Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60612R2
Full Title:	Combining Laser Capture Microdissection and Microfluidic qPCR to Analyze Transcriptional Profiles of Single Cells: A Systems Biology Approach
Section/Category:	JoVE Genetics
Keywords:	Single-cell gene expression; laser capture microdissection; microfluidic qPCR; Gene expression; opioid dependence; anatomic specificity; amygdala; gut microbiome; glia
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Additional Information:	
Question	**Response**
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TITLE:

Combining Laser Capture Microdissection and Microfluidic qPCR to Analyze Transcriptional Profiles of Single Cells: A Systems Biology Approach to Opioid Dependence

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KEYWORDS:

single cell gene expression, laser capture microdissection, microfluidic qPCR, opioid dependence, anatomic specificity, amygdala, gut microbiome, glia

SUMMARY:

This protocol explains how to collect single neurons, microglia, and astrocytes from the central nucleus of the amygdala with high accuracy and anatomic specificity using laser capture microdissection. Additionally, we explain our use of microfluidic RT-qPCR to measure a subset of the transcriptome of these cells.

ABSTRACT:

Profound transcriptional heterogeneity in anatomically adjacent single cells suggests that robust tissue functionality may be achieved by cellular phenotype diversity. Single-cell experiments investigating the network dynamics of biological systems demonstrate cellular and tissue responses to various conditions at biologically meaningful resolution. Herein, we explain our methods for gathering single cells from anatomically specific locations and accurately measuring a subset of their gene expression profiles. We combine laser capture microdissection (LCM) with microfluidic reverse transcription quantitative polymerase chain reactions (RT-qPCR). We also use this microfluidic RT-qPCR platform to measure the microbial abundance of gut contents.

INTRODUCTION:

Measuring the gene expression profiles of single cells has demonstrated extensive phenotypic heterogeneity within a tissue. This complexity has complicated our understanding of the biological networks that govern tissue function. Our group and others have explored this phenomenon in many tissues and conditions[1–6]. These experiments not only suggest that regulation of gene expression networks underlie such heterogeneity, but also that single-cell resolution reveals a complexity in tissue function that tissue-level resolution fails to appreciate.

Indeed, merely a small minority of cells may respond to a specific condition or challenge, but the impact of those cells on overall physiology may be substantial. Additionally, a system biology approach that applies multivariate methods to high dimensional datasets from multiple cell types and tissues can elucidate system-wide treatment effects.

We combine LCM and microfluidic RT-qPCR to obtain such datasets. We take this approach here in contrast to gathering single cells via fluorescence-activated cell sorting (FACS) and using RNA sequencing (RNA-seq) to measure their transcriptome. The advantage of LCM over FACS is that the exact anatomic specificity of single cells can be documented with LCM, relatively and absolutely. Further, while RNA-seq can measure more features that RT-qPCR, microfluidic RT-qPCR is less expensive and has a higher sensitivity and specificity[7].

In this representative experiment, we investigated the effects of opioid dependence and naltrexone-precipitated opioid withdrawal on rat neuronal, microglia, and astrocyte gene expression in the central nucleus of the amygdala (CeA) and gut microflora abundance[4]. Four treatment groups were analyzed: 1) Placebo, 2) Morphine, 3) Naltrexone, and 4) Withdrawal (**Figure 1**). We found that opioid dependence did not substantially alter gene expression, but that opioid withdrawal induced the expression of inflammatory genes, *Tnf* in particular. Astrocytes were the most affected cell type. The gut microbiome was profoundly impacted by opioid withdrawal as indicated by a decrease in the *Firmicutes* to *Bacteroides* ratio, which is an established marker of gut dysbiosis[8,9].

PROTOCOL:

This study was carried out in accordance with the recommendations of Animal Care and Use Committee (IACUC) of Thomas Jefferson University and Drexel University College of Medicine. The protocol was approved by the Thomas Jefferson University and Drexel University College of Medicine IACUC.

1. Animal model

1.1. Insert two 75 mg slow-release morphine sulfate pellets or two placebo pellets subcutaneously in adult male Sprague-Dawley rats.

1.1.1. Use a gown and gloves appropriately for minor sterile surgery. Shave the rat dorsum with clippers if necessary.

1.1.2. Apply vet ointment to the animal's eyes. Anesthetize the rat with approximately 20 s of isoflurane inhalation. Anesthesia is confirmed by loss of consciousness.

1.1.3. Make a midline incision in the rat dorsum with bead-sterilized blunt scissors and separate the dermis from the body wall with a bead-sterilized probe. Insert the pellets under the dermis with bead-sterilized forceps. Suture the incision closed with a sterile needle.

NOTE: The entire procedure takes about 5 min per rat. Fresh sterile gloves are used for each rat.

1.1.4. Place the rat into an isolation cage for postsurgical recovery. Check for a heartbeat and regular respiratory rhythm. Observe the rat until consciousness is regained. Assess for postsurgical pain.

1.1.5. Assess the rats 8 h postsurgery and every 12 h after for recovery and infection. Place rats in a cage with the rest of the cohort when they are fully recovered from surgery, about 24 h postsurgery.

1.2. Give an intraperitoneal naltrexone injection (75 mg/kg) to the G and the Withdrawal cohorts following 6 days of morphine exposure.

NOTE: There were four rat cohorts in this representative experiment (see **Figure 1**).

2. Sample harvesting

2.1. Harvest the brain 6 days following the pellet insertion or 24 h following the naltrexone injection.

2.1.1. Place the animal in an isoflurane chamber for approximately 30 s or until loss of consciousness occurs, indicated by a lack of motion and decreased respiratory rate.

2.1.2. Use a sharp guillotine to rapidly decapitate the animal.

2.1.3. Dissect the brain out from the animal's skull.

2.1.4. Use a sharp handheld razor to make the following gross incisions to the removed brain: First, slice off the cerebellum and discard. Second, separate the brainstem from the forebrain with a transverse incision. Third, hemisect the forebrain and/or brainstem with a midline sagittal incision.

2.1.5 Place the forebrain and brainstem into a plastic tissue-embedding mold with 3–4 cm of Optimal Cutting Temperature compound (OCT) in the bottom of the mold. Cover the rest of the sample with OCT.

2.1.5. Immediately place the plastic tissue-embedding mold with the sample covered with OCT into a bath containing dry ice and methanol. Do not let the methanol spill into the tissue-embedding mold. Keep the embedding mold with the brain sample in the methanol-ice bath until tissue collection is finished (~10–15 min maximum).

2.1.6. Place the brain sample into a -80 °C freezer as soon as possible.

2.2. Harvest the gut samples concurrently.

2.2.1. Following rapid decapitation, make a midline incision in the animal's abdomen with a scalpel.

2.2.2. Find the cecum and sever its connection to the ascending colon.

2.2.3. Squeeze the cecal contents into a 15 mL conical tube.

2.2.4. Immediately place the conical tube on dry ice and put into a -80 °C freezer as soon as possible.

NOTE: The small intestine contents can also be collected using the same methods as a negative control.

3. Slicing

3.1. Slice the hemisected rat forebrain using a cryostat.

3.1.1. Remove the plastic embedding mold with the forebrain from the -80 °C freezer and place into a -20 °C cryostat.

3.1.2. Remove the OCT-embedded hemisected forebrain sample from the embedding mold. Use a razor to slice the corners of the plastic embedding mold vertically if necessary. Mount the forebrain for rostral to caudal coronal slicing on a cryostat chuck using OCT.

