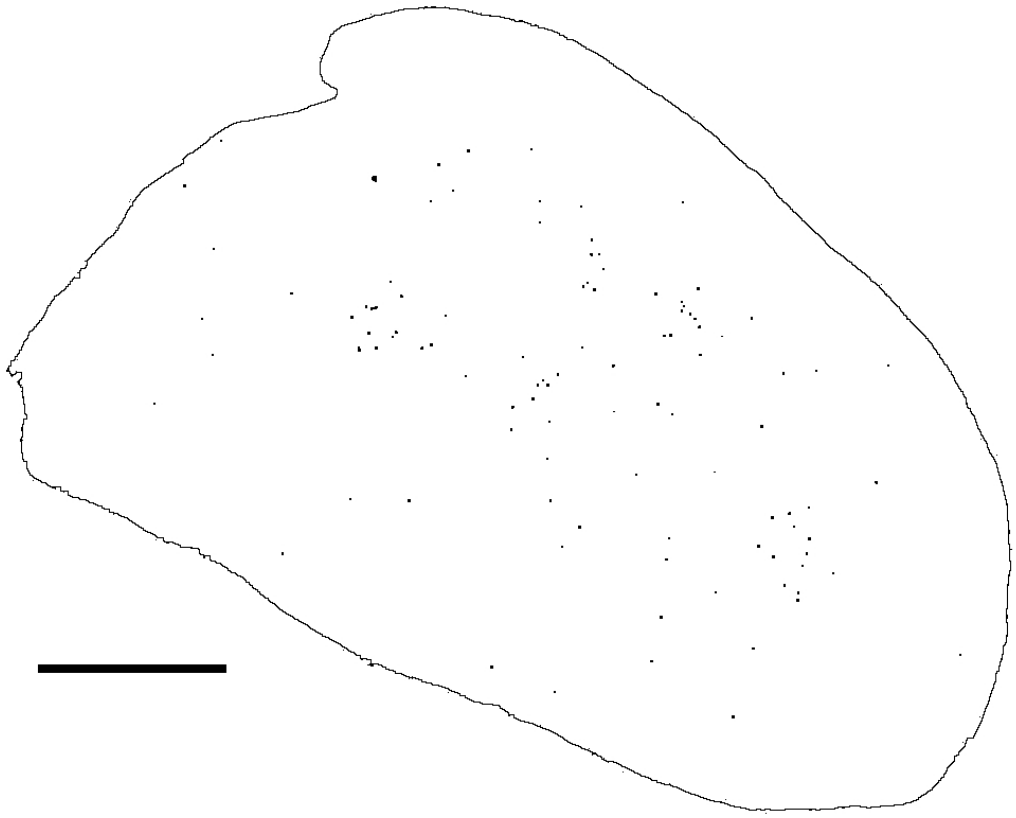

Figure 4.18: Locations of donor cell lodging in representative spleen section

A single spleen section from a mouse receiving 5 × 10^5 sham-radiolabelled donor cells was photographed in a series of overlapping microscope images at 40× magnification, on the DAPI and CMRA fluorescence channels. The outline of the DAPI-stained spleen section is shown and the locations of lodged CMRA-positive donor cells are marked (not to scale, inflated ≈ 5 fold for visual clarity). Scale bar shows 1 mm.

To visualise the donor cell lodging patterns in three-dimensions, a 50 µm spleen section from a mouse 22 h after receiving 5×10^5 sham-radiolabelled cells was examined using confocal microscopy. As seen in *Figure 4.19*, the lodged donor cells were not present only in a single plane, but formed three-dimensional clusters with a radius of 100–150 µm. It was thus important to recognise that the number of donor cells visible within a thin tissue cross-section did not take into account those donor cells present above or below the plane, and that fields of view containing donor cells were more likely to be surrounded by others in a three-dimensional cluster.

To assess the distribution of donor cells throughout the recipient mouse, tissues (spleen, liver, lungs, small intestine, thymus, heart, bone marrow from both femurs and a 50 µL sample of blood collected near hepatic vein) were collected from a single mouse 22 h after adoptive transfer of 5×10^5 radiolabelled donor cells. The tissues were sonicated in PBS to liquefy the samples, and aliquots of the pulverised tissue were added to scintillation cocktail. The radioactivity of the samples was read on a LS3801 Liquid Scintillation Counter (Beckman Coulter, Inc.) using the ^{3}H counting window for 2 min or, until the 2σ error was < 2.0 (whichever was the earlier). Radioactivity was mainly recovered from the small intestine (50%), spleen (20%), lung (9%), liver (8%) and thymus (7%) with <3% recovered from the bone marrow and blood samples, as expected for adoptive transfer of T lymphocytes (Tseng *et al.*, 1989).

