

A method for detecting functional activity related expression in gross brain regions, specific brain nuclei and individual neuronal cell bodies and their projections

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ABSTRACT

We have developed a system to visualize functionally activated neurons and their projections in the brain. This system utilizes a transgenic mouse, *fos-tau-lacZ* (*FTL*), which expresses the marker gene, *lacZ*, in neurons and their processes after activation by many different stimuli. This system allows the imaging of activation from the level of the entire brain surface, through to individual neurons and their projections. The use of this system involves detection of neuronal activation by histochemical or immunohistochemical detection of β -galactosidase (β gal), the product of the *lacZ* gene. Furthermore, the underlying brain state of the *FTL* mice determines the basal levels of expression of β gal. Here we describe in detail our protocols for detection of *FTL* expression in these mice and discuss the main variables which need to be considered in the use of these mice for the detection and mapping of functionally activated neurons, circuits and regions in the brain.

INTRODUCTION

A central aim in neurobiology is to determine the neural components and circuitry which are responsible for a given brain function. Traditionally, the connections a neuron makes have been identified using techniques such as electrophysiological recordings, histochemical techniques, anterograde or retrograde tracers, and functional imaging. While these approaches have particular and powerful benefits, they also all have drawbacks that limit their usefulness in the mapping of functionally activated circuits. For example, electrophysiology and functional imaging do not visualize connectivity, and tracers are not targeted to functionally activated neurons.

One approach to determine which parts of the brain are involved in a particular function is to look for markers of neuronal activation. One group of markers, the

immediate early genes, code for inducible transcription factors and are rapidly transcribed following neuronal stimulation. Of these genes, *c-fos* is the most studied and is induced by a wide range of different stimuli, eg depolarization, calcium influx, many different neurotransmitters (1-4). However, since *c-fos* is expressed exclusively in the cell nucleus, its localization does not provide information of the connectivity or the morphology of activated neurons within the nervous system. We have generated transgenic mice in which an axon targeted β -galactosidase (β gal) reporter system (5) is under the regulation of the promoter of the *c-fos* gene (6). In these *fos-tau-lacZ* (*FTL*) transgenic mice, neurons that express *c-fos* will target β gal expression to axons and dendrites via the tau sequences in the fusion protein. This permits direct visualization of neuronal cell bodies and their projections using a simple enzymatic assay (6) or immunohistochemistry. Thus, the *FTL* transgenic mice have certain advantages in imaging functionally activated areas and circuits in the central nervous

system, in that it allows the identification of cell bodies and their projections and therefore may aid in imaging functionally activated circuitry.

Here we describe a number of different methods we have used to visualize activated neurons and their projections, activated brain nuclei, and the brain surface using the *FTL* mice. Depending on the resolution and brain area to be assessed, different types of immunological or histochemical detection of β gal can be used. We present our current protocols for each of these methods.

MATERIALS AND METHODS

Animals

Mice were males or female transgenic heterozygote *FTL* mice aged between 2 and 3 months. Animals were housed in a room with a 12-hour light-dark cycle. All experimental procedures adhered and were approved by the Institutional Animal Care and Use Committee of the University of Melbourne. Food and water were supplied *ad libitum*. For analysis of β gal expression under basal conditions, mice were taken from their home cages and immediately injected intraperitoneally with a lethal overdose (100 μ l) of Lethabarb (Virbac, Peakhurst, Australia). After the animals were deeply anaesthetised, they were transcardially perfused with 12 ml of chilled 5% sucrose in H₂O followed by the 25 ml of chilled 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4; PFA). After 15 min, brains were removed and post-fixed in fresh 4% PFA for 15 min at 4°C, transferred to 20% sucrose in phosphate buffered saline (PBS) and equilibrated for 48 hr at 4°C. Brains were then frozen in Tissue-Tek (Sakura, Tokyo, Japan) and cryostat sections cut (50 or 100 μ M) and collected in wells of 24 well tissue culture plates (1-2 sections/well) in PBS.