NOTE: Anatomic landmarks to identify the CeA include the optic tract and stria terminalis (**Figure 2B**). The optic tract branches from the optic chiasm and tracks dorsal-lateral as the brain is sliced rostral to caudal. When the optic tract has a morphology similar to what is seen in a rat brain atlas bregma -2.12 mm[10], test slices may be viewed under a microscope. The optic tract and stria terminalis morphology can be checked in a rat brain atlas[10] to identify the bregma and whether the CeA surrounds the stria terminals.

3.1.3. Slice 10 µm thick coronal sections from the hemisected forebrain rostral to caudal until sections containing the CeA are reached.

NOTE: The width and height of the sections are approximately 200 mm.

3.1.4. Collect 10 µm sections containing the CeA, or the preferred brain region, by thaw-mounting 10 µm sections onto plain glass slides. Immediately place the glass slides onto a metal pan resting on dry ice. Put the slides with brain sections into a -80 °C freezer as soon as possible.

NOTE: Multiple slices may be placed on the same slide. If using a different cell type stain for slices on the same slide, leave about 100 mm between slices so a hydrophobic pen can be used to separate cell type-specific antibody solutions on the slide. Leave about 20 mm from the edge of

the slide on each side of the slice.

4. Immunofluorescence staining

4.1. Stain the forebrain sections for the brain cell of choice (e.g., neuron, microglia, astrocyte, etc.) using immunofluorescence.

4.1.1. Remove one or more slides with 10 µm sections of the CeA from the -80 °C freezer.

4.1.2. Fix the slides with 75% ethanol for 30 s. Remove the excess liquid.

4.1.3. Block the slices for 30 s with 2% bovine serum antigen (BSA) in 1x phosphate buffer saline (PBS). Wash 1x with PBS.

4.1.4. Add a primary antibody solution to the slide for 2 min. Wash 1x with 2% BSA solution.

NOTE: The primary antibody solution is composed of 2% primary antibody, 1% RNase Out, and 96% of same BSA PBS solution for the blocking step above (step 4.1.3). The representative experiment used an anti-NeuN antibody, an anti-Cd11β antibody, and an anti-GFAP antibody in the following quantities: 3 µL of primary, 1.88 µL of RNA inhibitor, and 145.12 µL of BSA solution.

4.1.5. Add the secondary antibody solution to the slide for 3 min. Wash 1x with PBS.

NOTE: The secondary antibody solution is composed of 1 µL of goat anti-mouse 488 nm fluorescent tag (1:500), 2.5 µL of RNA inhibitor, 1.3 µL of DAPI (1:10,000), and 196.5 µL of 2% BSA.

5. Ethanol and xylene dehydration series

5.1. Dip the slides into 75% ethanol for 30 s. Immediately after, dip the slides in 95% ethanol for 30 s. Immediately after, dip the slides into 100% ethanol for 30 s. Immediately after, dip the slides into a second container containing 100% ethanol for 30 s.

5.2. Following the ethanol dehydration series, dip the slides into freshly poured xylene for 1 min. Immediately after, dip the slides into a second container of xylene for 4 min.

5.3. Remove the slides from the xylene bath and let air dry in the dark for 5 min.

5.4. Place the slides in a desiccator for 5 min to dry further.

6. Laser capture microdissection

6.1. If stained, place the slide into the microscope and find the region of interest (CeA) using anatomic landmarks (i.e., the optic chiasm and stria terminalis).

6.2. Use fluorescence to identify the stained cell type and its nucleus in the region of interest. Choose one cell or multiple cells if doing single cell pooled samples. Mark the cells of interest using LCM software.

6.3. Place the LCM cap on top of the slice on the region of interest.

6.4. Use test shots of an infrared (IR) laser to adjust the IR laser strength, size, and duration so that the LCM cap adhesive melts only over the area of the selected single cell. This ensures that no other cells will be collected other than the cells selected.

NOTE: In this representative experiment, 10 cell pools of the same cell type were used as a single sample to limit the cell-to-cell variability in gene expression between samples with the same treatment. However, this method can be used for true single cell experiments[1,3].

6.5. Select the individual cells to be collected for analysis using the LCM software tools (**Figure 2C**). Cells selected must be in the anatomic area of the CeA (or the brain region of choice) based on the rat brain atlas and the bregma[10]. Cells should be at least 3 µm from the adjacent stained nuclei.

6.6. Fire the IR laser to collect the identified single cells.

6.7. Place the cap in the quality control (QC) station and view it to ensure that only the desired cells were selected. If other cells were mistakenly selected, an ultraviolet laser can be used to destroy the unwanted cells while the cap remains in the QC station.

6.8. Take a photo of the tissue section from where the cell was collected to document its anatomic specificity. Record the distance of the slice from the bregma if appropriate using a rat brain atlas as a reference[10].

6.9. Remove the LCM cap from the QC station, attach the sample extraction device, and pipette 5.5 µL of lysis buffer onto the sample.

NOTE: The lysis buffer solution consists of 0.5 µL of lysis enhancer and 5 µL of resuspension buffer.

6.10. Fit the ExtracSure device onto a 0.5 mL microcentrifuge tube and place on a hotplate at 75 °C for 15 min.

6.11. Spin down the sample and lysis buffer for 30 s at low speed (0.01–0.02 x *g*) and place the collected sample into a -80 °C freezer.

7. Single-cell microfluidic RT-qPCR

7.1. Preamplification of single cell mRNA for 96.96 Dynamic Array Chip

7.1.1. Combine the forward and reverse mRNA qPCR gene primers for all the genes being assayed in a primer pool for preamplification (500 nM concentration each primer). For example, 1 µL of 100 µM primers in 80 µL plus 120 µL of DNA Suspension Buffer.

NOTE: The primer sequences used for the representative experiment can be found in O'Sullivan et al.[4].

7.1.2. In a new 96 x 96 PCR plate, add 1 µL of 5x VILO to each well.

7.1.3. Remove LCM single cell samples from the samples stored at -80 °C, let thaw briefly, centrifuge briefly at a low speed (0.01–0.02 x *g*), and add 5.5 µL of the lysed single-cell sample to the PCR plate. Each sample is added to its own well.

7.1.4. Place the PCR plate with the samples and VILO into the thermocycler and heat at 65 °C for 1.5 min. Spin the plate for 1 min at 1,300 x *g* at 4 °C and place the plate on ice.

7.1.5. Add 0.15 µL of 10x cDNA synthesis master mix, 0.12 µL T4 Gene 32 protein, and 0.73 µL of DNA suspension buffer to each well.

7.1.6. Place the PCR plate into the thermocycler and run the following protocol: 25 °C for 5 min, 50 °C for 30 min, 55 °C for 25 min, 60 °C for 5 min, 70 °C for 10 min, 4 °C to end.

7.1.7. Add 7.5 µL of Taq polymerase master mix to each well.

7.1.8. Add 1.5 µL of the primer pool (see above) to each well.

7.1.9. Place the PCR plate in the thermocycler and run the following preamplification protocol: 95 °C for 10 min, followed by 22 cycles of 96 °C for 5 sec, 60 °C for 4 min.

7.1.10. Add 0.6 µL of exonuclease I reaction buffer 10x, 1.2 µL exonuclease I, and 4.2 µL of DNA suspension buffer to each well.

7.1.11. Place the PCR plate in the thermocycler and run the following protocol: 37 °C for 30 min, 80 °C for 15 min.

7.1.12. Add 54 µL of TE buffer to each well. Spin the PCR plate at 1,300 x *g* at 5 min. Store at 4 °C if immediately continuing to next step. Store at -20 °C if waiting more than 12 h for next step.

