SHORT TECHNICAL NOTE

An improved method for preparing thick sections for immuno/histochemistry and confocal microscopy and its use to identify rare events

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Summary
Detection of rare events within solid tissues by immunocytochemistry is aided by imaging thick sections. Sections of 40–100 μm thickness of paraformaldehyde-fixed solid tissue can be prepared by use of a vibrating microtome and when immunolabelled these sections can be imaged in a confocal microscope. This approach provides excellent preservation of the structure of the sample and imposes minimal antigenic damage. In studies of the invasion of the bovine intestinal epithelium by Salmonella, this method has allowed detection of individual invading bacteria within large samples. The thick vibrating microtome sections were also used for the detection of rare apoptotic cell nuclei identified by TUNEL staining.

Introduction
In order to obtain the maximum resolution of cellular details of biological samples with a confocal microscope, it is essential that specimen preparation should induce minimum alteration to cellular structure. Whilst cold methanol or acetone have been used routinely as a fixation step for cells which have been maintained in tissue culture, considerable extraction of the cellular components takes place during this process. By contrast, fixation with a low concentration of formaldehyde (freshly prepared from paraformaldehyde and generally referred to as a solution of paraformaldehyde) in a buffered solution gives excellent retention of cellular structure. When followed by permeabilization with Triton X-100 or saponin, immunolabelling is possible with a wide range of antibodies. Not surprisingly, this has become a standard method for preparing tissue culture cells for confocal microscopy. Preparing sections of solid tissue presents a more difficult task. Routine histological preparation methods based around formalin fixation and paraffin embedding modify many antigens to the point where they are not detectable by the appropriate antibodies. By contrast, frozen sections retain good antibody labelling but offer only poor cellular structure. Thus, the full potential of the confocal microscope to image antigens within tissue sections requires a processing method which retains both the structural integrity of the tissue and the reactivity of antigens.

As part of an ongoing study into the pathogenesis of salmonellosis, we are characterizing bacterial intestinal invasion with the aim of identifying the eukaryotic cells targeted by the invading organisms and describing changes induced within these cells during infection. However, identifying bacteria which have invaded intestinal mucosa can be a difficult task because of the low numbers of bacteria located within the mucosa. The ability of the confocal microscope to image within thick (40–100 μm) sections of tissue increases the chances of detection of rare events, such as low numbers of bacteria in any one section of tissue. Paraaffin embedding is not appropriate for several of the antibodies to be used in this study, and so an alternative approach has been applied. Infected intestinal mucosa was fixed with paraformaldehyde, and the tissue was sectioned without any further processing using a vibrating microtome (Leica Microsystems, Milton Keynes, U.K.). Vibrating microtomes are predominantly used for sectioning unfixed brain tissue for neuroanatomical studies. Here we report
that they are also ideal for preparing thick sections of paraformaldehyde fixed tissue for imaging in the confocal microscope.

Materials and methods

Preparation of sections

Bovine ligated ileal loops were infected with *Salmonella dublin* as described previously (Watson et al., 1995). Biopsies of bovine ileal mucosa, which had been infected with *S. dublin* for 120 min, were fixed for 60 min in 4% paraformaldehyde, in phosphate buffered saline (PBS) at room temperature and stored in 1% paraformaldehyde in PBS at 4 °C. For cutting sections, pieces of approximately 10 × 3 × 3 mm were dissected, the serosal muscle layers were removed and the tissue was mounted onto the vibrating microtome with superglue. To provide support for the tissue during sectioning, it was covered in a small amount of a molten solution of 1.5% agar held at 45 °C. Sections of 50 μm were cut and transferred to droplets of PBS on Parafilm by lifting them carefully with a paint brush which had been trimmed down to one bristle. The tissue section was easily dissociated from the agar by gentle agitation with the brush.

Immunolabelling

Sections were permeabilized in PBS containing 0.1% Triton X-100 for 30 min followed by blocking in PBS containing 0.5% bovine serum albumin (PBS-BSA) for 60 min. *Salmonella* were detected using *Salmonella* group D agglutinating rabbit serum (Murex Biotech Ltd, Temple Hill, U.K.) diluted 1 : 200 in PBS-BSA. A mouse antibody recognizing alpha-tubulin (Sigma, Poole, U.K.) was used diluted 1 in 2000 in PBS-BSA. The sections were incubated for 60 min in both primary antibodies concurrently. Three 5 min washes in PBS were followed by incubation in both secondary antibody conjugates for 60 min diluted 1 : 200 in PBS-BSA. The anti-*Salmonella* antibody was detected with anti-rabbit IgG Alexa 568 and the anti-alpha tubulin was detected with anti-mouse IgG Alexa 488; both fluorescent conjugates were from Molecular Probes (Cambridge Bioscience, Cambridge, U.K.). The sections were washed three times in PBS and placed in drops of PBS on microscope slides. The PBS was replaced with Vectashield mountant (Vector Laboratories, Peterborough, U.K.) and the sections were sealed beneath coverslips with nail varnish.