The results of the pilot experiments demonstrated that the careful selection of experimental parameters based on data in the adoptive transfer and *in vitro* bystander effect literature produced a complete, working *in vivo* model. The donor cells were radiolabelled *ex vivo*, identified lodged in the spleen using the fluorescent probe at

the desired lodging density, and confirmed to be radiolabelled *in situ* using autoradiography. From here, the next step was to determine how to analyse the recipient mouse spleen tissues.

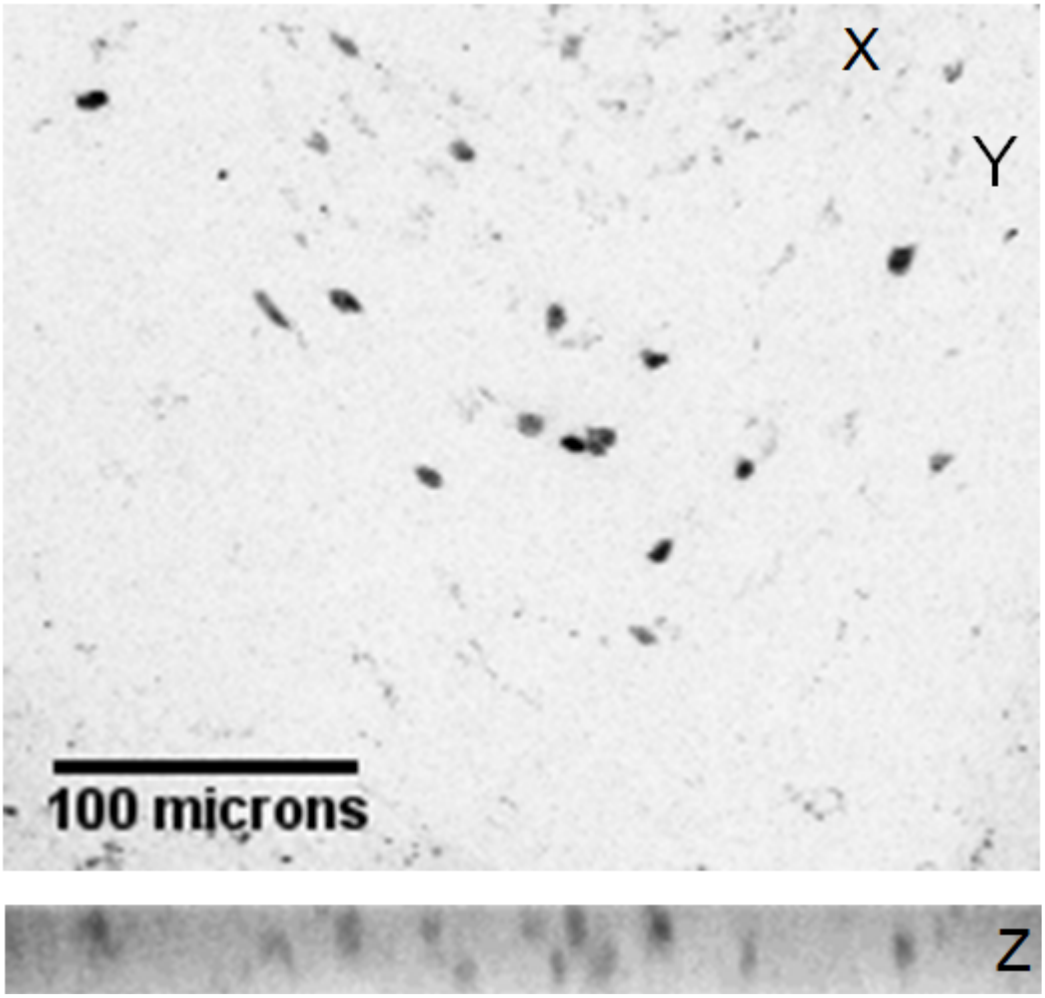

Figure 4.19: Z-stack projection of 3-dimensional cluster of lodging donor cells

A cluster of lodged donor cells in a 50 µm frozen spleen section from a recipient mouse taken 22 h after injecting with 5 × 10^5 sham-radiolabelled splenic T lymphocytes was identified by confocal microscopy. A z-series, imaging CMRA fluorescence was taken at 1.5 µm intervals through the depth of the section to a total depth of 30 µm. The mean fluorescence intensity through the stack was projected into a composite image (shown at top) and the colour was inverted for clarity (fluorescence intensity increasing from white to black). Numerous CMRA-positive cells (black) can be seen within the field. The same stack viewed side-on (shown at bottom) shows that the lodged donor cells are not present in a single plane, but form a three-dimensional cluster that likely extends several hundred micron above and below the frozen section. Scale bar = 100 µm for both images.