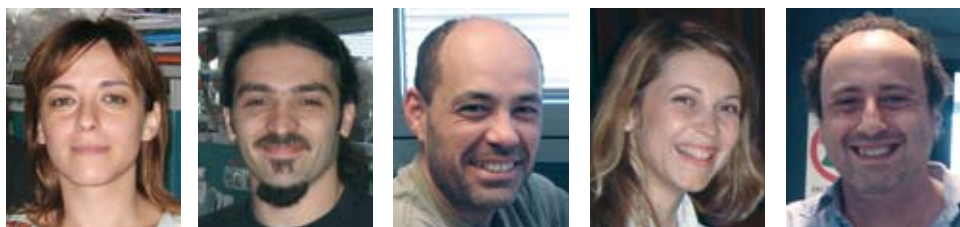


Gene expression profiling of laser capture–microdissected neuronal populations in the mammalian CNS



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Introduction

The central nervous system (CNS) contains an enormous variety of cell types which give rise to neuronal clusters, anatomically defined nuclei and subnuclei, which in turn organize into intricate and complex networks. The lack of adequate markers to discern unequivocally among this cellular heterogeneity renders the challenging task of dissecting out such neural networks and the cells that comprise them. We made use of an integrative multidisciplinary approach consisting of i) laser capture microdissection (LCM) which allows the collection of neuroanatomically defined, relatively pure neuronal cell populations, ii) global mRNA amplification with the μ MACS™ SuperAmp Kit, and iii) microarray technology in order to study and understand

the underlying molecular mechanisms of previously characterized and uncharacterized neuronal types. We used the TH-GFP/21-31 line of transgenic mice¹ for gene expression profiling of dopaminergic (DA) cells from the mesencephalon, that co-express green fluorescence protein (GFP). We also analyzed somatostatin-expressing hippocampal interneurons co-expressing enhanced GFP (EGFP), isolated from mice of the transgenic line GIN (GFP-expressing inhibitory neurons)².

Materials and methods

Tissue preparation and LCM

Adult TH-GFP/21-31 mice and GIN mice were

sacrificed by cervical dislocation. The brains were eased out of the skull, quickly washed in ice cold PBS, fixed, cryoprotected, and rapidly frozen in liquid nitrogen-cooled 4-methylbutane. For LCM coronal sections of 14 μ m from the mesencephalon and the hippocampal formation were cut in the cryostat. The mesencephalon sections were mounted on polyethylene naphthalate (PEN) membrane-coated slides (PALM Microlaser Technologies) and the hippocampal sections on SuperFrost plus glass slides (Menzel-Glaser).

For one set of experiments, 100 DA GFP-expressing cells and 100 non-DA cells were excised by laser microdissection and collected by laser pressure catapulting (LPC) from the substantia nigra compacta (SNc) of the mesencephalon sections from the TH-GFP/21-

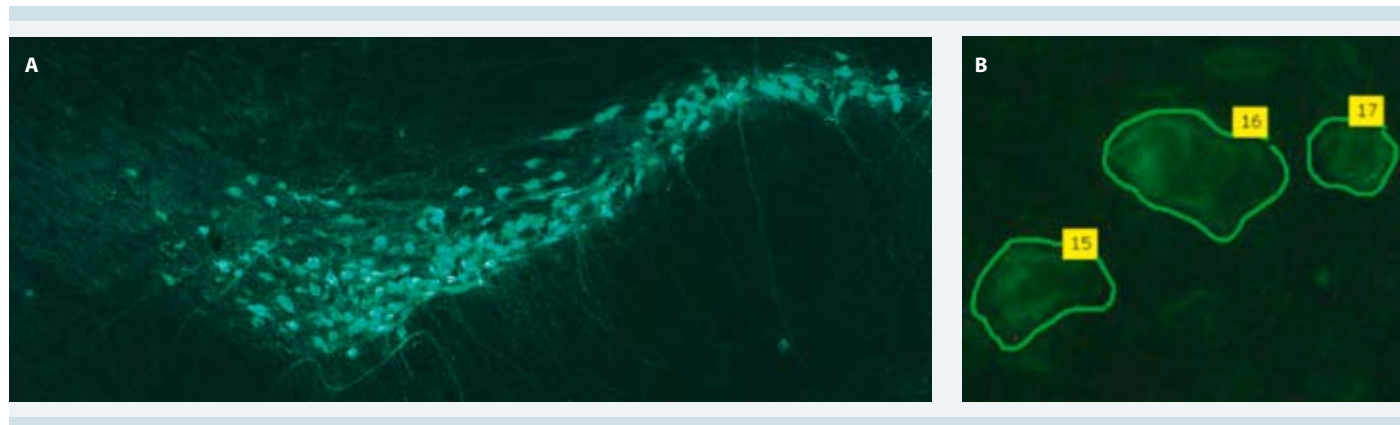


Figure 1 Isolation of GFP-expressing DA cells from the SNc of a TH-GFP/21-31 animal. **(A)** The SNc at a 10 \times magnification, **(B)** laser capture isolation of individual neurons in the SNc (40 \times).