β gal enzymatic assay

For detection of β gal enzymatic activity, PBS was aspirated and sections incubated in Stain buffer (5 mM MgCl₂, 5mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 0.4 mg/ml 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (Astral, Gynea, Australia)) for 24 hr at room temperature (RT). After staining, sections were rinsed in PBS and stored in 4%

PFA until mounting by placing into 0.5% gelatin in H₂O before transferring to slides coated with 0.5% gelatin, 10% potassium chromium alum. Sections were allowed to dry, dehydrated through graded alcohols to histolene, rehydrated through graded alcohols, rinsed in water and counterstained in 0.05% Nuclear Fast Red in 5% aluminium sulphate for 15 min. Sections were rinsed in H₂O, dehydrated again and mounted in Safety Mount (Fronine, Riverstone, Australia). Sections were analyzed microscopically and areas which were β gal positive were identified by comparison with an atlas of the mouse brain (7).

Immunohistochemistry

For β gal immunohistochemistry, cryostat sections, either free-floating or mounted, were incubated overnight at 4°C in rabbit anti- β gal antisera (MP Biomedicals, Solon, USA) diluted 1:20,000 in 10% CAS-Block (Zymed, San Francisco, USA), 0.1% Triton X-100 in PBS. After washing in PBS, sections were incubated for 1 hr in Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, USA) diluted 1:500 in PBS. Sections were washed in PBS; if free-floating, they were mounted onto gelatinized slides, and coverslipped in Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark). If already mounted, the sections were directly coverslipped in the same medium.

Alternatively, after washing in PBS, sections were incubated in biotinylated goat anti-rabbit IgG diluted 1:400 in 10% CAS-Block, 0.1% Triton X-100 in PBS for 1 hr. Immunoreactivity was visualized with StreptABComplex/horseradish peroxidase (DakoCytomation, Glostrup, Denmark), and Metal-Enhanced DAB (Pierce, Rockford, USA). Mounted sections, (or if free-floating, the sections were first mounted onto gelatinized slides) were air dried, dehydrated and coverslipped in Safety Mount.

Photographic imaging

Fluorescence photographs were obtained using an ImagePoint cooled CCD camera (Photometrics LTD, Tucson, AZ) and V for Windows imaging software (Digital Optics Ltd., Auckland, NZ). Images were processed with Adobe PhotoShop and Corel Draw software (Corel Corp., Dublin, Ireland). For confocal

microscopy, sections were imaged with a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany) fitted with a Bio-Rad 1024 confocal illuminating system (Bio-Rad, Sydney, Australia) using a x100 objective and the appropriate fluorescence filter.

RESULTS AND DISCUSSION

We have used a number of different detection methods for the visualisation of β gal in neurons of the mice. These methods are the histochemical detection of β gal activity, and immunocytochemical detection. For the immunohistochemistry, we have used both fluorescence and peroxidase detection methods. Each of these methods has particular advantages. As follows, we describe the images we obtain with these methods and their particular advantages with regard to different levels of resolution and sensitivity to detect *c-fos* related activation in the brain.

Histochemical detection of β gal

The most convenient method of detection of β gal is the histochemical detection of β gal enzymatic activity. In regions containing active β gal, the cleavage of the Xgal substrate results in the deposition of a fine blue crystalline precipitate. In sections of brain of the *FTL* mouse, this method of detection is suitable for analysis of whole brain sections at low and medium power (Fig. 1A and B). In many parts of the brain, β gal histochemistry can resolve the structures of individual neurons (Fig. 1B). However, in areas of high density of positive neurons, such as cortex, the pattern of staining using β gal histochemistry does not resolve individual neurons and axons. In these regions, there is a lot of staining within axons, and the blue precipitate formed in the histochemical reaction does not have enough precision to accurately resolve the very fine processes of many separate axons. In these regions, the staining will appear as blue cell bodies within a uniform blue pattern. A uniform blue pattern of staining is also seen in major tracts in the brain which are β gal positive, such as anterior commissure and corpus callosum.

The blue staining pattern is easy to detect macroscopically in the *FTL* mouse and it is simple to determine which brain structures have β gal activity, because the *FTL* product is found throughout the cell

body and neuronal processes. This is one of the principle advantages of the *FTL* mouse compared with immunohistochemistry for FOS, where only cell nuclei are labelled. Because the β gal expression fills the entire cell in the *FTL* mouse, the pattern of β gal expression often encompasses the brain structure which is activated (Fig. 1), whereas in sections stained for FOS, the FOS positive dots do not form such a clear pattern correlating with a particular brain structure. In *FTL* mice, brain tracts also stain for β gal if they are projecting from activated regions of the brain (6), whereas FOS immunohistochemistry is not detected in tracts as it is only expressed in the nucleus of neuronal cell bodies. Another advantage of β gal histochemistry is that there is no background staining. This means that positive staining is easy to detect and quantitate. Our preferred method of quantification employs the determination of a brightness area product over the brain region of interest (8).