7.2. Prepare the sample plate for the 96.96 Dynamic Array Chip.

7.2.1. In a new 96 well PCR plate, add 0.45 µL of 20x DNA binding dye and 4.55 µL of low ROX mastermix to each well.

7.2.2. Add 3 µL of preamplified sample to each well, spin the PCR plate at 1,300 x *g*, then put the plate on ice.

7.3. Prepare the assay plate for the 96.96 Dynamic Array Chip.

7.3.1. In a new 96 well PCR plate, add 3.75 µL of 2x GE assay loading reagent and 1.25 µL of DNA suspension buffer to each well.

7.3.2. Add 2.5 µL of 10 µM qPCR primer to each corresponding well. Spin the PCR plate at 1,300 x *g* for 5 min.

7.4. Load and run the 96.96 Dynamic Array Chip.

7.4.1. Prime the chip with control line fluid.

7.4.2. Place the chip in an IFC Controller HX and run the Prime (136X) script.

7.4.3. Add 6 µL of the sample from the PCR sample plate into the corresponding sample wells in the 96.96 Dynamic Array Chip.

7.4.4. Add 6 µL of the sample from the PCR assay plate into the corresponding assay wells in the 96.96 Dynamic Array Chip.

7.4.5. Use needles to pop any air bubbles in the wells of the 96.96 Dynamic Array Chip.

7.4.6. Place the 96.96 Dynamic Array Chip into the IFC Controller HX and run the Load Mix (136x) script.

7.4.7. Remove the chip from the IFC Controller HX, peel off the protective sticker, and place the 96.96 Dynamic Array Chip into a microfluidic RT-qPCR platform. Run the GE Fast 96 x 96 PCR protocol (30 cycles).

NOTE: The RNA quality and validity of the results are assessed by multiple methods, including assay validation via gel electrophoresis, melting temperature curves, sample and assay replicates, and standard dilution series plots. Additionally, transcriptional findings can be validated by independent methods on brain hemisection including Western blot and immunofluorescence assays.

8. Measuring the bacterial abundance with microfluidic RT-qPCR

8.1. Extract the bacterial DNA following the directions of the stool DNA extraction kit.

8.2. Estimate the bacterial DNA concentration using qPCR.

8.3. Add the extracted bacterial DNA to a new PCR plate. Add 1 µL of extracted bacterial DNA and 9 µL of DNA Suspension buffer.

8.4. Prepare the assay plate for the 48.48 Dynamic Array Chip (see steps 7.2.1–7.2.2)

8.5. Prepare the sample plate for the 48.48 Dynamic Array Chip (see steps 7.3.1–7.3.2)

8.5.1. In a new 96 well PCR plate, add 0.45 µL of 20x DNA binding dye and 4.55 µL of low ROX mastermix to 48 wells.

8.5.2. Add 3 µL of the sample from the PCR plate containing the bacterial DNA to the 48 wells and spin down the PCR plate at 1,300 x *g* for 5 min. Store at 4 °C.

8.6. Load and run the 48.48 Dynamic Array Chip (see steps 7.4.1–7.4.7).

REPRESENTATIVE RESULTS:

The selection of the single cells was validated both visually and molecularly. Visually, cellular morphology was viewed before cell collection. Cells collected were then viewed at the QC station and the cellular nuclei stain (DAPI) overlapped with the single cell selection marker fluorescence. **Figure 2A** shows representative images of a slide with hemisected rat forebrain containing the CeA. Subsequent images (**Figure 2 B**–**D**) show the selection of single cells and their removal from the tissue for transcriptomic analysis. Molecularly, the cell type-specific markers demonstrated increased expression in that cell type (**Figure 1C**). We looked at neurons, microglia, and astrocytes and measured the expression of *NeuN*, *Maf*, and *Gfap*, respectively. The figures were originally published in O'Sullivan et al.[4].

Further, controls can also be run in the microfluidic platform to validate the expression findings (e.g., analysis of other areas of the same tissue to demonstrate nucleus specificity). A separate tissue could also be compared to the desired sample to demonstrate primer specificity in the tissue of interest. Positive and negative control genes can also be included (e.g., genes known to either be absent from the selected tissue or expressed highly). Three or four housekeeping genes should also be included not only for data normalization purposes but also as a measure of experimental quality. These genes should demonstrate the lowest variance in expression across all samples and treatments. In this representative experiment, no alternate brain region was assayed, but housekeeping genes *Ldha* and *Actb* were used for normalization. *Gapdh* was used as an internal control.

Figure 3 displays some of the multivariate methods our group used to analyze our data. We found that astrocytes in the Withdrawal group were the most affected cell type. Based on these data in the context of other studies we speculate that astrocytes play a key role in inflammation in the CeA during opioid withdrawal and that this contributes to the physical and emotional symptoms that drive drug-seeking via negative reinforcement. We also show the gut microflora data (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Single-cell RT-qPCR workflow and transcriptional heterogeneity. (**A**) Experimental protocol (n = 4 for each condition) (**B**) Ten-cell pooled sample transcriptome measurement. (**C**) Bar plot displays median -ΔΔCt expression values. Neurons = purple; microglia = yellow; astrocytes = green. Error bars show standard error. *p < 0.05, ***p < 0.0003; Tukey's honest significance test. (**D**) The heat map shows the expression of all samples across 40 assayed genes. Rows are 10-cell pooled samples (930 neuronal samples, 950 microglial samples, 840 astrocyte samples as denoted); the numbers denote the sample clusters and the columns are the genes. The figure is modified from O'Sullivan et al.[4].

Figure 2: Laser capture microdissection images. (**A**) Four slices of dehydrated hemisected rat forebrain containing the CeA on a slide. Slices were placed on the slide exactly 10 µm from the previous slice. Left is anterior and right is posterior. The distance from the bregma can be estimated using a rat brain atlas and landmarks, including the optic tract and stria terminalis. (**B–D**) Sequence of images showing the selection of single cells in the CeA (**C**) and their removal from the tissue (**D**). Multiple LCM caps were used to select these cells. One cap is used to pick 10 cells of one cell type.

Figure 3: Representative results 1. (**A**) A cartoon schematic of a cell displaying the genes assayed and their location. The gene symbols labeled here are a reference for panel B. (**B**) The colored squares represent relative gene expression (median –ΔΔCt value) for the genes represented in panel A. The location of the squares represents the cellular localization or function of the corresponding protein. The panels display the relative gene expression represented by color across treatments and cell types. Yellow = high expression; blue = low expression; white = neutral expression. The figure is modified from O'Sullivan et al.[4].

Figure 4: Representative results 2. (**A**) Gene correlation networks. Pearson correlation was performed on the $-\Delta\Delta C_t$ values within a treatment and cell type. The nodes denote the genes and their color signifies the relative expression levels (the median -ΔΔCt value for each gene). The edges denote expression correlations and the thickness signifies the strength of the expression correlation (ρ). Correlations with a q-value <0.001 are displayed. Black edges = positive correlations; green edges = negative correlations. (**B**) Bar plots of select genes demonstrating significant differential gene expression. The statistics were calculated using nested ANOVA #p < 0.1, *p < 0.05, **p < 0.01, ***p < 0.0001 (n = 4 animals for all treatments). The figure is modified from O'Sullivan et al.[4].

Figure 5: Relative abundance of gut microflora. The barplots display the relative abundance of bacterial species ($-\Delta\Delta C_t$ values). #p < 0.1, *p < 0.05, **p < 0.008, ***p = 0.0009; two-way ANOVA; n = 4 animals for each treatment. The figure is modified from O'Sullivan et al.[4].

