Protocols for selective injection of tracer into the gastric mucosa and the gastric muscle of rat

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ABSTRACT

This protocol is for injection of neural tracers into the wall of the stomach to identify neurons innervating various tissues in the gastric wall including the gastric mucosa and the gastric muscle/myenteric plexus. This includes extrinsic neurons such as vagal efferent and afferent neurons, sympathetic and sensory neurons, as well as intrinsic neurons in the enteric plexuses, both locally and within other parts of the digestive tract, for instance the duodenum. It utilises tracer substances that are known to be taken up by the endings of neurons, including fast blue, which is taken up very efficiently by axons, but also fluorescent microspheres whose diffusion within tissues is limited so that they can be localized to particular tissues.

GUIDELINES

All animal procedures used in these protocols were approved by the Animal Experimentation Ethics Committee of the Florey Institute of Neuroscience and Mental Health and designed and performed in accordance with guidelines of the National Health and Medical Research Council of Australia.

MATERIALS

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Fast Blue **Polysciences Catalog #17740**

Red Retrobeads IX

Green Retrobeads IX

OPEN ACCESS

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Protocol status: Working
We use this protocol and it's working

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PROTOCOL MATERIALS

- Fast Blue **Polysciences, Inc. Catalog #17740** — Materials
- Lacrilube **Ellar Laboratories** — Step 3
- Flexigrid **Catalog #66024630** — Step 3
- Red Retrobeads IX — Materials, Step 8
- Green Retrobeads IX — Materials

SAFETY WARNINGS

There are no specific safety warnings relating to this protocol apart from the normal laboratory safety standards and protocols relating to use of chemicals, biological agents, sharp tools and laboratory animals.

BEFORE START INSTRUCTIONS

Before conducting any experiments using animals, ethics approval should be sought and obtained in accordance with local legal requirements and ethical standards as outlined in national and international guidelines for the use of animals in research.

Be aware that this protocol includes several critical steps that require some skill, practice and care, including surgical procedures and performing microinjection of tracer that is limited to specific tissue layers within the stomach and associated structures. Production of beveled glass injection pipettes is also a skilled process that requires care and specialized equipment, although these may also be purchased from scientific suppliers at the time of writing.

1 Sprague Dawley rats (RRID: RGD_10395233) of 200-500 g, both sexes, are used. They are fasted overnight and operated in the morning.

2 The rat is anesthetized with a mixture of medetomidine (0.5 mg/kg) and ketamine (75 mg/kg), given intraperitoneally, using a 27 gauge needle. Carprofen (5 mg/kg) analgesic injection is given subcutaneously prior to surgery.

3 The rat is placed on a warm (30^{o}C) operating table and operated on under aseptic conditions. The upper abdomen is clipped to remove the fur, swabbed with chlorhexidine followed by 70% ethanol and covered with sterile protective film (Opsite Flexigrid^{TM}). All aqueous solutions are sterilized by ultrafiltration. The

rat's eyes are protected from drying with Lacrilube. Lacrilube **Polysciences Inc**

Flexigrid **Polysciences Inc Catalog #66024630**

4 The skin and abdominal muscle in the mid-line, from the xyphoid process caudally for 1.5 cm, are opened separately. The stomach is exteriorized and kept constantly moist with physiological saline.

5 Neuronal tracers (usually Retrobeads^{TM}, Lumafluor Inc.) are injected into the external muscle or mucosa at multiple sites with 30 - 100 nL volumes injected at each site using beveled glass micropipettes (tip diameter 60-90 µm) disinfected by immersion in 70% ethanol. The injection is made with a glass micropipette rather than a conventional metal needle as the beveled glass micropipette is finer (60 µm versus 300 µm) and so does less damage, and it also makes it possible to use the small volumes required.

6 For injection into the gastric muscle in the antrum, corpus, or fundus, the micropipette is introduced at a very low angle (approx. 10 degrees) and the tip is pushed in to be at the level of the myenteric plexus.

7 For injections into the muscle of the pyloric sphincter, the pipette tip is advanced and the tip inserted into the constricted region of muscle between the stomach and duodenum, while avoiding the many blood vessels supplying the sphincter taking care not to disrupt any of them.

8 When the mucosa is to be injected, the stomach is opened along the greater curvature, beginning at the gastric groove (junction of fundus and corpus) and extending to the antrum. The stomach is opened along the incision, and cotton buds are used to remove any gastric content. The stomach is partly everted to reveal the mucosa and viewed using an operating microscope. Micropipettes are inserted into the mucosa using a low angle of approach. The tip can generally be seen through the mucosa. The successful injection is monitored visually under a surgical microscope.

