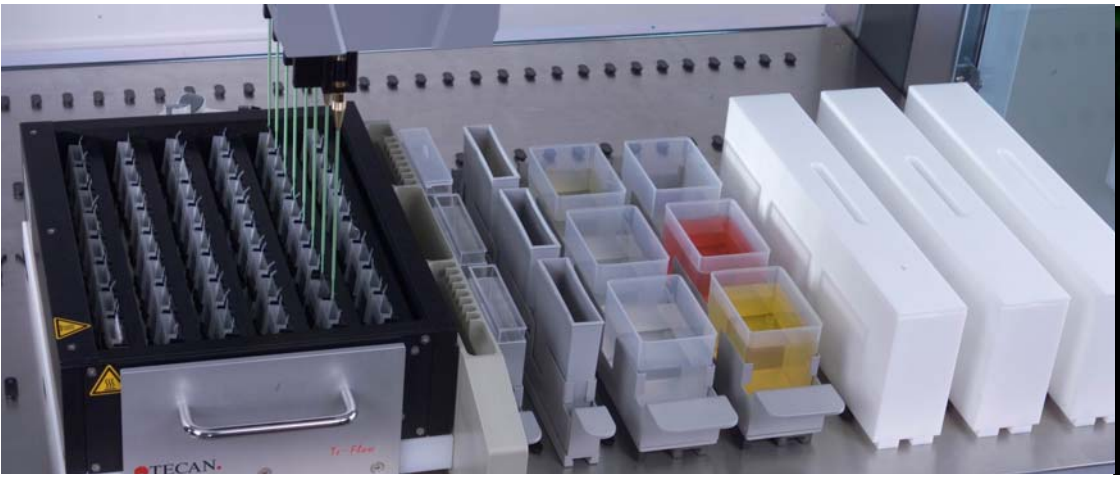
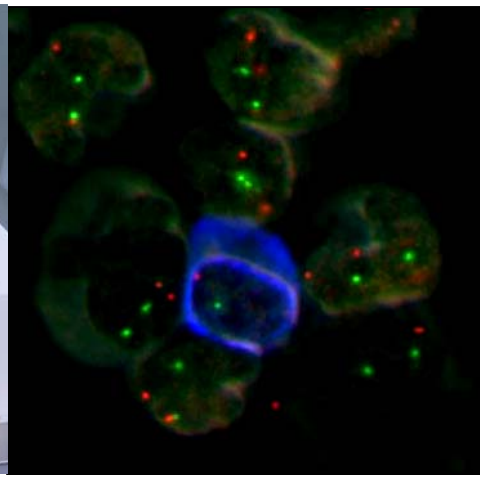
Semi-Automated FISH analysis of cells on slides

Automated Post-Hybridization in a FISH protocol of leukaemia cells from blood with the Tecan GenePaint system





Introduction

Fluorescence *In situ* hybridization (FISH) is a technique that can be used for the analysis of genomic aberrations in tumor cells. The resolution level of FISH is by far better than chromosome banding which is also widely used for the study of chromosome aberrations in cancer. In addition to the improved resolution FISH allows for the analysis of genomic aberrations not only on metaphase chromosomes but also in interphase cells ("interphase cytogenetics"). This improves the sensitivity for the detection of genomic aberrations in many tumors. In fact, in some leukaemias the incidence of chromosomal aberrations is almost twice as high as compared to chromosome banding. The reason is that, for FISH no dividing cells are needed, i.e. there is no need for short term in-vitro culture for cells to obtain metaphase spreads. This is a key advantage in tumors with a low in-vitro proliferative index such as some leukaemias and lymphomas. The principle of FISH for the study of genomic aberrations is the evaluation of the number and localization of fluorescence signals in the cell nucleus or on metaphase chromosomes. Aberrant numbers or local distributions indicate numerical or structural chromosomal aberrations.

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 genomic aberrations can be analysed within a short time. In addition, reproducibility and robustness, accuracy and sensitivity are increased by automation.