DISCUSSION:

Single-cell biology has demonstrated the heterogeneity of cellular phenotypes and robustness of tissue function. These findings have provided insight into the organization of biological systems

at both macro and micro scales. Here, we describe the combination of two methods, LCM and microfluidic qPCR, to obtain single-cell transcriptome measures that provide anatomic specificity and transcriptional accuracy at a relatively low cost (**Figure 1**). Our group takes a systems biology approach and often measures multiple tissues in the same animal. We find these methods to be both flexible and fruitful in determining how biological systems respond to various challenges at the transcriptional level. Additionally, we use these methods in the anatomic mapping of cellular phenotypes in baseline conditions.

We provide data and modified figures from a recent publication exploring how the CeA responds to opioid dependence and withdrawal[4]. In this example, we used the same microfluidic qPCR platform to measure the relative abundance of the gut microflora. The methods and workflow are summarized in **Figure 1** and were originally published in O'Sullivan et al.[4]. Major findings from high-throughput microfluidic RT-qPCR can be subsequently validated by protein measures such as Western blot or immunofluorescence[4].

A major challenge of this systems biology approach is determining specific causal biological mechanisms. Fuzzy logic is a validated solution that we have employed with success to infer agents in gene regulatory network behavior[2]. Animal model manipulation may also be employed to provide insight into systemic mechanisms. For example, the same protocol provided herein with the addition of a rat cohort with a gastric vagotomy will yield data that provide insight into the flow of information via the vagus nerve.

DISCLOSURES:

The authors declare that they have no competing financial interests.

ACKNOWLEDGEMENTS:

The work presented here was funded through NIH HLB U01 HL133360 awarded to JS and RV, NIDA R21 DA036372 awarded to JS and EVB, and T32 AA-007463 awarded to Jan Hoek in support of SJO'S.

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Figure 1

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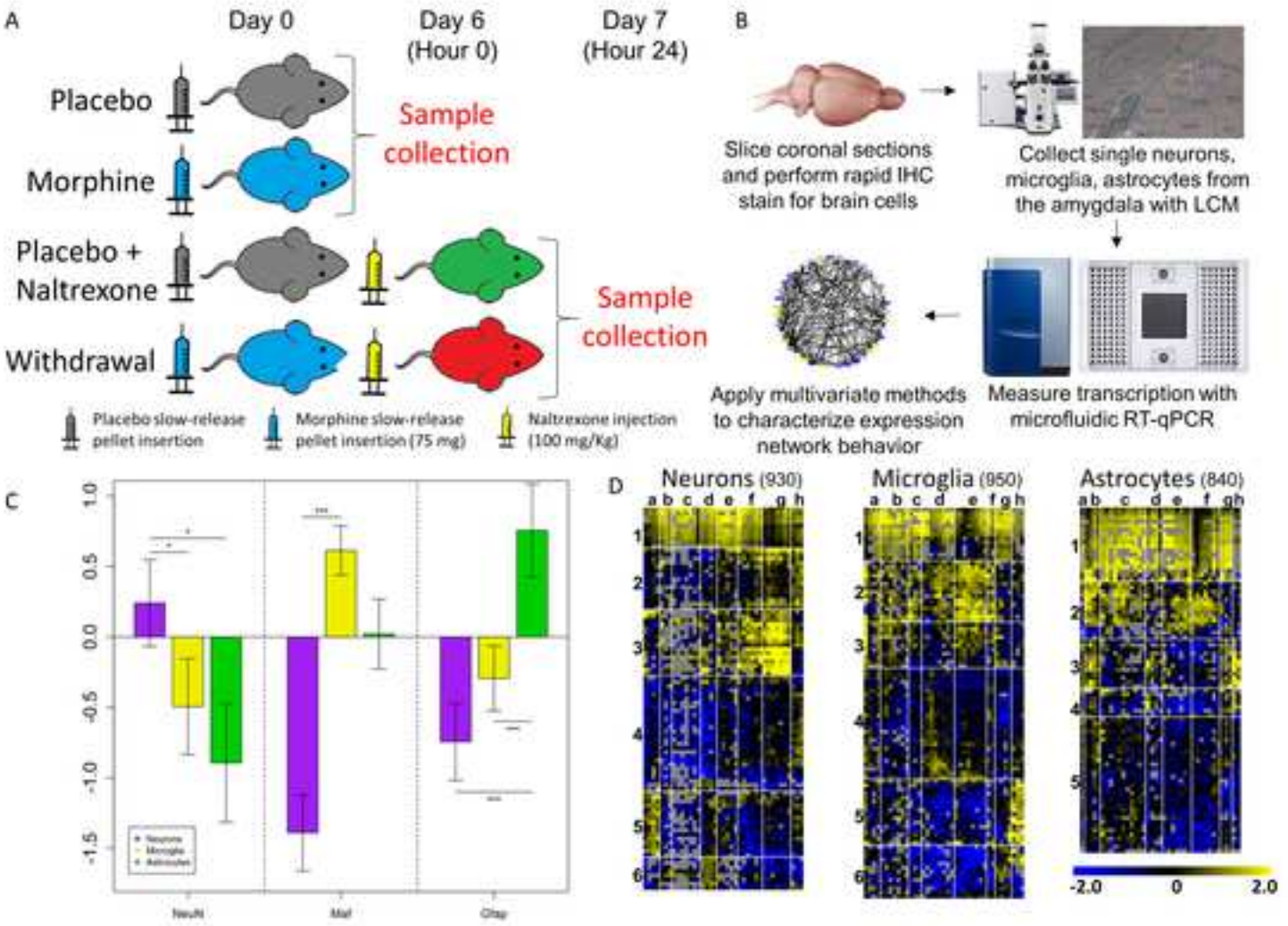

Figure 2

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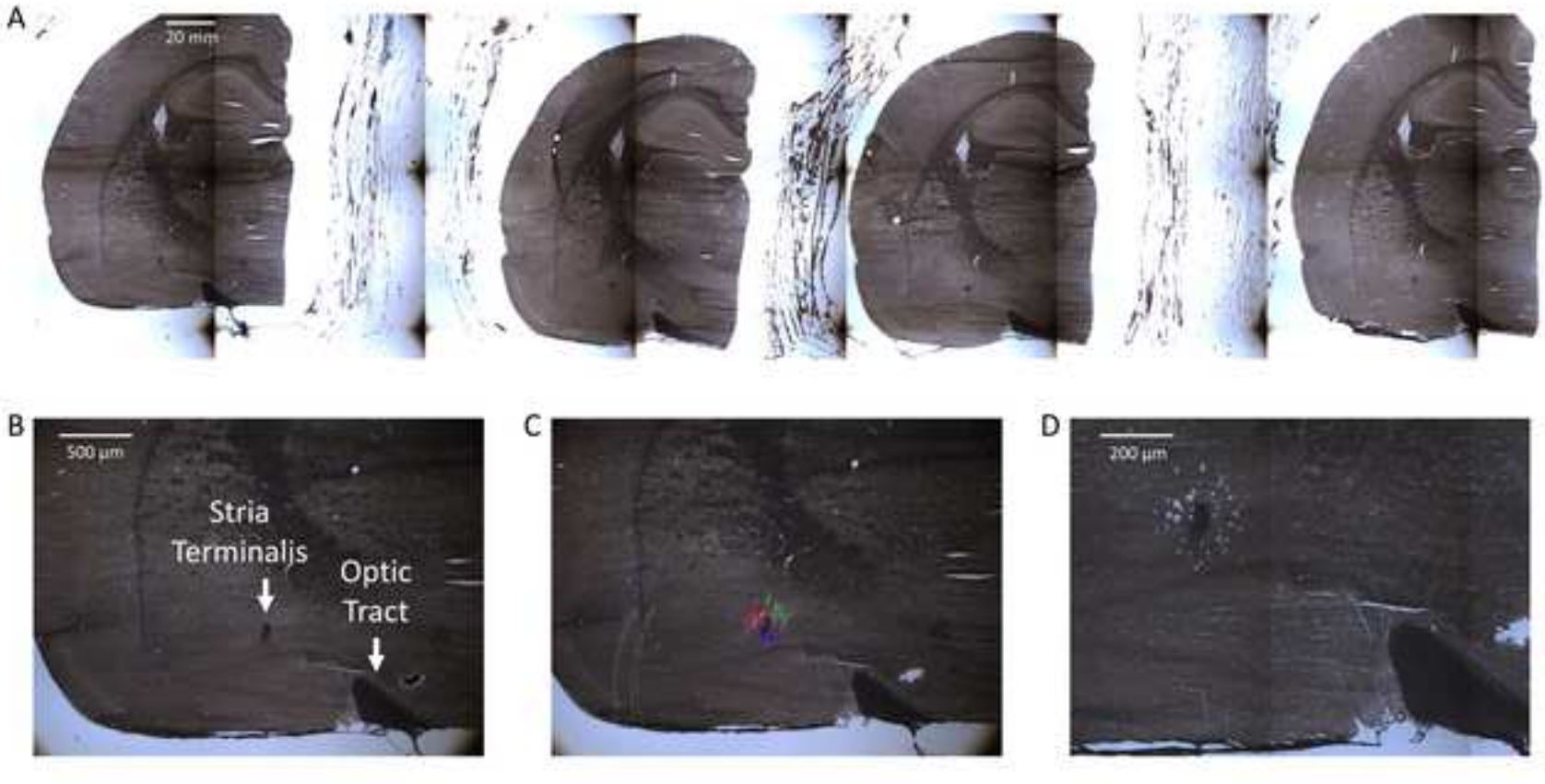

