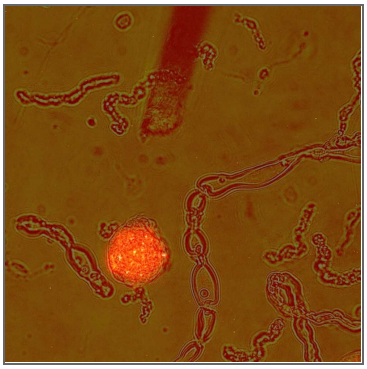

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Protocol status: Working
We use this protocol and it's working

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Protocol for RNAseq analysis of identified gastric vagal afferent neurons

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ABSTRACT

This protocol describes methods to dissociate nodose ganglion neurons from rats, identify and specifically collect neurons previously labeled using retrograde tracing, and then perform short read RNAseq analysis on pooled samples of small numbers of neurons or single neuronal cells. The parameters of the analysis are designed to allow deep sequencing (pooled cell samples, high numbers of reads per sample) to enable robust identification of genes expressed at low copy numbers that are important for neuronal function, such as ion channels and G-protein coupled receptors. It also includes a basic description of the sorts of differential expression analysis that can be performed on sequencing/transcriptomic data of this type.

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.

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Keywords: RNAseq, low copy number genes, retrograde tracing, vagal sensory neurons, nodose ganglion, transcriptomics, differential expression analysis

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MATERIALS

- Leibovitz's L-15 Medium, no phenol red **Thermo Fisher Catalog #21083027**
- Collagenase Type 1 **Worthington Biochemical Corporation Catalog #LS004197**
- Trypsin-EDTA solution **Sigma Catalog #T4049**
- Deoxyribonuclease I from bovine pancreas **Sigma Aldrich Catalog #D4527**
- Fluorodish **World Precision Instruments Catalog #FD35-100**
- SMART-Seq HT kit **Takara Bio USA, Inc. Catalog #634456**
- Nucleomag NGS clean-up and Size Select **Takara Bio USA, Inc. Catalog #744970.5**
- High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**
- Qubit dsDNA HS kit **Life Technologies Catalog #Q32851**
- Nextera XT DNA Library Preparation Kit **illumina Catalog #FC-131-1096**
- Pentobarbital

PROTOCOL MATERIALS

Qubit dsDNA HS kit **Life Technologies Catalog #Q32851** Materials

Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1096**

Materials, Step 25

High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**

Materials, Step 22

Leibovitz's L-15 Medium, no phenol red **Thermo Fisher Catalog #21083027**

Materials, Step 3

Pentobarbital Materials, Step 2

Fluorodish **World Precision Instruments Catalog #FD35-100** Materials, Step 13

Nucleomag NGS clean-up and Size Select **Takara Bio Inc. Catalog #744970.5**

Materials, Step 22

Collagenase Type 1 **Worthington Biochemical Corporation Catalog #LS004197**

In Materials and 2 steps

Trypsin-EDTA solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T4049**

Materials, Step 7

Deoxyribonuclease I from bovine pancreas **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D4527**

In Materials and 2 steps

SMART-Seq HT kit **Takara Bio Inc. Catalog #634456** Materials, Step 20

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 difficult dissections that must be done quickly, trituration steps and cell sampling that require skill, practice, and optimization under specific local conditions, as well as steps that require an RNAse free and uncontaminated workspace.

Dissociation of neuronal cells is a difficult process that requires skill. If not optimized, cell yield will be low, and cell viability will be compromised. Poor sample quality will result in wastage of expensive reagents and poor outcomes from expensive sequencing runs. Care should be taken to optimize conditions and practice skills before experimental samples are collected and processed for sequencing.

Tissue harvesting

1 Vagal neurons innervating either the muscle layers or the mucosa in various stomach regions in Sprague Dawley rats (RRID: RGD_10395233), are identified using injections of fluorescent beads as described in a separate protocol (see below)

Protocol

NAME

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

CREATED BY

Martin Stebbing

PREVIEW

2 Rats that have received tracer injections into the stomach 7-14 days previously are euthanized by overdose with Pentobarbital (100 mg/kg i.p.) Pentobarbital **Takara Bio Inc.**

3 Nodose ganglia are immediately dissected out and placed in ice-cold Leibovitz's L-15 medium (Gibco/Thermo Fisher, Cat n^{o}21083-027). Connective tissue around the ganglia is removed, and a

longitudinal slit is made in the capsule with a scalpel blade.