Using the TECAN GenePaint System a highly automated FISH application procedure has been developed (Fig. 1).
This system is based on two main components:
1.The TECAN liquid handling workstation 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 a thermostated Chamber Rack.

Experimental Procedure

Tumor Cell Preparation
Cell samples originating from the tumor of choice are purified (i.e. by ficoll density gradient) and fixed by routine cytogenetic procedures. The cell suspension can be stored for long periods of time. For FISH analysis the cells are washed and collected on glass slides commonly used for cytogenetics or other procedures.

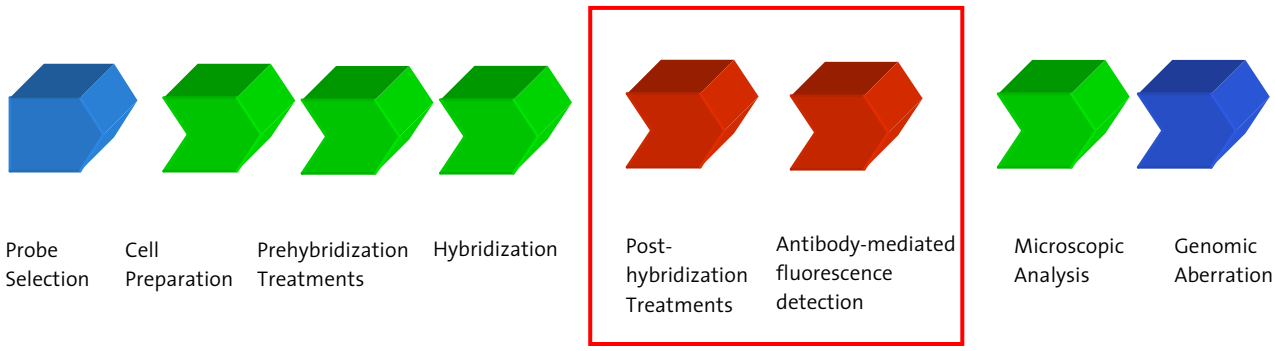
Slide and probe assembly
The slides containing the tumor cells are pretreated (i.e. by pepsin digestion) if required, fixed and subsequently subjected to FISH. Digoxygenin- and Biotin-labelled DNA probes derived from YAC, PAC, BAC, cosmid or plasmid clones are combined with salmon sperm and Cot DNA according to routine FISH protocols and dissolved in hybridization solution which is subsequently applied to the slide. After overnight hybridization at 37°C the slides are subjected to posthybridization treatment in the TECAN robotic system.

Post-hybridization treatments
After hybridization, stringency washes are carried out with 50% Formamide / 2xSSC and 0.5xSSC solution at room temperature. These stringency washes remove unbound DNA probe.
The automated procedure guarantees minimal inter-experiment variability resulting in superior signal quality as compared to the same post hybridization procedure performed hands-on.

Antibody-mediated fluorescence detection
At ambient temperature, blocking steps serve to reduce non-specific background. After the blocking step, antidigoxigenin antibodies labelled with Cy-3 and avidin labelled with FITC are applied to the washed slides. An optional signal amplification step may be applied to improve signal strength of weak probes (i.e. plasmids).
After several automated washing steps to remove unbound antibody, the slides are removed and mounted with antifade solution for microscopy. The antibody incubation and washing steps can be programmed to a specific time and temperature optimized for the respective antibody. The automated procedure of antibody application, incubation and washing leads to highly accurate experimantal procedure with minimal variability and shows an improved signal to noise ratio as compared to previous non-automated experience.

Combination of FISH with immunophenotyping (FICTION)
In addition, the routine FISH procedure can be combined with other experimental procedures such as immunophenotyping / immunocytology of the cells under investigation. This adds specificity to the procedure since the cells investigated for chromosomal aberrations can be further characterized for the morphological and immunological background (see Figure 6).

Workflow fluorescence in situ hybridization



FISH post-hybridization steps

performed by the GenePaint system

Figure 1: GenePaint Chamber Rack holding 48 Flow-Through chambers. The Liquid Handling arm of the robotic system is equipped with eight dispensing tips. It assures automatic and parallel pipetting of all solutions.