31 mice (fig. 1).

For the other experiment, three samples from different GIN mice were collected using LCM, each containing 300 EGFP-expressing interneurons from the hilus of the hippocampal formation.

As a control for the whole process, several pieces of plain membrane from a PEN slide were laser-microdissected, catapulted into a cap, and processed like the other samples.

RNA isolation, amplification, and fluorescence labeling

Isolation of mRNA, millionfold amplification, and labeling of the resulting cDNA with Cy3-dCTP and Cy5-dCTP (PerkinElmer) was performed using the μ MACS™ SuperAmp™ Kit and a thermoMACS™ Separator, according to the recommended protocol (for details on the SuperAmp Technology see page XX).

Preparation of reference RNA

10 μ g of universal mouse reference RNA (Stratagene) were indirectly labeled with Cy5 and used for all hybridizations of the interneuron samples.

Microarrays

Hybridization of 3.0 μ g labeled DNA (for both dyes) was performed using our in-house cDNA platform which contains 7000 clones in triplicates. For the experimental set-up see table 1. Scans were made with the Axon 4100 scanner and the GenePix version 5.0 software. Data were normalized and analyzed with the R Bioconductor package Limma.

Results and discussion

To evaluate this new method, we performed two sets of experiments (table 1). In the first set, RNA was amplified from 100 DA neurons and from 100 non-DA neurons of the SNc in the mesencephalon to be hybridized against each other on a microarray. Experiments 1A and 1B represent a technical replicate as the same pools of cells were used for two independent amplifications followed by cDNA labeling with Cy3 or Cy5 respectively (dye swap).

For the experiments 2A, 2B, and 2C, 300 hippocampal interneurons were collected independently for each experiment. The RNA was isolated, amplified, labeled with Cy3, and hybridized versus Cy5-labeled universal mouse reference RNA on a microarray.

An overall control sample, containing only

Experiment 1	Cy3	Cy5
1A	100 GFP-expressing DA cells	100 non-DA cells
1B	100 non-DA cells	100 GFP-expressing DA cells

Experiment 2	Cy3	Cy5
2A	300 somatostatin-expressing interneurons	Universal mouse reference RNA
2B	300 somatostatin-expressing interneurons	Universal mouse reference RNA
2C	300 somatostatin-expressing interneurons	Universal mouse reference RNA

Table 1 Experimental set-up: Cells were laser capture–microdissected and global RNA was amplified using the μ MACS™ SuperAmp™ Kit for subsequent microarray analysis. In experiment 1, DA and non-DA cells were isolated from the SNc of the TH-GFP/21-31 line of transgenic mice. In a dye-swap experiment, DA cells were hybridized versus non-DA cells. In experiment 2, somatostatin-expressing interneurons were isolated from the hippocampus of three GIN mice. The biological replicates were hybridized against universal mouse reference RNA.

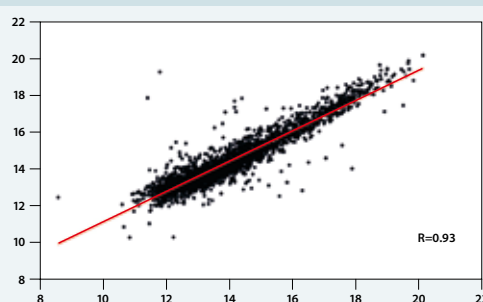


Figure 2 Experiment 1: Reproducibility of gene expression profiling experiments (1A and 1B), starting from 100 laser-captured cells from the SNc. The plot shows the correlation ($R=0.93$) of a dye-swap experiment. The samples were prepared by two independent amplifications, starting from common cell pools, followed by cDNA labeling with Cy3 or Cy5, respectively. Amplified RNAs of DA versus non-DA cells were hybridized on microarrays.