Figure 3

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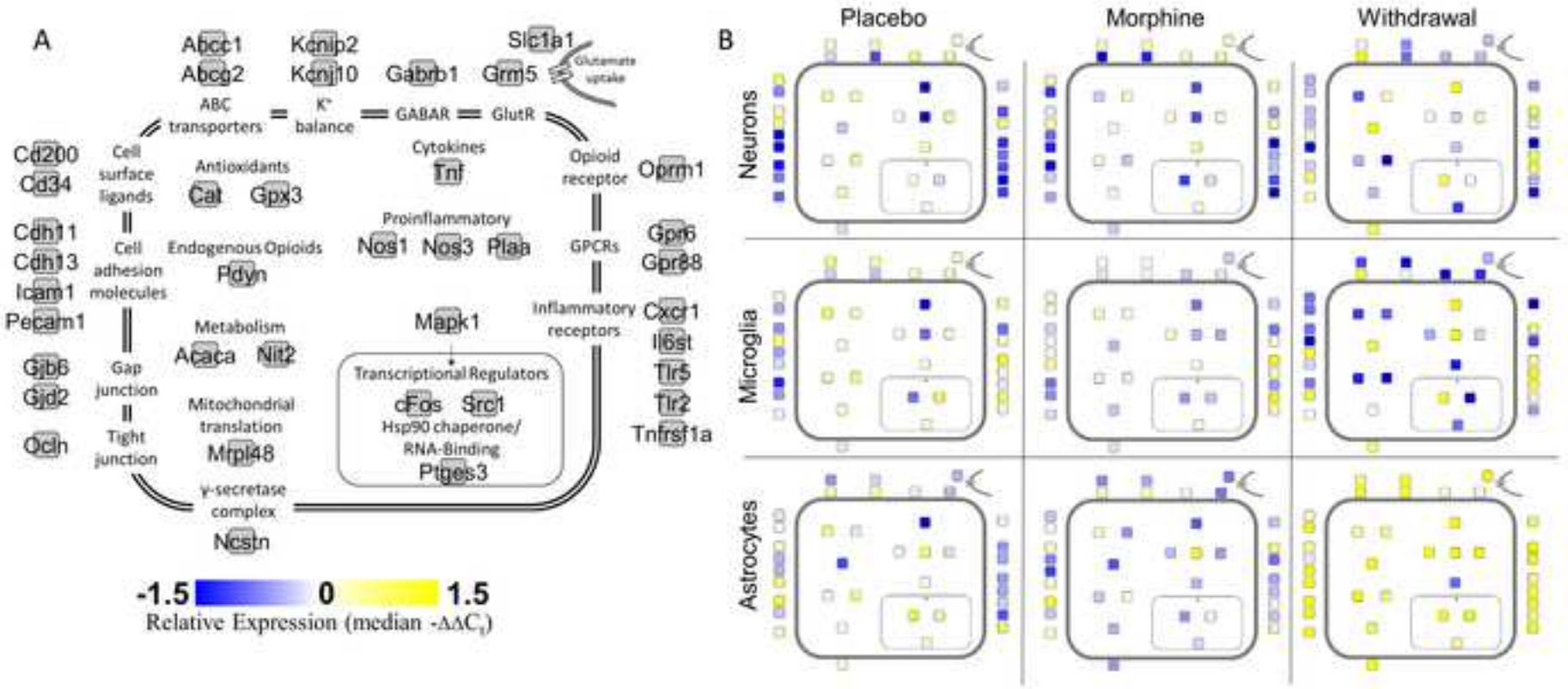

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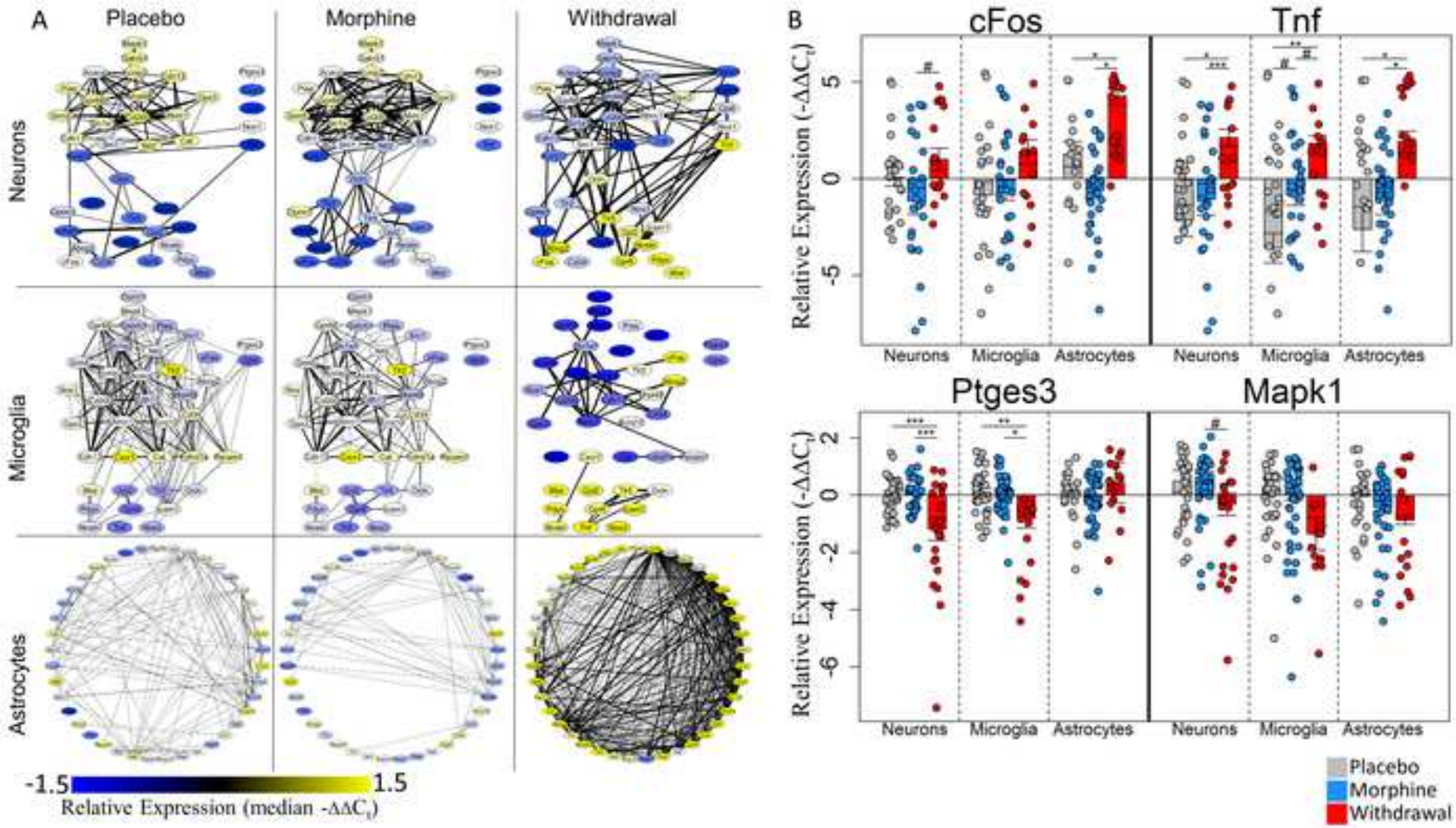