Leibovitz's L-15 Medium, no phenol red **Takara Bio Inc. Catalog #21083027**

4 The stomach is also dissected out, opened flat, and fixed in 4% PFA in PBS overnight at 4^{o}C for later examination of injection sites (see protocol quoted in step 1)

Nodose Ganglion dissociation

5 Left (LNG) and/or right (RNG) nodose ganglia are digested separately with 2 mg/mL collagenase type I (Worthington, Cat n^{o} LS004196/7) in L-15 medium at 37^{o}C with orbital shaking (120 rpm) for 45 minutes.

Collagenase Type 1 **Takara Bio Inc. Catalog #LS004197**

6 The resulting samples are spun down at 400 x g for 5 min at room temperature.

7 Samples are further digested with 2 mg/mL collagenase type I and 0.075% Trypsin-EDTA (Sigma, Cat n^{o}T4049) in L-15 medium at 37^{o}C with orbital shaking (120 rpm) for 45 minutes.

Collagenase Type 1 **Takara Bio Inc. Catalog #LS004197**

Trypsin-EDTA solution **Takara Bio Inc. Catalog #T4049**

8 Samples are next spun down at 1000 x g for 5 min at 4oC and washed in L-15 medium containing 100 µunits/mL DNAase type I (Sigma, Cat no D-4527).

Deoxyribonuclease I from bovine pancreas **Takara Bio Inc. Catalog #D4527**

9 Samples are again spun down at 1000 x g for 5 min at 4^{o}C.
Ganglia are washed for the second time in L-15 medium containing 100 µunits/mL DNAase type I

Deoxyribonuclease I from bovine pancreas **Takara Bio Inc. Catalog #D4527**

10 Nodose ganglion samples are spun down at 1000 x g for 5 min at 4^{o}C.

11 Nodose ganglion samples are resuspended in L-15 medium containing 100 µunits/mL DNAase type I and triturated with flame-polished glass Pasteur's pipettes coated with 1% bovine serum albumin. Ganglia are passed up and down the stem of pipettes with decreasing inner diameter until achieving a homogeneous cell suspension.

12 Nodose ganglion cell suspensions are spun down at 1000 x g for 5 min at 4^{o}C.

13 Dissociated nodose ganglia cells are resuspended in L-15 medium without enzymes and placed on coverslip bottom 35 mm culture dishes (Fluorodish, WPI). Cells are left to settle down for 20 minutes before manual cell picking. Fluorodish **Takara Bio Inc. Catalog #FD35-100**

Cell Collection

14 Neurons are viewed on an inverted fluorescence microscope with red and green filters and labeled neurons identified by the characteristic red fluorescent cytoplasmic puncta within their cytoplasm. These puncta are extremely bright and extremely small, so that focusing up and down causes a characteristic 'sparkling effect' as the beads come in and out of focus.

15 Only clearly isolated single cells with a defined plasma membrane are chosen for sampling. The area around the cell is first cleared of debris and other cells, using a small diameter 'sweeping' pipette attached to a micromanipulator, and the position of each cell to be sampled is recorded using the vernier scale on the microscope stage.

16 After applying positive pressure to it via an attached tube and valve, a sampling pipette beveled at 45 degrees is introduced into the bath with the bevel parallel to the surface of the dish using a micromanipulator. The pipette aperture is placed over the cell to be sampled before a very slight positive pressure is applied to pull the cell and only the cell into the pipette.

17 When pooled cell samples are being collected, up to 12 labeled cells are collected into the same pipette in a similar fashion. With any indication that debris or unlabeled cells have been inadvertently sampled, the sample should be discarded.

18 Unlabeled control cells are collected in a similar way, except that clearing of surrounding cells is not performed before sampling.

19 The collected cell(s) is/are expelled from the pipette with slight positive pressure into an RNAse free microfuge tube and the pipette tip is broken within the tube to ensure any adherent cells are also collected. After a brief spin to aggregate the sample, the volume of solution collected is estimated and the sample is snap frozen on dry ice and kept at -80^{o}C until use.

mRNA isolation, amplification cDNA purification and quality a...