Red Retrobeads IX **Polysciences Inc**

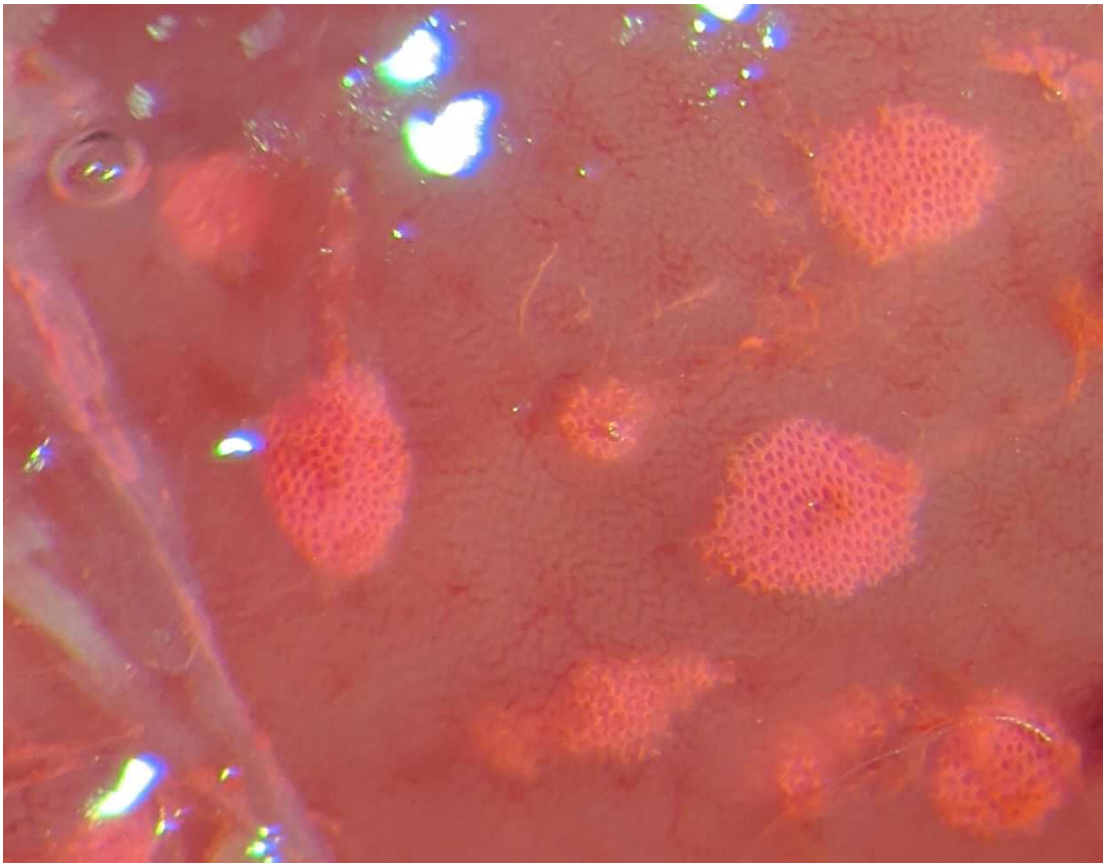

Image of red tracer bead injection sites within the gastric mucosa. Note the injected beads outlining the glandular structures confirming they are within the mucosal tissue.

9 In control experiments, the nerve supply to stomach is interrupted by section the vagus nerve close to the stomach before injection of tracers into the stomach wall are carried out. In other control experiments, tracers were placed in the lumen of the stomach or in the abdominal cavity.

10 The stomach incision is closed with sterile 5.0 (USP) silk suture. The abdominal wall is closed with sterile 2.0 (USP) polydioxanone absorbable suture. Skin incision is closed with surgical staples.

11 The anesthesia is reversed by administration of atipamezole hydrochloride (1 mg/kg) intraperitoneally.

12 The rats are monitored visually during recovery (about 1.5-2 h).They are then returned to an animal holding room and monitored daily.

13 7 to 14 days after tracer injection, the animals are deeply anesthetized with pentobarbital sodium and killed for tissue harvesting.

14 Once the rat is fully anesthetized (confirmed by lack of response to vigorous skin pinch) the rat is perfused via cardiac puncture with phosphate buffered saline followed by 4% paraformaldehyde. The nodose ganglia and stomach are removed and placed in 4% paraformaldehyde overnight before washing 3x 10 minutes in PBS.

15 Neuronal tracer Injection sites in the stomach are photographed and tissue is stored in PBS/0.1 % sodium azide until further use. See step 17.

16 Nodose ganglia are dissected to remove all connective tissue before being placed on microscope slides with fluorescence mounting medium (Dako) and cover-slipped with slight compression.

17 Nodose ganglia are imaged using a confocal fluorescence microscope (Zeiss LSM800 Airyscan). Large tile scans are collected at various focal planes (z-stack) in order to cover the entire ganglia in both red and green channels. Identification of tracer labelled nodose neurons as well as determination of their cell body area size are determined using Zen Blue 3.0 software (Zeiss).

18 The injection sites in stomach samples are dissected out, mounted on microscope slides in wells constructed using rubber take to create wells for imaging thick tissue and cover-slipped. Injection sites are scanned at low power using confocal microscopy as in step 16.

19 To further examine injection sites in the stomach, specimens may be placed in 30% sucrose in PBS overnight, then in in 30% sucrose/PBS: Optimal cutting temperature (OCT) compound (50:50) overnight and finally placed in cryomolds wells in 100% OCT compound before being frozen using iso-pentane cooled in liquid nitrogen. Sections are then cut (20-30 µm thick) using a Cryostat and mounted on slides before imaging on a fluorescence microscope equipped with a color camera, Zeiss Axio-observer/Zeiss HRMc.