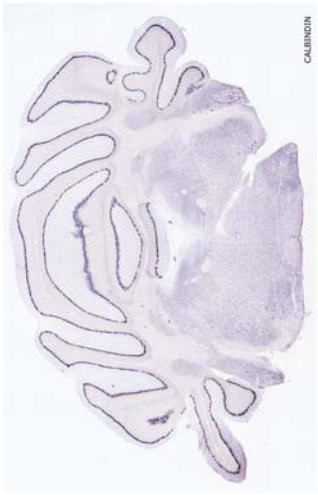
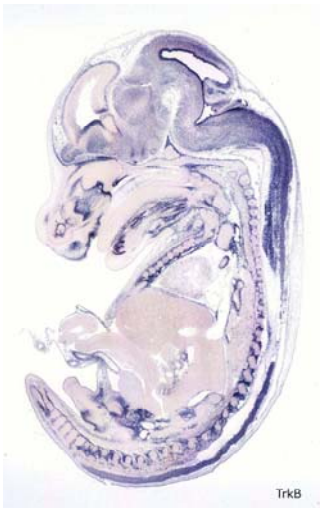
Fully automated In Situ Hybridization

High-throughput In Situ Hybridization with the GenePaint system





Introduction

In situ hybridization is a technique that is used to determine gene expression patterns by localising mRNA transcripts in cells and tissues. The cellular resolution of ISH is by far larger than that of RNA blotting, differential display and microarrays, techniques that are also widely used for gene expression analysis. The reason is that, for in situ hybridization, tissues are not dissected for extraction of RNA, but mRNA transcripts are localised in intact tissue sections, thereby visualising gene expression in individual cells or clusters of cells within their tissue context.
The signal obtained shows where a gene is 'expressed', i.e. where it is active. Gene activity depends on promoters, which are DNA sequences that drive the transcription of genes into mRNA. Thus, in situ hybridization is a powerful tool to determine the cell- and tissue-specific activity of promoters. In addition, genes and their products are usually functional in the cells where they are expressed. Therefore, in situ hybridization is also used to determine the function of genes of interest. In summary, in situ hybridization is a technique to characterise gene function and promoter activity by localising mRNA transcripts in cells and tissues.

The experimental procedure of in situ hybridization is a multi-step process, which, without automation, is extremely labour-intensive, and therefore it is very susceptible to human errors. By automation and adaptation to high-throughput, many genes can be analysed within a short time. In addition, reproducibility and robustness, accuracy and sensitivity are increased by automation.

TECAN and the Max-Planck-Institute of Experimental Endocrinology in Hannover, Germany, have collaborated to develop a system for the automation of in situ hybridization: the GenePaint System (Fig. 1).
This system is based on two components:

1. the TECAN GENESIS Robotic Sample Processor that controls the pipetting of solutions, timing of incubations, hybridization and detection reactions
2. the GenePaint Components consisting of a newly developed Flow Chamber and thermostated Chamber Rack.

Experimental Procedure

Tissue Sample Preparation
Tissue samples originating from the organism of choice (human, animal, plant or microbes) are fixed and embedded in a suitable matrix for sectioning, such as paraffin wax or Hydro-Matrix. Paraffin-embedded or frozen tissue samples can be used in the procedure. They are sectioned using a microtome or cryostat, and sections of 10-30µm thickness are collected on adhesive-coated glass slides commonly used for microscopy.

Slide assembly
The slides are assembled into the GenePaint Flow Chambers (Fig. 4), each consisting of an aluminium frame, 75µm thick spacers, a glass backplate and two metal brackets. All of these components can be sterilised by autoclaving. The slides remain in the flow chambers until the end of the procedure. This way the tissue sections are liquid-covered at all times and are thus protected from damage and desiccation.
The assembled flow chambers are placed into the GenePaint Thermorack (Fig. 2 and 3), where they are in direct and permanent contact with the heat exchange wall (Fig. 3).