20 mRNA amplification and cDNA synthesis of sampled dissociated neurons is performed using SMART-Seq HT kits (Takara Bio USA, Inc., Cat n^{o}634456) according to the manufacturer instructions.

SMART-Seq HT kit **Takara Bio Inc. Catalog #634456**

21 Briefly, lysis buffer containing RNAase inhibitors is added while cells are still frozen. One step PCR reactions containing both first strand synthesis SMARTscribe reverse transcriptase and SeqAmp DNA polymerase are set up with the following protocol:

1) 42^{o}C for 90 min;

2) 95^{o}C for 1 min;

3) 98^{o}C for 10 sec;

4) 65^{o}C for 30 sec;

5) 68^{o}C for 3 min;

(Steps 3-5 for 14 cycles [pooled cell samples] or 16 cycles [single cell samples] depending on the sample type);

6) 72^{o}C for 10 min.

22 cDNA is purified using a magnetic bead kit (Nucleomag NGS clean-up and Size Select, Macherey-Nagel GmbH & Co., Cat n^{o} 744970.5) according to the manufacturer instructions. cDNA quality is assessed by loading purified cDNA into High sensitivity D5000 screen tapes (Agilent Technologies, Cat n^{o} 5067-5592) and analyzing them using a 2200 Tapestation system. cDNA concentration is determined using Qubit dsDNA HS Assay kits (Invitrogen, Cat n^{o}: Q32851) according to manufacturer instructions.

Nucleomag NGS clean-up and Size Select **Takara Bio Inc. Catalog #744970.5**

High Sensitivity D5000 ScreenTape **Takara Bio Inc. Catalog #5067-5592**

cDNA fragment library generation and sequencing

23 These steps are performed by a service provider, namely the Monash Medical Genomics facility according to our requested protocol. Samples are transported on dry ice directly to the facility.

24 Briefly, all samples are quantified again at the facility using Qubit, and 600 pg of amplified cDNA is used for library preparation as recommended by the SmartSeq protocol.

25 Apart from this, the samples are processed according to the standard protocol using the Illumina Nextera XT DNA Library Kit (Ref Guide 15031942)

Nextera XT DNA Library Preparation Kit **Takara Bio Inc. Catalog #FC-131-1096**

26 The libraries are next QC'd by Qubit and Bioanalyzer. They should be similar with a mean size of ~360 bp at a concentration of 15 ng/uL. Prior to sequencing libraries are also quantified by qPCR and size adjusted qPCR concentration is used for sequencing.

27 Library pools are prepared so that both control and labeled cell samples are run together with between 12 and 150 barcoded samples combined in each sequencing run. This sequencing design should yield around 30-40 million reads per pooled cell sample or 5-10 million reads per single cell sample (approximately 400-500 million, or 1,100 million total reads depending on the sequencer), so that expression of low copy number genes such as GPCRs can be quantified.

28 For sequencing, denatured libraries at a loading concentration of 2.0pM (NSQ 550) or non-denatured libraries at a loading concentration of 1000 pM (NSQ 2k) are used and clustering is performed onboard for each high output sequencing run (NextSeq v2.5 75bp runs on an NSQ550 sequencer or v3.0 100 bp runs on an NSQ2k P3 sequencer) together with 1% PhiX control.

29 Criteria for success include a high number of resulting reads per sample, and >90% of reads with >Q30, PhiX sequencing control detected with error rate <0.5%, Phasing <0.4 and Prephasing <0.2.

RNAseq analysis

30 Differential Expression Gene (DEG) analysis is performed using the open source, web-based Galaxy platform.

31 Raw fragment sequencing data (fastq files) is mapped to the latest rat genome assembly (mRatBN7.2, Feb 2021, Sanger Institute) using the alignment tool *HISAT2*.

32 The number of reads mapped to each gene is determined with the light-weight read counting program *Featurecounts*.

33 A single count matrix containing all samples is generated and the differential gene expression of nodose neurons projecting to any two different stomach locations is determined by the Edge R method. Counts are normalized by the trimmed mean of M (log2 fold change between the two samples) values. A threshold of 1 count per million reads is used to filter out low count expression genes when using the whole mRatBN7.2 genome assembly. No low count filter is applied when DEG analysis is performed on smaller subsets of genes from mRatBN7.2 (i.e. GPCRs, ion channels, etc…).