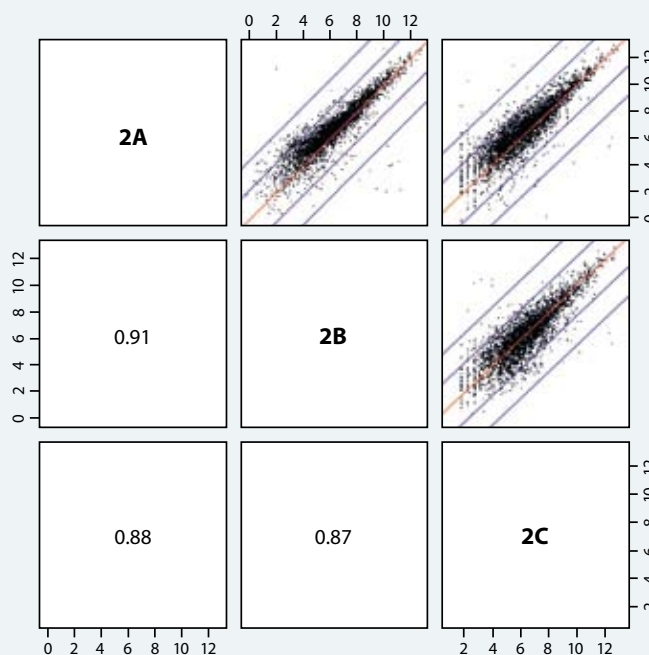


Figure 3 Experiment 2: Pair-wise scatter plots showing the correlation between biological replicates. Three samples from different GIN mice (2A, 2B, and 2C) were collected independently by LPC, each containing 300 interneurons. Amplified RNA of the samples was hybridized versus universal mouse reference RNA.

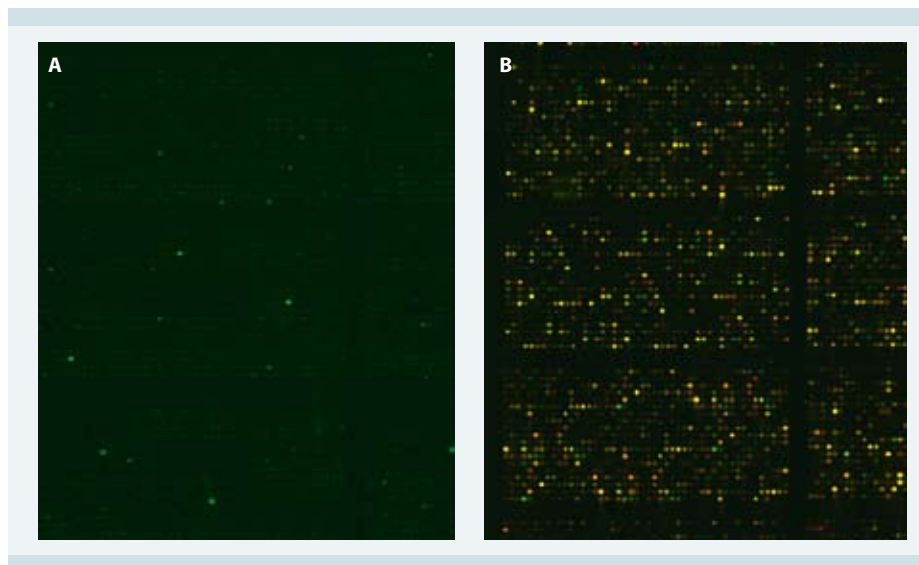


Figure 4 (A) Membrane control microarray slide: The green spots represent bacterial genomic DNA. (B) Representative microarray slide probed with labeled cDNA from DA cells (Cy3, green signals) and non-DA mesencephalic cells (Cy5, red signals).

membrane from a PEN slide, was processed as all the other samples.

The RNA from 100 or 300 laser capture-microdissected neuronal cells was isolated, amplified, and labeled using the μ MACS™ SuperAmp™ Kit. Considering the very low amount of starting material, the cDNA yields at the end of the PCR reaction were good, typically ranging from 30 to 60 ng/ μ L in a total volume of 60 μ L. As only 200 ng of cDNA are needed for

the Klenow reaction, there is enough material left over to perform further experiments starting from the same PCR source.

In order to assess the validity of the system, pair-wise scatter plots and the corresponding Pearson correlation coefficient (R) of gene expression profiles were calculated for all experiments. The high linear correlation coefficient (R=0.93) between experiment 1A and experiment 1B (dye swap) shows the reproducibility of the experimental approach

and the amplification process (fig. 2). Known expression of marker genes for the DA cells is consistent with our cDNA microarray analysis providing an insight into the relative sensitivity of the experimental procedure (manuscript in preparation). Amongst others, our microarray profiles for DA neurons showed an enriched expression of the DAT transporter³ and GIRK2^{4,5} which are well known markers for DA cells of the SNc.

In the other experiment, good values of the correlations for the three interneuronal samples indicate a reasonable reproducibility between the biological replicates (fig. 3).

In the membrane control sample the positive spots that appeared were caused by bacterial genomic DNA present on our cDNA slides as control spots (fig. 4A). From a technical point of view, the slides were clean and showing a very low background noise (fig. 4B).

These results show, that LCM in combination with the SuperAmp Kit is a reliable and powerful method to perform gene expression profiling from very low numbers of microdissected cells.

References

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