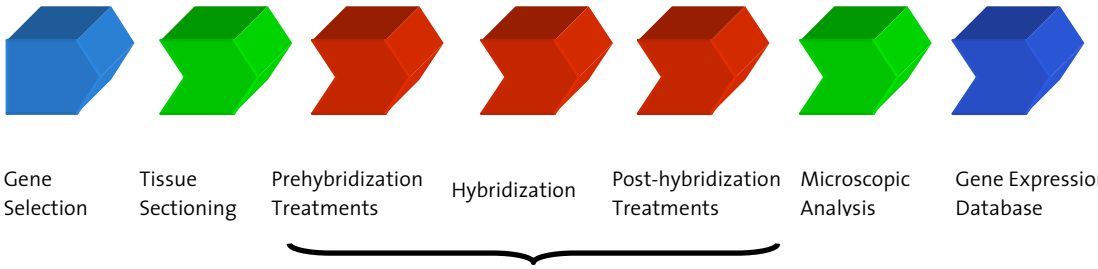
Prehybridization treatments
To prepare the tissue sections for hybridization with the probe, they are submitted to a series of pre-hybridization treatments such as paraffin-removal, peroxide treatment, proteinase incubation and post-fixation. The solutions are prepared and placed in heatable or ambient temperature reservoirs on the Genesis Robot platform. The Genesis Robot performs all steps automatically by pipetting the solutions into the slide chambers according to the programmed script. A wide variety of scripts can be used, corresponding to the desired in situ hybridization protocol.

Probe preparation and hybridization
Digoxygenin-labelled riboprobe is produced by in vitro transcription using a DNA clone of the gene of interest as a template. The riboprobe is diluted in hybridization buffer to a typical concentration of 100ng/µl and applied to the preheated slides. This application of probe to the slides can be performed manually or automatically. Hybridization is carried out at a suitably stringent temperature (typically 50-55oC, corresponding to 5-10oC below the Tm of the individual riboprobe), for a period of several hours to overnight.

Post-hybridization treatments
After hybridization, stringency washes are carried out, typically at a temperature between 55 oC and 65 oC. These stringency washes remove unbound riboprobe.
For these washing steps, the Genesis Robot pipets preheated SSC/formamide solutions from their heatable reservoirs into the GenePaint slide chambers. The GenePaint chamber rack and the slides are also heated according to the desired washing temperature.

Antibody-mediated detection
At ambient temperature, several blocking steps serve to reduce non-specific background. After the blocking steps, antidigoxigenin antibodies are applied to the slides. Typically these antibodies consist of Fab fragments that are linked to alkaline phosphatase, peroxidase or another enzyme for colourimetric detection. An optional signal amplification step helps to detect transcripts of weakly expressed genes.
After several washing steps to remove unbound antibody, the substrate for colour reaction is applied and slides are incubated until the desired signal intensity is reached. The reaction can be timed manually or programmed to a specific time. Then the reaction is stopped and the colour precipitate is fixed to be preserved for slide mounting and microscopy.