Figure 5

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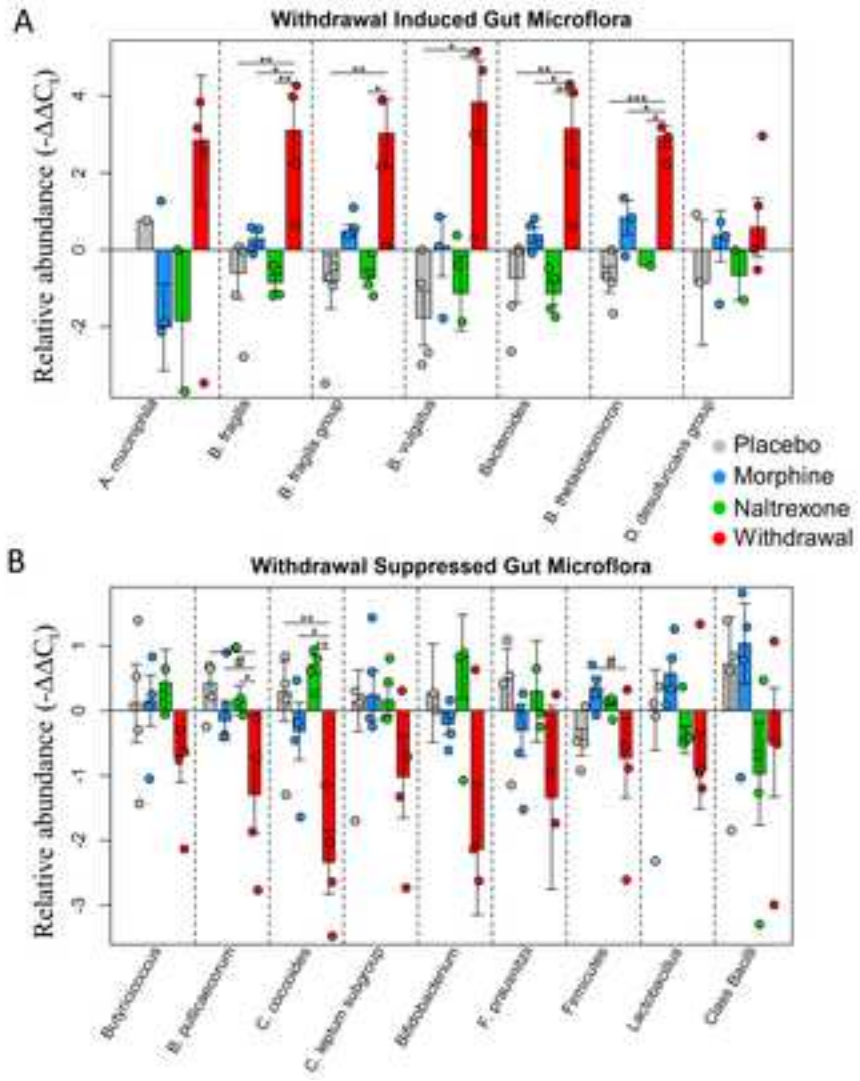

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
20X DNA Binding Dye	Fluidigm	100-7609	NA
2x GE Assay Loading Reagent	Fluidigm	85000802-R	NA
48.48 Dynamic Array IFC for Gene Expression	Fluidigm	BMK-M-48.48	NA
96.96 Dynamic Array IFC for Gene Expression	Fluidigm	BMK-M-96.96	NA
Anti-Cd11β Antibody	Genway Biotech	CCEC48	Microglia Stain
Anti-NeuN Antibody, clone A60	EMD Millipore	MAB377	Neuronal Stain
ArcturusXT Laser Capture Microdissection System	Arcturus	NA	NA
Biomark HD	Fluidigm	NA	RT-qPCR platform
Bovine Serum Antigen	Sigma-Aldrich	B4287	
CapSure Macro LCM Caps	ThermoFisher Scientific	LCM0211	NA
CellDirect One-Step qRT-PCR Kit	ThermoFisher Scientific	11753500	Lysis buffer solution components
DAPI	ThermoFisher Scientific	62248	Nucleus Stain
DNA Suspension Buffer	TEKnova	T0221	
Exonuclease I	New Englnad BioLabs, Inc.	M0293S	NA
ExtracSure Sample Extraction Device	ThermoFisher Scientific	LCM0208	NA
Fisherbrand Superfrost Plus Microscope Slides	ThermoFisher Scientific	22-037-246	Plain glass slides
GeneAmp Thin-Walled Reaction Tube	ThermoFisher Scientific	N8010611	
GFAP Monoclonal Antibody	ThermoFisher Scientific	A-21294	Astrocyte Stain
Goat anti-Mouse IgG (H+L), Superclonal™ Recombinant Secondary Antibody, Alexa Fluor 488	ThermoFisher Scientific	A28175	Seconadry Antibody
IFC Controller	Fluidigm	NA	NA

RNaseOut	ThermoFisher Scientific	10777019	
SsoFast EvaGreen Supermix with Low Rox	Bio-Rad	PN 172-5211	Rox master mix
SuperScript VILO cDNA Synthesis Kit	ThermoFisher Scientific	11754250	Contains VILO and SuperScript
T4 Gene 32 Protein	New Englnad BioLabs, Inc.	M0300S	NA
TaqMan PreAmp Master Mix	ThermoFisher Scientific	4391128	NA
TE Buffer	TEKnova	T0225	NA

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Title of Article: Combining Laser Capture Microdissection and Microfluidic qPCR to Analyze Transcriptional Profiles of Single Cells: A Systems Biology Approach to Opioid Depedence

Author(s): Sean O'Sullivan, Beverly A.S. Reyes, Rajanikanth Vadigepalli, Elisabeth J. Van Bockstaele, James S. Schwaber

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Dear Editor,

The rebuttal letter below outlines the comments by the editor and reviewers addressed by the authors.

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Comment 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Revision: Complete.

Comment 2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Revision: Uploaded

Comment 3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Revision: All symbols and brand names removed.