Nuclei containing fragmented DNA were detected by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) staining using the ApopTag *in situ* apoptosis detection kit (Intergen Europe, Oxford, U.K.) according to the manufacturer’s instructions. The TUNEL stain was performed after the tissue had been permeabilized and blocked and before the addition of any primary antibodies or propidium iodide counterstain. Nuclei were labelled with a 1 μg mL⁻¹ solution of propidium iodide for 2 min after incubation in DNase-free RNase for 15 min at 37 °C. Sections were imaged in conventional XY mode using a Leica TCS confocal microscope (Leica Microsystems, Milton Keynes, U.K.) equipped with Ar and Kr lasers.

Results

Labelling with anti-alpha-tubulin clearly stained the cytoplasmic tubulin network of cells throughout the sections of bovine ileal mucosa (Fig. 1). The enterocytes exhibited a well-defined arrangement of tubulin, which allowed delineation of the enterocyte monolayer, individual enterocytes and their nuclei. In the lamina propria the cell morphology was variable, which reflects the more heterogeneous cell population. In some areas of the lamina propria the intensity of labelling was uneven. *Salmonella* were readily seen within infected mucosa from both of two calves used. The majority of bacteria were within the enterocyte monolayer and lamina propria either singly, or in microcolonies which contained up to 20 bacteria. The majority of lamina propria bacteria were within the top half of the villi, and were distributed from the underside of the enterocyte monolayer to just above the central lacteal. No bacteria were observed within the lacteal itself. There were relatively few bacteria on the luminal surface of the enterocyte monolayer. Although the relationship between bacteria and the intracellular tubulin labelling made it clear that some were present within cells, in a number of cases it was not possible to determine whether the bacteria were located in an intra- or extracellular position. We are currently investigating markers of cell membranes to allow better definition of bacterial location and hence their route of invasion.

TUNEL-positive cells occurred mainly in the lamina propria and lacteals, and for the majority of cells the marginalization of condensed chromatin typically associated with cell death by apoptosis was observed (Fig. 2). There was no association of TUNEL-positive cells with *Salmonella*. No problems were encountered with nonspecific labelling of cells at the edge of the section.

We have also demonstrated the successful application of this methodology to cells maintained in tissue culture in suspension (data not shown). These cells were fixed, embedded within a block of gelatin, and then sections of the gelatin prepared as described for solid tissues. When imaged in XZ mode, immunolabelling was demonstrated throughout the section, confirming the permeability of the gelatin sections.
Discussion

We report an approach for preparing both cells and tissues for confocal microscopy by combining two well-established techniques, namely preparation of samples by fixation with paraformaldehyde and permeabilization with Triton and preparation of thick sections using a vibrating microtome. Minimal changes to the samples occur and the imaging of rare events within relatively large samples of well-preserved tissue is possible. Clearly, as the nature of the tissue is changed and/or the section thickness is increased, immunolabelling may not be uniform throughout the sample, but the parameters chosen for this work have shown good penetration of the immunolabelling with low non-specific background labelling.

It proved possible to store intestinal mucosa samples after the initial fixation and we found no deterioration in subsequent immunolabelling of intestinal tissue after storage in 1% paraformaldehyde at 4°C for several months. When imaging the sections it was possible to detect some attenuation of the signal when focusing down into the section from the upper surface, but this was not sufficient to restrict study of the centre region of the section.

The application of this approach to our studies on Salmonella pathogenesis has allowed the distribution of bacteria within infected intestinal mucosa to be defined.

These results confirm previous observations of Salmonella-infected bovine intestines using conventional immunohistochemistry of NBF-fixed, wax-embedded sections and transmission electron microscopy (Watson et al., 1995; Frost et al., 1997). However, unlike the previous studies, this technique can now be extended to describe the phenotype of the intestinal cells associated with the invading Salmonella. The excellent preservation of tissue architecture will also allow the effect of Salmonella on the cell cytoskeleton and subsequent vacuolar trafficking within enteroctyes to be characterized. Such studies have up until now been limited to infection of cultured cells in vitro.

Salmonella can induce cell death in both enteroctyes and phagocytes during infection in vitro and the mechanism has been variously described as being similar to, or distinct from, apoptosis (Guilloteau et al., 1996; Monack et al., 1996; Kim et al., 1998; Brennan & Cookson, 2000; Watson et al., 2000). Salmonella have also been reported to induce apoptosis in vivo, after infection of murine ileal mucosa for 1 h (Monack et al., 2000). In the present study, there was no association of Salmonella with TUNEL-positive cells in bovine intestinal mucosa after 2 h of infection. The number of TUNEL-positive cells was relatively low, and their detection was greatly aided by using thick sections. The preservation of tissue structure allowed the majority of individual TUNEL-positive cells to be characterized as having nuclear morphology.
typical of cell death by apoptosis. Salmonellosis manifests as two distinct disease syndromes in mice and calves and this may explain the differences in induction of cell death in our results compared to previous work in mice. Future work will address this by further characterizing the effect of Salmonella infection on the number and distribution of TUNEL-positive cells at various times after infection of bovine intestinal mucosa and correlating this to the induction of intestinal inflammatory disease.

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References