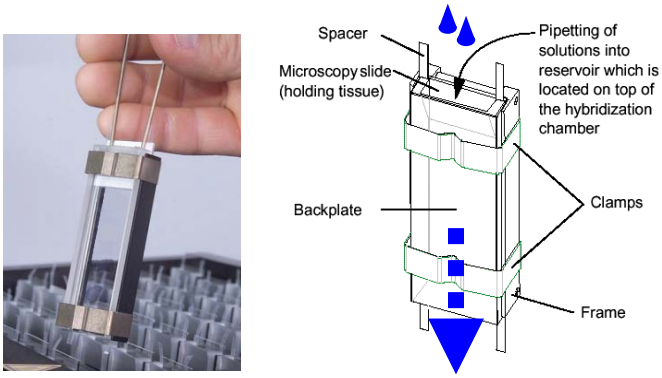
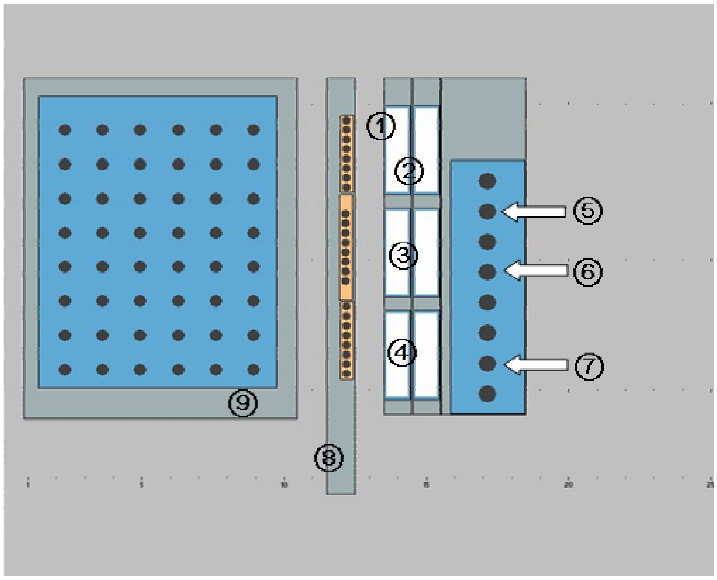


Figure 2: GenePaint Flow Chamber with slide carrying cells.
Schematic drawing shows how the slide is assembled in the flow chamber.



① = 1xPBS
② = 4x SSC
③ = 50% Formamide / 2x SSC
④ = 0.5x SSC
⑤ = „Blocking" = 3% BSA in4xSSC / Tween 0.1%
⑥ = „Detection" = Fluorescence-AK in 200µl 1% BSA in 4xSSC/Tween 0.1%
⑦ = DAPI
⑧ = tip wash station
⑨ = Te-Flow chamber rack holding up to 48 slides

Figure 3: In the here described FISH experiments a GenePaint system with one chamber rack and 4 pipetting tips has been used. The worktable layout with the solutions for stringency washing and fluorescence detection is shown (schematically).

GenePaint robotic system

GenePaint Components
A Tecan robotic system has been equipped with one GenePaint chamber rack and various liquid reservoirs to give the complete GenePaint System (Fig. 3). All post-hybridzation experiments has been carried out at ambient temperature.
Each glass slide carrying cells under investigation is assembled into a GenePaint Flow Chamber, consisting of an aluminium frame, 75µm thick spacers, a glass backplate and two metal brackets (Fig. 2). Up to 48 flow chambers (i.e. slides) can be placed into the GenePaint Chamber Rack (Fig 1).

Tecan Software
The Tecan software 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 post-hybridization, from stringency washes to antibody-mediated chromogenic reaction, are carried out by the robotic system with no human intervention and supervision.

Throughput
The typical throughput of the here presented GenePaint system (Fig 3) is 200-250 slides per week. Two automated post-hybridization protocols are frequently running on the system. The automated protocols run 2 and 4 hours, respectively.
On a Freedom EVO 200/8 platform (about 2m wide) up to four GenePaint Chamber Racks can be accommodated, resulting in a maximun throughput of up to 192 slides per experiment (equivalent to 400-600 slides per week).