β gal immunohistochemistry

An approach which offers good resolution of individual neurons and processes involves immunohistochemistry with antibodies to β gal and detection either with peroxidase-labeled or fluorophore-labeled secondary antibodies. Immunohistochemistry with peroxidase-labeled secondary antibodies results in similar resolution of staining compared to β gal histochemistry. There are some advantages and disadvantages of this technique compared with histochemistry. The primary advantage is that there is an increase in resolution of cell bodies and processes in regions of the brain containing a high density of *FTL* positive neurons, in particular in cortex (9) (Fig. 1C, D shows amygdala region). A disadvantage is that there is low level background staining, so that the areas of negative to low expression are not as clean and clear compared with β gal histochemistry.

The alternative immunohistochemical detection employs fluorophore-labeled secondary antibodies (Fig. 1E). We have used this detection in the past to visualize neurons in diverse regions of the brain (6, 8-9). Very high resolution images can be obtained using confocal microscopy of thin optical sections through the neuron. For construction of neurons in three dimensions, a series of images through the depth of section can be taken and

the images pooled to reconstruct a composite image of the neuron (6).

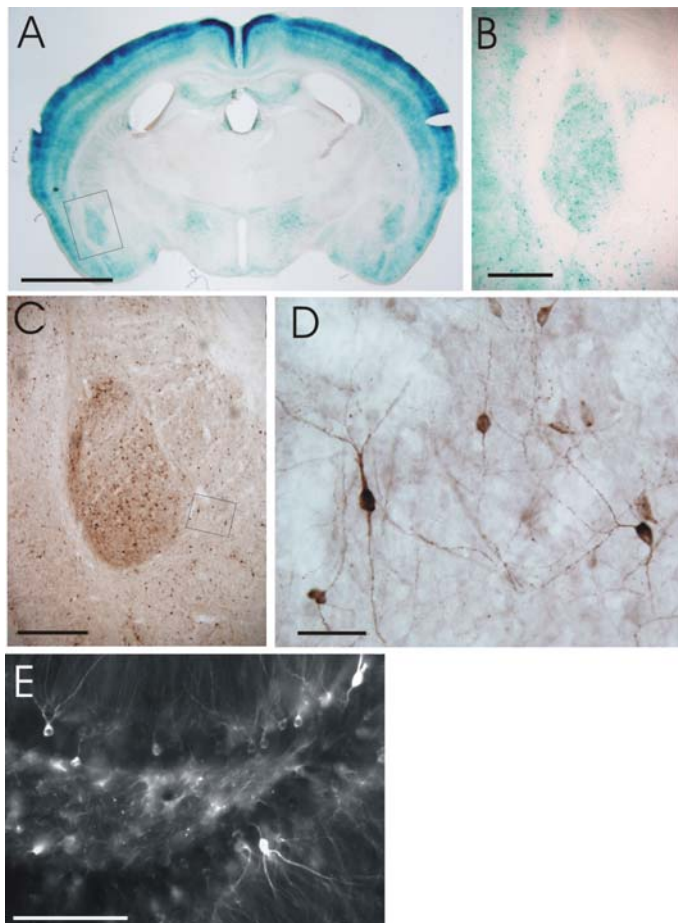


Fig. 1: Examples of different modes of detection of activated *FTL* expression in brains of *FTL* mice. (A) and (B) show detection of *FTL* expression in neurons using β gal histochemistry. (A) shows a low power view of a coronal section of the brain (approximate Bregma - 1 mm) from an *FTL* mouse which had received a mild aversive stimulus (all sections were from mice which had received a footshock of 0.5 mA for 2 seconds). (B) shows higher power view of region boxed in (A), and which shows the basolateral complex of the amygdala. (C) and (D) shows detection of *FTL* expression in neurons using β gal peroxidase immunohistochemistry. (C) shows similar view as (B), and contains basolateral complex of amygdala. (D) shows higher power view of region boxed in (C). (E) shows *FTL* expression in dentate gyrus neurons using β gal fluorescence immunohistochemistry. Scale bars: A, 2 mm; B and C, 400 μ m; D, 50 μ m; E, 100 μ m.