Comment 4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Revision: Added: “This study was carried out in accordance with the recommendations of Animal Care and Use Committee (IACUC) of Thomas Jefferson University and Drexel University College of Medicine. The protocol was approved by Thomas Jefferson University and Drexel University College of Medicine IACUC.”

Comment 5. Please mention how animals are anesthetized and how proper anesthetization is confirmed.

Revision: Added to step 1.1.2: “1.1.2 Anesthetize rat with approximately 20 seconds of isoflurane inhalation. Anesthesia confirmed with loss of consciousness.” And “2.1.1 Place animal in isofluorane chamber for approximately 30 seconds or until loss of consciousness occurs indicated by lack of motion and decreased respiratory rate. “

Comment 6. Please use a single space between numerical values and their units.

Revision: Complete

Comment 7. Please do not abbreviate journal titles for references.

Revision: Complete.

Comment 8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Revision: Complete

Comment 9. Please provide at least 10 references to support your manuscript.

Revision: Added two additional references.

Reference 6" "Papalexi, E. & Satija, R. Single-cell RNA sequencing to explore immune cell heterogeneity. *Nature Reviews Immunolology.* **18**, 35–45 (2018)."

And reference 10: "Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates: Hard Cover Edition*. (2006)."

Reviewer 1:

Comment 1. Abstract: What do you mean by "…tissue responses to various conditions at resolution."? Do you mean 'at resolution of the single cell', or 'upon resolution of the condition', as in ameliorating the effects of opioid withdrawal? Please specify the usage of the word "resolution".

Revision: "Single-cell experiments investigating the network dynamics of biological systems demonstrate cellular and tissue responses to various conditions at biologically meaningful resolution."

Comment 2. Line 62: please add that this study is in a rat model.

Revision: added "rat"

Comment 3. Line 75: Please indicate the anatomical location of the pellet. Include instructions on how to insert the morphine and placebo, trochar/needle size, concentration and volume of morphine.

Revision: added: "1.1 Insert 2 75 mg slow-release morphine sulfate pellets or 2 placebo pellets subcutaneously."

1.1.1 Shave rate dorsum with clippers if necessary

1.1.2 Anesthetize rat with ~approximately 20 seconds of isoflurane inhalation. Anesthesia confirmed with loss of consciousness.

1.1.3 Make a midline incision in the rat dorsum with blunt scissors and separate dermis from body wall with probe. Insert pellets under dermis with forceps. Suture the incision closed."

Comment 4. Line 92: Please indicate the size/dimensions of the brain slices (length, width, height).

Revision: added: “3.1.4 Slice 10 µm thick coronal sections from hemisected forebrain rostral to caudal until sections containing the central nucleus of the amygdala (CeA) are reached. Width and height of sections approximately 200 mm.”

Comment 5. Line 93: "Hemisect the forebrain and/or brainstem" - please indicate if this is a sagittal or transverse plane hemisection. Does it make any difference for the LCM?

Revision: Now reads: “2.1.4 Use a sharp hand-held razor to make the following gross incisions to the removed brain. Slice off the cerebellum and discard. Separate brainstem from forebrain with a transverse incision. Hemisect forebrain and/or brainstem with a midline sagittal incision.”

Comment 6. Line 98: How long do you leave the OCT embedded tissue in the dry ice-methanol?

Revision: Now reads: “2.1.6 Immediately place plastic tissue embedding mold with sample covered by O.C.T. into bath containing dry ice and methanol. Do not let methanol spill into tissue embedding mold. Keep embedding mold with brain sample in methanol-ice bath until tissue collection is finished (approximately 10-15 minutes maximum).”

Comment 7. Line 122: Why do you need a razor to remove the forebrain from the plastic mold? Do you need to trim off the OCT? OCT embedded tissue should readily pop-out of a plastic cryomold.

Revision: Now reads: “3.1.2 Remove O.C.T. embedded hemisected forebrain sample from embedding mold. Use razor to slice the corners of the plastic embedding mold vertically if necessary. Mount forebrain for rostral to caudal coronal slicing on cryostat chuck using O.C.T.”

Comment 8. Line 125: How do you know when you have reached the CeA? Please provide some anatomical landmarks.

Revision: Added: “3.1.3 Anatomic landmarks to identify the CeA include the optic tract and stria terminalis. The optic tract branches from the optic chiasm and tracks dorsal-lateral as the brain is sliced rostral to caudal. When the optic track has a morphology similar to what is seen in rat brain atlas bregma -2.12 mm, test slices may be viewed under the microscope. Optic tract and stria terminalis morphology can be referenced in a rat brain atlas to determine the bregma and if CeA surrounds the stria terminals.”

Comment 9. Line 129: Please specify any special characteristics of the glass slides, such as plain, uncoated, charged, or poly-L-lysine coated. Typically plain slides are used for LCM and coated/charged slides are used for IHC/IF.

Revision: Now reads: “3.1.5 Collect 10 µm sections containing the CeA, or preferred brain region, by thaw-mounting 10 µm sections onto plain glass slides. Multiple slices may be placed on same slide. If

using a different cell type stain for slices on the same slide, leave about 100 mm between slices so hydrophobic pen can be used to separate cell type-specific antibody solutions on the slide. Leave about 20 mm from the edge of the slide on each side of the slice. Immediately place glass slides onto metal pan resting on dry ice. Put slides with brain sections into -80° C freezer as soon as possible."

Comment 10. Line 139: This note describes separating multiple sections on a slide with a hydrophobic pen (Pap pen). Please add a note to section 3.1.3 describing the placement of multiple tissue sections on a slide. Include information regarding the usable area of the slide for LCM (e.g. not to close to the edges or either end of the slide).

Revision: Now reads: "3.1.5 Collect 10 µm sections containing the CeA, or preferred brain region, by thaw-mounting 10 µm sections onto plain glass slides. Multiple slices may be placed on same slide. If using a different cell type stain for slices on the same slide, leave about 100 mm between slices so hydrophobic pen can be used to separate cell type-specific antibody solutions on the slide. Leave about 20 mm from the edge of the slide on each side of the slice. Immediately place glass slides onto metal pan resting on dry ice. Put slides with brain sections into -80° C freezer as soon as possible."

Comment 11. Line 144: Do you remove the BSA blocking solution prior to adding the primary antibody? If so, do you rinse the slide or just remove the BSA?

Revision: Now reads: "4.1.3 Block slices for 30 s with 2% bovine serum antigen (BSA) in phosphate buffer saline 1x (PBS). Wash 1x with PBS."

Comment 12. Line 152: How many times do you wash the slide with 2% BSA?

Revision: Now reads: "4.1.4 Add primary antibody solution to slide for 2 min. Primary antibody solution is composed of 2% primary antibody, 1% RNase Out, and 96% of same BSA PBS solution for blocking step above. We used anti-NeuN antibody, anti-Cd11β antibody, or anti-GFAP antibody in the following quantities: 3 µL of primary, 1.88 µL of RNA inhibitor, and 145.12 µL of BSA solution. Wash 1x with 2% BSA solution."

Comment 13. Line 156: How many times do you wash the slide with PBS?

Revision: Now reads: "4.1.5 Add secondary antibody solution to slide for 3 min. Secondary antibody solution is composed of 1 µL of goat anti-mouse 488 nm fluorescent tag (1:500), 2.5 µL RNA inhibitor, 1.3 µL of DAPI (1:10000), and 196.5 µL of 2% BSA. Wash 1x with PBS."

Comment 14. Line 173: Please insert the phrase "IF stained" between "Place slide…" to ensure that the reader understands you are using the IF stained slides for LCM.