Results

The GenePaint Systems routinely allows for high-throughput FISH experiments. This type of analysis is particularly well suited for the study of disease in which a large range of different probes need to be applied and multiple cases / samples need to be studied such as B-cell chronic lymphocytic leukemia (B-CLL). B-CLL is the most common leukemia among adults in western coutries but despite this high incidence the knowledge on genetic abnormalities underlying B-CLL pathogenesis is very limited. This is mainly related to the low in-vitro mitotic activity of the tumor cells which is leading to poor quantity and quality of metaphase spreads and has made classical cytogenetic studies by chromosome banding difficult. On the other hand, analyses based on molecular genetic techniques such as Southern blot analysis or polymerase chain reaction (PCR) have been limited by the fact that only for few genetic regions affected in B-CLL candidate genes are known. To overcome both, the low chromosome yield from B-CLL cells and the lack of candidate genes to be analyzed, FISH was shown to be instrumental in the genetic analysis of B-CLL. By FISH genetic abnormalities can be detected on the single cell level also in non-dividing cells circumventing the need to obtain chromosomes from B-CLL cells. The genetic regions to be analyzed can be detected with probes available from genome wide libraries without the need of prior candidate gene identification. Since many different aberrant regions need to be studied in multiple samples automation oof the FISH procedure is instrumental for the analysis of large patiente cohorts. This forms the basis for both basic scientifc projects related to the pathogenesis and clinical applications. By FISH chromosome aberrations can be detected in more than 80% of B-CLL cases with a disease specific probe set. In contrast to other types of low-grade lymphoma, the by far most frequent type of genetic abnormalities in B-CLL are deletions of genetic material. The most frequent deletion cluster regions affect band 13q14, followed by 11q22-q23, 17p13 and 6q21. Common gains of chromosomal material are trisomies 12q, 8q and 3q. Translocations, in particular affecting the immunoglobulin heavy chain gene locus (*IgH*) at 14q32, which are frequently observed in other types of lymphoma, are rare events in B-CLL. Genes affected by the aberrations in B-CLL appear to be *p53* in band 17p13 and *ATM* in a subset of cases with 11q22-q23 deletions. However, for most of the frequently affected genomic regions in B-CLL the search for candidate genes is ongoing. In addition to the delineation of critical regions, FISH allowed the accurate evaluation of the

incidence of chromosome aberrations in B-CLL. This provided a valid basis for correlation of genetic abnormalities with clinical disease manifestations and outcome. Deletions of 17p13 (*p53*) and 11q22-q23 have proven to be among the most important independent prognostic factors identifying subgroups of patients with rapidly progressive disease and inferior survival. In addition, deletion 17p13 (*p53*) has been shown to predict for non-response to therapy with purine analogs such as fludarabine. Therefore, the study of genetic abnormalities has lead to a better understanding of the molecular pathogenesis and clinical course of B-CLL.

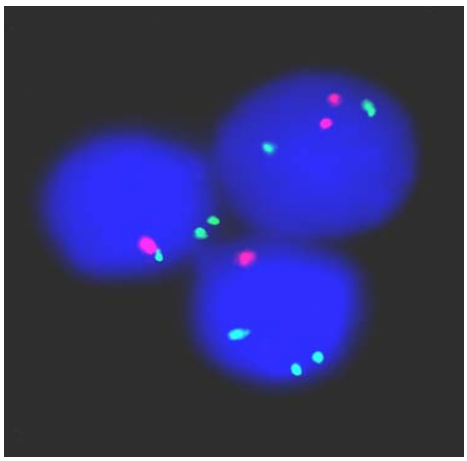


Figure 4: Examples of FISH images demonstrating genomic aberrations in CLL
Trisomy 12q (three green hybridization signals) and monoallelic deletion 13q14 (single red signal) in two of three nuclei in a B-CLL specimen. A single cell reflecting the normal disomic status of the two regions is shown for comparison.