This method is reliable and has the advantage of good signal and low background. It is also very useful for double labeling studies to further identify the labeled functional circuits (9). Some problems can arise if the titer of either primary or secondary antibody is not fully optimized, which will result in either a low signal or high background. In addition, this method is normally only suitable for analysis of small regions of the brain,

because the fluorescent signal is difficult to detect at low power. In some situations, where the *FTL* expression is particularly strong, this technique can be used for the analysis of entire brain sections (8).

Whole brain histochemistry

The overall patterns of expression in the cortex of *FTL* animals can be simply examined using whole-brain histochemical staining. We have used whole brain staining to look at patterns of *FTL* expression in response to visual stimuli, and in particular to examine light responsive regions of the cortex (9). In animals which have been housed in the dark for several days, and thus have had no light input, the primary visual cortex can be clearly distinguished as a roughly circular patch of white on the brain surface (Fig. 2A). In animals housed under normal light conditions, this area is filled with blue staining (Fig. 2B).

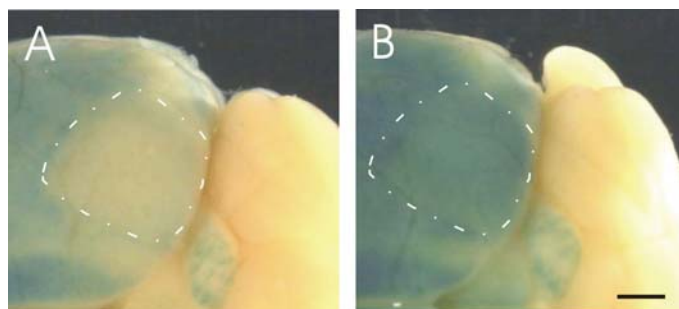


Fig. 2: Detection of *FTL* expression on the surface of the brain using whole brain β gal histochemistry. Shown are side views of posterior cortex of brain from an *FTL* mouse which had been (A) housed in the dark for 3 days and (B) housed in an environment with a 12 hr dark/12 hr light cycle. The primary visual cortex is circled. Scale bar = 250 μ m.

Behavioral state of mice

In our studies, we have found β gal expression in the *FTL* mouse brain to be a sensitive indicator of brain state. For example, if *FTL* mice are taken immediately from their home cage and their brains analyzed for β gal expression, some variation in expression of β gal is found between individual mice, in particular in cortical regions and hypothalamus. We believe this is due to different degrees of brain activation, and have found that if mice are given periods of exposure to an enriched environment (10), both the level of β gal staining decreases and overall variation in staining between individual mice decreases (11). This is most likely due to decrease in stress levels of the mice following environmental enrichment. We note this because it is

important to point out that the behavioural state of the mice will influence their β gal expression patterns, and this needs to be taken into consideration for any experiments where these mice are used to determine which parts of the brain are activated following any given stimulus. In particular, any stimuli which may stress the mice will also affect expression in stress-responsive systems of the brain.

ACKNOWLEDGMENTS

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PROTOCOLS

1. Breeding the mice

We currently breed the *FTL* mice as a heterozygous colony by out breeding with C57/Bl6 mice. As heterozygotes, the mice are indistinguishable from C57/Bl6 mice with regard to viability and behaviour. It may also be possible to use homozygous *FTL* mice, but we have not investigated this possibility in any depth.