Workflow in situ hybridization



GenePaint System

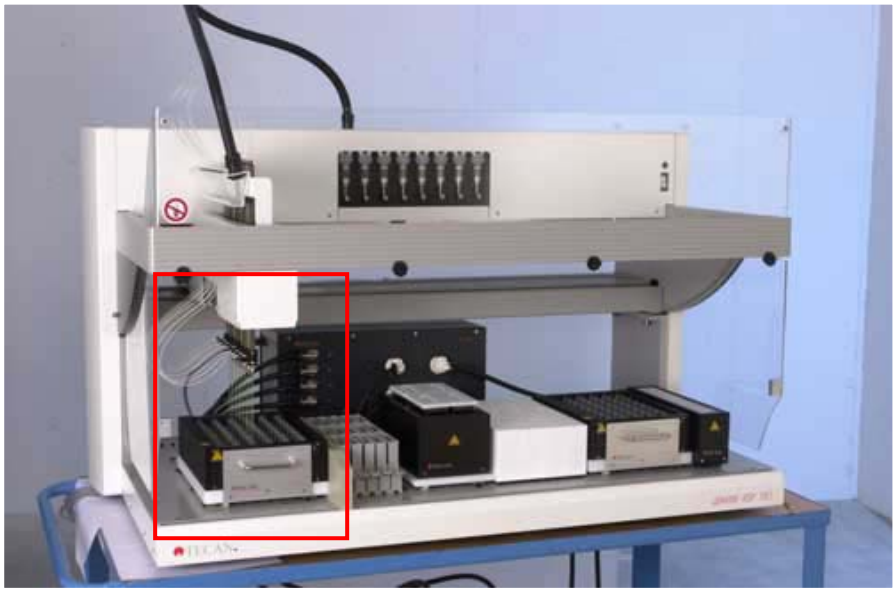


Figure 1: GenePaint System on a GENESIS RSP 150/8 Platform. Visible from left to right are: GenePaint Chamber Rack, 15 small reagent reservoirs, four medium-size heatable reagent reservoirs, three large reagent reservoirs, another GenePaint Chamber Rack, and a heatable rack for Eppendorf tubes.

Figure 2: GenePaint thermostated Chamber Rack holding 48 flow chambers located on the GENESIS plaform. The eight pipets on the robotic arm are also visible.

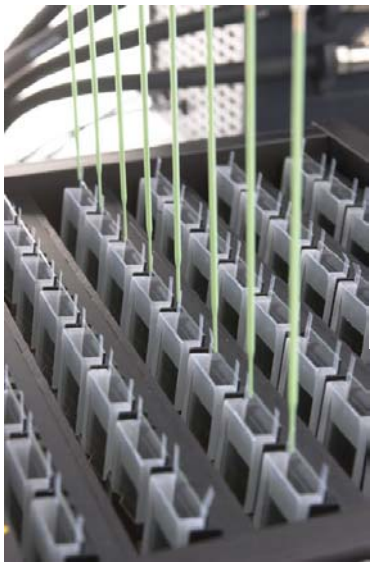


Figure 3: Close-up of GenePaint Flow Chambers located in the GenePaint Chamber Rack. At their backs the flow chambers are in direct contact with the heat exchange wall. The eight pipet tips above one row of flow chambers are visible in green.

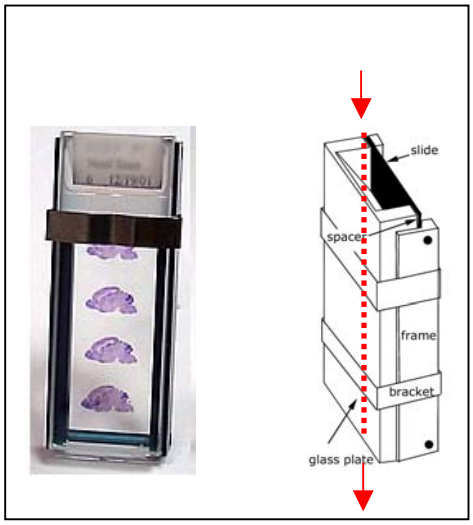


Figure 4: GenePaint Flow Chamber with slide carrying tissue sections (purple) after *in situ* hybridization.
Schematic drawing shows how the slide is assembled in the flow chamber.

Performance

Components
A Genesis RSP 150/8 platform (about 1.5m wide) is equipped with GenePaint components to give the complete GenePaint System (Fig. 1).
Each glass slide carrying tissue sections is assembled into a GenePaint Flow Chamber, consisting of an aluminium frame, 75µm thick spacers, a glass backplate and two metal brackets (Fig. 4). Up to 48 flow chambers (i.e. slides) can be placed into the GenePaint Chamber Rack (Figs 2 and 3).