Revision: Complete

Comment 15. Lines 183-186: Are you collecting a single cell on each LCM cap? Or are you collecting

multiple cells, all of the same cell type, on one cap? The use of the terms "single-cell" and "single-cells" appear to be used interchangeably throughout the manuscript. In this manuscript, the authors are microdissecting multiple cells of a specific cell type, and pooling 10 cells together as a 'specimen'. The use of the terms 'single-cell' and 'single-cells' is somewhat misleading. A better term for this study is 'specific cell types'. A true single-cell analysis means only one cell is microdissected per LCM cap and used for downstream analysis. From the reference paper, Frontiers in Neurosci 2019 "We gathered 1060 neurons, 1070 microglia, and 1060 astrocytes from the CeA of rats that were either given placebo pellets (Placebo, n = 4), morphine pellets (Morphine, n = 4), or experienced 24 h of acute naltrexone-precipitated morphine withdrawal (Withdrawal, n = 4) (Figure 1A). 33,088 individual PCR reactions occurred using the microfluidic BioMarkTM platform (Fluidigm©) (Figures 1A,B). Strict quality control was employed to limit inaccuracies producing a dataset containing 13,650 individual data points. In total, the expression of 46 gene transcripts across 930 neurons, 950 microglia, and 840 astrocytes were analyzed (Figure 1C)."

Revision: Added: “Note: In this representative experiment, 10-cell pools of the same cell type were used as a single sample to limit the cell-to-cell variability in gene expression between samples of the same treatment. However, this method can be used for true single-cell experiments as we have done previously.”

Comment 16. Line 190: Please describe how you place the cap back on the slide from the QC station. Can you use the ArcturusXT software or do you have to manually place the cap on the slide? Older versions of the ArcturusXT software do not allow cap movement from the QC station back to the slide.

Revision: Now reads: 6.7 Place cap in quality control (QC) station and view it to observe that only desired cells were selected. If other cells were mistakenly selected, ultraviolet laser can be used to destroy these cells while cap remains in QC station.”

Comment 17. Line 193: The bregma is an anatomical location on the skull. How do you match/measure the distance from this skull location to a piece of tissue?

Revision: Cited a rat brain atlas throughout to clarify this point. Also added: “6.8 Take a photo of the tissue section from where to the cell was collected to document anatomic specificity. Record distance of slice from bregma if appropriate by reference to rat brain atlas.”

Comment 18. Line 204: Specify the rpm for the centrifuge speed.

Revision: Done

Comment 19. Line 219: Specify the rpm for the centrifuge speed.

Revision: Done

Comment 20. Line 294: list the specific step numbers for preparing the assay plate, rather than simply stating "same as above".

Revision: Added: 8.3 Prepare assay plate for 48.48 Dynamic Array (steps 7.2.1 – 7.2.2)

Comment 21. Line 304: list the specific step numbers for loading and running the array chip, rather than simply stating "same as above".

Revision: Added: “8.4 Prepare sample plate for 48.48 Dynamic Array (steps 7.3.1 – 7.3.2)”

Comment 22. Lines 315-315: The phrase "Originally published in O'Sullivan et al. 2019" should have a reference citation.

Revision: Done

Comment 23. The figures are very low resolution making interpretation difficult. Please provide high resolution images.

Revision: Done. Split Figure 3 into two separate figures.

Comment 24. Line 337-343 Figure 1 legend 1: Part B Single cell data - does this truly represent just one cell, or is the data from 10 cell pools. See comment #15 above regarding the term single-cell versus a collection of individual cells. Please make sure the reader can understand how many cells are being analyzed for each assay, and in each figure panel. Part D: Please specify how many samples and genes are shown in the heatmap. Please specify the meaning of the numbers in parenthesis in the heatmap titles.

Revision: Legend now reads: “**Figure 1. Single-cell RT-qPCR workflow and transcriptional heterogeneity.** Modified from O’Sullivan et al. 2019 **(A)** Experimental protocol (n=4 for each condition) **(B)** 10-cell pooled sample transcriptome measurement. **(C)** Bar plot displays median -ΔΔCt expression values. Neurons are purple, microglia are yellow, astrocytes are green. Error bars show standard error. *p<0.05, ***p<0.0003 Tukey’s honest significance test. **(D)** Heat map shows expression of all samples across 40 assayed genes. Rows are 10-cell pooled samples (930 neuronal samples, 950 microglial samples, 840 astrocyte samples as denoted); numbers denote sample clusters) and columns are genes.”

Comment 25. Line 349 Figure legend 2: Please specify if the image shows microdissection of a collection of individual cells on one cap or if multiple caps were used to collect one cell. The phrase "showing the selection of single cells" implies that multiple cells, of the same type, were collected on one LCM cap.

Revision: **Figure 2. Laser Capture Microdissection Images. (A)** Four slices of dehydrated hemisected rat forebrain containing CeA on a slide. Slices are were placed on slide exactly 10 µm from previous slice. Left is anterior and right is posterior. Distance from bregma can be estimated using rat brain atlas and landmarks including the optic tract and stria terminalis. **(B-D)** Sequence of images showing the selection of single-cells in the CeA (C) and their removal from the tissue (D). Multiple LCM caps were used to select these cells. 1 cap is used to pick 10 cells of one cell type.“

Comment 26. Lines 351-362 Figure legend 3: Part A does not make sense. Please provide a descriptive figure legend for panel A. Panels B and C are too small and low resolution to be able to interpret. It is difficult to discern the differences in the thick and thin correlation lines in panel C. Panel C: the correlation coefficient should be denoted as the Greek symbol rho (ρ), rather than the letter "q".

Revision: This was clarified: "Figure 4. Representative Results 2. Modified from O'Sullivan et al. 2019. **(A)** Gene correlation networks. Pearson correlation was performed on the $-\Delta\Delta C_t$ values within a treatment and cell type. Nodes denote genes and their color signifies relative expression levels (median -ΔΔCt value for each gene). Edges denote expression correlations; thickness signifies strength of expression correlation (ρ). Correlations with a q-value < 0.001 are displayed. Black edges are positive correlations and green edges are negative correlations. **(B)** Bar plots of select genes demonstrating significant differential gene expression. Statistics were calculated using nested ANOVA (#p<0.1, *p<0.05, **p<0.01, ***p<0.0001 n=4 animals for all treatments)."

Reviewer 2:

Comments: Minor Concerns:

process. As this is a methods paper, the authors could give more detail of the microfluidic PCR. Additionally, some data on RNA quality would also be helpful.

Revisions: The microfluidic RT-qPCR platform Biomark™ is explained in great detail elsewhere. We do not believe a description here adds to the quality of this methods paper.

We assess the quality of our RNA and experiments through a variety of methods. These include validation of primer sequences via electrophoresis gel, melting temperature curves, sample and assay replicates, and standard dilution series plots. After discussion, we concluded that the inclusion of raw data from these quality control measures were beyond the scope of this methods paper.

We added the following note: did add the following note: "Note: RNA quality and result validity is assessed by multiple methods including assay validation via gel electrophoresis, melting temperature curves, sample and assay replicates, and standard dilution series plots. Additionally, transcriptional findings can be validated by independent methods on brain hemisection including Western blot and immunofluorence assays."

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Letter from Editor:

Frontiers Neuroscience Production Office <neuroscience.production.office@frontiersin.org> Jul 29, 2019, 10:47 AM

to me

Good afternoon Dr. O'Sullivan,

Thank you for your email.

You do not need our permission to use your own figures from your published article.
We just ask that you cite the figures correctly in the methods paper.

Kind regards,
Folakemi

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