2. Phenotyping the mice

We find that phenotyping *FTL* mice for β gal activity is simpler than genotyping to determine the transgenic status of the mice. We phenotype by detecting β gal activity in tail biopsies, as follows:

Tail Assay. Cut off tail tips (0.3-0.5 cm) and cut in half along the long axis, remove the bone fragments and collect into PBS in a 24 well tissue culture plate (or similar). Tails can be collected from 3-4 week old mice at weaning; however, the tail phenotype in mice of this age can be weak and variable in some mice. This variable detection is overcome by incubating tails in Stain buffer with a high concentration of 5-Bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal). Thus, Remove PBS from tails and add high X-gal Stain buffer (5 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mg/ml X-gal (Astral, Sydney, Australia)); ~500 μ l per well is sufficient. Incubate overnight at room temperature using rotation or agitation. Check for positive hair follicles, i.e. the presence of blue staining of the base of the hair follicles, using a stereomicroscope (Fig. 3). There can also be blue staining in the bone (which is why it is advisable to remove the bone) which is due to endogenous β gal activity, and can be present in both transgenic and non-transgenic animals (Fig. 3). The presence of bone fragments interferes with the penetration of the buffer into hair follicles underneath the bone and visualizing the hair follicles when the assay is scored.

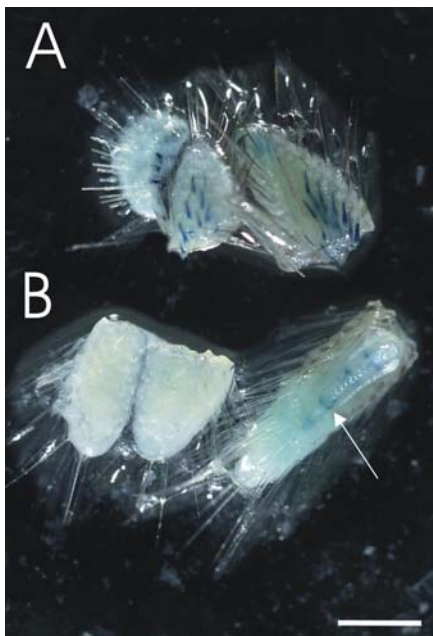


Fig. 3: Tail assay for phenotypic identification of *FTL* positive transgenic mice. Small sections of tail were clipped from mice and incubated in Stain buffer as described in protocols. **(A)** shows a stained tail from an *FTL* positive transgenic mice with clear blue staining in hair follicles. **(B)** shows stained tails from *FTL* negative mice, with arrow indicating some endogenous β gal staining in bone. Scale bar = 2 mm.

3. Staining brain sections for β gal activity

1. *β gal histochemistry.* This procedure is recommended for low to intermediate resolution analysis of brain sections. It has the advantage of low background and strong signal, is easy to detect micro- and macroscopically, but has lower resolution compared to β gal immunohistochemistry at the level of single neurons and fine morphological detail.

Perfusion fixation: Inject the mouse intraperitoneally, with 100 μ l of Lethobarb (Virbac, Peakhurst, Australia). When the mouse is deeply anesthetized (no response to paw pinch) open the chest cavity, locate the right atria of the heart and make a small incision. Using a 10 ml syringe and 25G needle transcidentally perfuse 12 ml of 5% sucrose in H₂O through the left ventricle of heart followed by 25 ml of freshly prepared 4% PFA. Perfusion first with sucrose in H₂O was used to decrease tissue shrinkage (12). Monitor the success of perfusion by appearance of tissue during and after the perfusion. Indications that the perfusion is successful are tissue (such as liver, brain and kidney) clears of blood, the lungs do not expand, no liquid emerges through the nose or mouth and the whole carcass becomes stiff. Dissect brains and postfix in at least 50 ml of the same fixative for 15 min at 4°C. Wash twice in PBS to remove the fixative, cyroprotect and snap-freeze specimens, as described below.

Cryoprotection and freezing: place brains in 20% sucrose in PBS for 24-48hr at 4°C to equilibrate the specimens. Remove brains from the sucrose and embed in an embedding medium for frozen tissue specimens such as Tissue-Tek OCT (Sakura Tokyo, Japan). Snap-freeze on isopentane cooled by liquid nitrogen.

Sectioning: Cryostat sections can be collected directly on to coated glass slides (15-50 μ m sections) as described above, or as free-floating (50-100 μ m) sections. Freezing microtome sections (30-100 μ m) can also be collected as free-floating sections.

Slide-mounted sections, four per slide, are directly mounted onto 0.5% gelatine / 5% chromium potassium sulphate coated slides. Taking up the section into in a drop of PBS on the slide ensures bubbles and folds in the brain sections are minimised. The sections are dried at room temperature for a minimum of 2 hours. When well air-dried the slides are placed into a coplin jar, rinsed once in PBS and incubated with enough Stain buffer, containing a standard concentration of 0.4 mg/ml X-gal (~50 ml) to cover the slides.