Temperature Control
The flow chambers and thus the slides are in direct contact with the heat exchange wall (Fig. 3). This wall is hollow and perfused by temperature controlled water which is provided by a circulator bath (one or several?).
The direct contact between the heat exchange wall and the slides carrying the specimen results in a highly efficient thermal transfer.
Temperatures typically used for different steps in the in situ hybridization procedure range from room temperature to 65 oC. The thermorack and temperature-controlled reagent reservoirs cover a range from 10 oC to 80 oC, thereby including the required temperatures.
Within this range, the accuracy of temperature control in each individual flow chamber is ±0.5 oC; and the variation of temperature across the whole thermorack is ±1.0 oC. This highly accurate temperature control ensures highly consistent and reproducible results both within one experiment and between different experiments.

GEMINI Software
The Genesis Gemini software controls the temperature by regulating the circulator bath(s) and valves. The temperature change is very fast: an increase or decrease of 20 oC to 30 oC can be achieved within 5 min or less.
In addition to temperature, the Gemini software also controls the pipetting of reagents in each step, the volume pipetted, the incubation time and the number of cycles, i.e. the required number of repetitions of each step. After each step the pipette tips are automatically cleaned to avoid cross contamination of reagents.
The control of all parameters is executed and tracked according to a user-defined work list, which is based on the individual in situ hybridization protocol. Each work list can be saved as an individual program file and different program files can be selected and run as needed. In this way, all steps of in situ hybridization, from prehybridization treatments to addition of probes, hybridization, stringency washes and antibody-mediated chromogenic reaction, are carried out by the robotic system with very little human intervention and supervision.

Throughput
On a Genesis RSP 200/8 platform (about 2m wide) up to four GenePaint Chamber Racks can be accommodated, resulting in a throughput of up to 192 slides per experiment. With this level of throughput thousands of genes can be analysed within a few months, thereby opening the opportunity to use in situ hybridization as an efficient functional genomics tool.

Results

In the group of Prof. Gregor Eichele at the Max-Planck-Institute of Experimental Endocrinology, several GenePaint Systems are routinely used to carry out high-throughput in situ hybridization experiments. In an ongoing project, a large number of genes selected from a mouse genome database is submitted to tissue-specific gene expression analysis by in situ hybridization. The results are presented in a gene expression database at www.genepaint.org. One focus is on the nervous system and brain2, another on the embryo1. Recently, the systematic expression analysis of all identifiable murine orthologues of human chromosome 21 genes and the resulting gene expression atlas were published in Nature1. The *in situ* hybridization results obtained are highly specific expressions patterns without background staining or tissue damage and completely reproducible.

Some prominent examples of the MPI group's work are presented in Figures 5, 6 and 7.

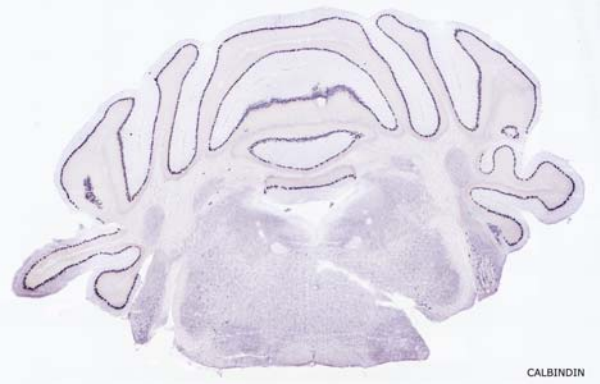


Figure 5: A 20-µm coronal cryostat section of an adult mouse brain was hybridized with a riboprobe of the gene coding for Calbindin-28K, a gene encoding a calcium-binding protein. Strong expression (blue/purple) is visible in the Purkinje neurons of the cerebellum.