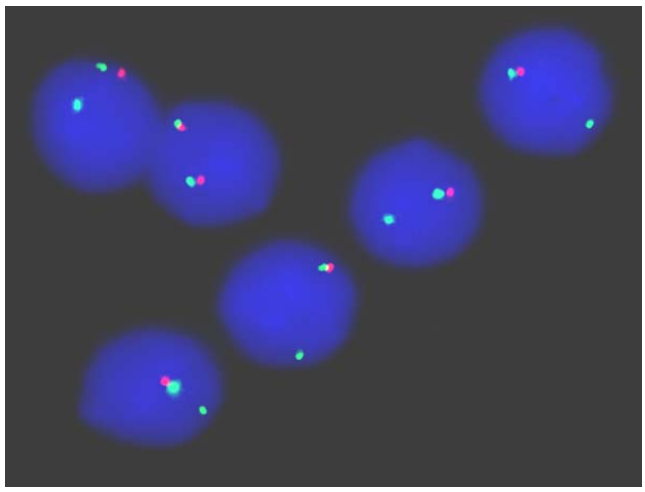


Figure 5: Examples of FISH images demonstrating genomic aberrations in CLL
CLL with monoallelic 11q22-q23 deletion as demonstrated by the single red signal in five of the six nuclei shown. Two green signals of an internal control probe hybridizing to an adjacent disomic genomic region proof a high hybridization efficiency. The single cell with two red signals likely represents a non-leukemic cell from the blood specimen.

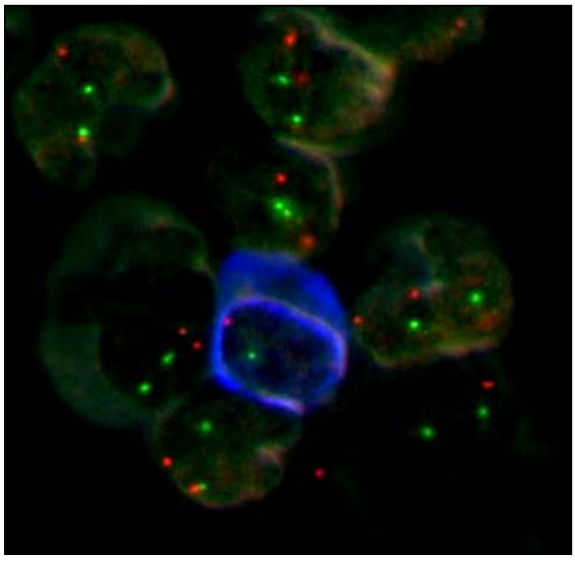


Figure 6: An example of FISH combined with immunocytology (FICTION) for the study of multiple myeloma: Deletion of chromosome band 13q14 and deletion of chromosome band 17p13 in a patient with IgG kappa-myeloma. A myeloma cell is labeled blue by a kappa-light chain fluorescence antibody. Note two red and two green signals in the surrounding normal bone marrow cells.

Conclusion

The TECAN GenePaint System is very well suited to automated high-throughput FISH, enabling the analysis of up to 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. The quality of the hybridization signals is consistently superior to similar experiments performed hands-on. In addition, there is a significantly more efficient work process reducing the time and manpower needed for the analysis of multiple samples.
These features make the TECAN GenePaint System a valuable choice for the study of genomic aberrations in tumor cells for which large scale genomic analysis is of great importance. The unique advantage of FISH hybridization over other techniques such as PCR, chromosome banding or blotting techniques is the enumeration and localization of genomic copy numbers in intact cells and tissues, giving a much higher level of information. Large numbers of genomic regions can be screened for aberrations in various tumor entities. The aberrations detected may hold a key role in basic cell physiology and pathophysiology. In particular for the study of the clinical relevance of genomic aberrations large numbers of tumor samples need to be analyzed. To this end automation by the TECAN system is a key tool.
Different protocols can easily be transferred into the Tecan software, so that the GenePaint System is programmed to carry out automated immunophenotyping and FISH or other additional procedures.
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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