For free-floating sections, one to two sections are collected into PBS in 24-well tissue culture plates. Sections can be used immediately for detection of β gal activity or left overnight at 4°C in PBS. For histochemical staining, sections are incubated in 500 μ l Stain buffer per well.

Notes regarding staining

We normally stain for 24 hr at room temperature using rotation. However the incubation times can be shorter or longer depending on how much activity you want to visualize. If enzymatic activity is high the yellow stain solution will gradually become colorless, as the components of the solution are being catalyzed. If this happens, fresh Stain buffer should be added or the original volume you incubate in should be increased.

The enzymatic activity of the slide-mounted sections is stopped by removing the Stain buffer, rinsing in PBS and air drying the sections. The activity of the free-floating sections is stopped by removing the Stain buffer and adding ~500 μ l 4% PFA per well. The free-floating sections can be stored in PFA indefinitely. When ready to mount place the sections into 0.5% gelatin in H₂O and mount onto 0.5% gelatin / 5% chromium potassium sulphate coated slides and air-dry. Dried sections are dehydrated through graded alcohols to histolene and mounted in Safety Mount (Fronine, Riverstone, Australia).

Sections can be counterstained, for example, with Nuclear Fast Red, which contrasts well with the blue precipitate formed by the enzymatic activity. If counterstaining in an aqueous counterstain the air-dried sections must first be delipidated by taking sections from histolene through graded alcohols to water. Sections can then be counterstained in 0.05% Nuclear Fast Red in 5% aluminum sulphate for 15 min, rinsed in H₂O, dehydrated through graded alcohols to histolene and mounted in Safety Mount (Fronine, Riverstone, Australia).

1. Immunohistochemistry. We use both peroxidase and fluorescence immunohistochemistry for detection of β gal. Immunoperoxidase detection is good for intermediate resolution analysis. It is easy to see both microscopically and macroscopically, but has some background. It has generally better resolution of individual neurons and processes compared with β gal histochemistry. Fluorescence immunohistochemistry has very good resolution of cells and processes, has relatively low background, but is generally only suitable for examining small regions of the brain. For β gal immunoreactivity, we currently use the same perfusion, fixation, cryoprotection and sectioning conditions as those for β gal histochemistry. For immunohistochemistry we use 20-50 μ m sections.

Staining: After air-drying slide-mounted cryostat sections for 2-4 hours, remove dried embedding material around the sections using a cotton-tipped applicator and water. Wax pens do not adhere well to slides if embedding material is still present around the sections; do not wet the sections at this stage. When slides have been cleaned use a wax pen, DakoCytomation Pen (DakoCytomation Glostrup, Denmark), to draw an encircling margin around each section.

Incubate sections in 10% CAS-Block (Zymed, San Francisco, USA) in 0.1% Triton X-100 in PBS at RT for 30 min in a humidified chamber. For all incubations, 50 μ l is adequate to cover a single section. Remove the solution and add rabbit anti- β gal primary antibody (MP Biomedicals, Solon, USA) diluted 1:20,000 in the same solution. Incubate overnight in a humidified chamber at RT. The same solution with no added antibody is applied to the no primary control sections. Wash slides 3 times for 5 min in PBS.

For detection of immunoreactivity using peroxidase apply anti-rabbit biotinylated secondary antibody (Vector, Burlingame, USA) diluted 1:400 in 10% CAS-Block, 0.1% Triton X-100 in PBS and incubate for 1 hr at RT. Wash slides 3 times for 5 min in PBS. Add StrepABComplex/horseradish peroxidase (DakoCytomation Glostrup, Denmark), prepared according to kit instructions, for 1 hr at RT. Immunoreactivity is visualized using Metal-Enhanced DAB (Pierce, Rockford, USA).

For staining using fluorescence detection, incubate for 1 hr at RT in Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, USA) diluted 1:500 in PBS for 1 hr at RT. Rinse with PBS and coverslip slides with Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark).

Notes on β gal antibodies. There are many β gal antibodies available and we have only tried a small selection. We have found the rabbit antibody from MP Biomedicals and a monoclonal antibody from Promega are reliable and have low background staining.