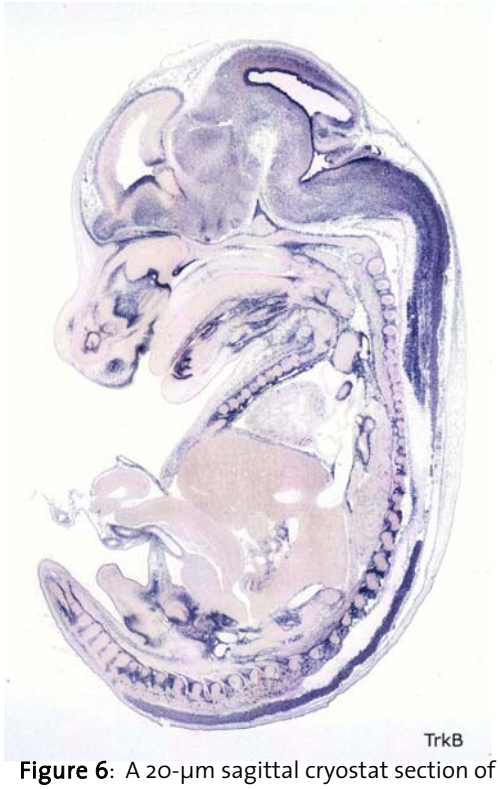


Figure 6: A 20-µm sagittal cryostat section of a 14.5 day-old mouse embryo was hybridized with a riboprobe of the gene encoding the neurotrophic tyrosine kinase type 2 receptor (Ntrk2). Widespread but specific expression is seen in brain and spinal chord, various cranial and axial skeletal structures and in spinal ganglia (tail region).

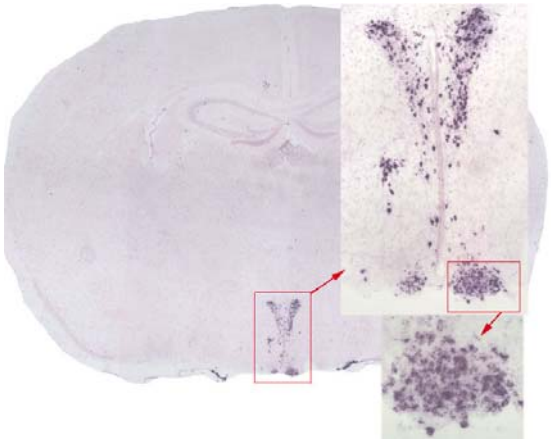


Figure 7: A 20-µm cryo-section of a mouse brain was hybridized with a riboprobe of the gene coding for vasopressin, an antidiuretic hormone. The gene is highly expressed in the hypothalamus. The expression signal appears dark purple/blue. Enlargements show cellular resolution.

Conclusion

The TECAN GenePaint System is completely suited to automated high-throughput in situ hybridization, enabling the analysis of 400-600 slides per week. The newly developed components, the GenePaint Flow Chamber and GenePaint Thermostated Chamber Rack ensure highly consistent and reproducible results of excellent quality.
These features make the TECAN GenePaint System the first choice for Functional Genomics, in which large scale gene expression analysis is of great importance. The unique advantage of in situ hybridization over destructive gene expression techniques such as microarrays is the localization of transcripts in intact cells and tissues, giving a much higher level of information. Large numbers of genes can be screened for a desired expression pattern, for example in specific regions of the brain. Such genes might encode potential drug targets or hold a key role in basic brain physiology and pathophysiology.
Different protocols can easily be transferred into the GEMINI software, so that the GenePaint System is programmed to carry out automated Immunohistochemistry (IHC), i.e. the localization of proteins in tissue sections. Also, the GenePaint System has already been successfully used for Fluorescence In Situ Hybridization (FISH), i.e. for physical mapping of chromosome markers.
The high sensitivity, robustness and reproducibility make the GenePaint System particularly suitable for all these applications and offers unique opportunities in the growing field of Functional Genomics and Proteomics.

Literature

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