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Standardisation of FISH-procedures: Summary of the Second Discussion Workshop

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1. Introduction

Fluorescence *in situ* hybridization (FISH) is widely used to assess chromosomes and the localization of genetic elements in tissues, nuclei of cultured cells, and spreads of metaphase chromosomes. FISH procedures have become routine in many laboratories of fundamental and applied research as well as in medical diagnostics, e.g., in cytogenetics and pathology. The commercial availability of probes and labelling kits might suggest that FISH is a routine technique without further need for improvement. However, the 1st Discussion Workshop on Standardisation of FISH-Procedures held at Schloss Elmau in 2002 [2] resulted in a quite different view on FISH. FISH procedures vary grossly from laboratory to laboratory, and are far from optimal for many questions in modern biology and medicine. Therefore, scientists, clinicians and members of companies for FISH probe production and companies for microscopic instrumentation and software development were invited for a second discussion workshop with the aim to improve the dialogue between customers and suppliers.

Here, a report on the "2nd Discussion Workshop on Standardisation of FISH-Procedures" held at the Karl-Benz House in Ladenburg, Germany, July 10–11, 2003 is given. This meeting was organised by Christoph Cremer (Heidelberg), and Michael Hausmann and Martin Werner (Freiburg). It was supported by the Gottlieb Daimler and Karl Benz foundation which dedicates its efforts in promoting interdisciplinary science and research and the discourse between university and industry. In this sense the workshop was convened to discuss recent developments, problems of routine applications, and future requirements in the intriguing subject of specific fluorescence DNA labelling. The 28 participants very lively supported the discussion and elabo-

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rated aspects for methodological research and requirements to FISH probe, microscope, and software manufacturers.

2. Lectures: FISH probes, procedures, and standardisation

After opening remarks by Martin Werner, Michael Hausmann summarized some conclusions of the first discussion workshop (see [2]).

Christoph Cremer tried to define the potential of novel FISH procedures. He showed the possibilities of multicolour FISH and its impact on the investigations of the architecture of the cell nucleus [1]. However, to expand the application of FISH for studying the nuclear architecture and supra-molecular chromosome organisation, it appears necessary to understand the physics of the FISH procedure itself. Moreover, he suggested that nowadays computer simulations may have some potential to better understand the result of an imaging process from the labelling procedure to the light microscopic image. There are further aspects in the present FISH methods which make it highly desirable to develop and promote novel techniques. Such novel techniques include:

(1) development of methods which allow to perform FISH much faster than presently routinely used approaches;
(2) development of methods which allow to perform FISH without substances which may be hazardous to human health; since a large part of FISH-applications is performed by women with a special risk potential, the elimination of unnecessary hazardous substances such as chaotropic agents should be mandatory;
(3) developments of novel light microscopy techniques to allow gene expression microscopy;
(4) development of appropriate image analysis procedures for analysis of FISH-labelled nuclei;
(5) development of "Virtual Microscopy" tools for optimization of molecular cytogenetics in cell nuclei;
(6) development of methods which allow to perform FISH with specifically chosen pools of synthesized oligo-nucleotides;
(7) development of methods which allow to perform FISH not only using denatured DNA sequences but also in combination with double stranded targets;
(8) development of FISH methods for labelling and detection of short target sequences with only a few possible fluorochrome attachment sites.

In the following discussion a central point was the acceptance of novel protocols by the end-user, a problem to which especially companies are continuously confronted. There is an obvious difference between the methodological feasibility and accepted implementation.

In her talk Evelin Fiedler, Abbott Diagnostics, presented the strategy of her company to produce not only single probe kits but also disease specific combinations for instance for solid tumours or haematological neoplasia. Multicolour approaches for breakpoint labelling allow an easy classification of cells by counting spots of pure colour and mixed colour. The aim of the company is to specify certain probe kits for certain cancer cells and to standardize procedures by certification, e.g., ISO 9001 or FDA approval.

Michael Vetter of Qbiogene distributed a "universal FISH protocol" to show the companies effort to make FISH more easy and reliable. In his talk he showed that protocol adaptations are necessary due to the labelling technology, on the other hand there are also restrictions due to the end-users' acceptance of methodological modifications or developments. Usually the company makes practical tests with new probes on different types of samples (e.g., metaphase chromosomes, blood cells and paraffin embedded tissues), with a final optimization of the probe and protocol at a beta-test site. Nonetheless, an optimal and universal protocol seems to be impossible since too much factors influence the result, e.g., the type of fixation, the probes, the type and quality of the specimen, etc. Development of an optimal FISH assay requires multi-centre studies and quality assurance ring trials. At the moment a company can only provide the most easy, versatile protocol to the applicants; but such a protocol can only be an "average" one, while the final optimization needs to be done by the individual laboratory.

The following discussion pointed out that on one hand standardisation by the companies is required which makes probes more expensive. On the other hand the end user is looking for cheaper probes to reduce costs in diagnostics.

Although many end-users so far limit themselves to only qualitative, visual microscopic inspection of a FISH labelled specimen, quantitative image analysis may be a future indispensable component for standardisation of FISH evaluation and comparableness of diagnostic results.

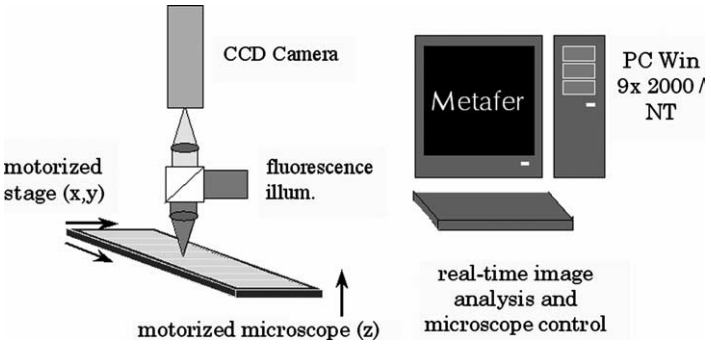

Fig. 1. Set-up of slide scanning platform Metafer.

Interphase FISH scoring by visual inspection is a tedious and error-prone procedure that is subject to inter-observer variability. Andreas Plesch, MetaSystems, presented an automated FISH scoring system that standardizes the FISH analysis. The basic principle of automated slide scanning is to move the slide in a regular meander-like pattern. Each field of view is captured and analyzed. Depending on the result of the analysis individual objects (cells) within a field may be identified as objects of interest and will be further analyzed and stored in an image gallery. After the scan the on-screen image gallery can be used to review the detected cells and to reject unsuitable cells or to do corrections. Any given cell can automatically be relocated under the microscope for direct visual inspection.

Metafer (Metasystems) is a fully automated system (Fig. 1) based on a fluorescence microscope (Carl Zeiss Axioplan2 Imaging Mot) with motorized focus, motorized filter cube revolver, and a motorized scanning stage (Maerzhaeuser). It is controlled by a microcomputer system (Pentium IV, 2.6 GHz, Windows XP operating system). The focus motor of the microscope provides a focus resolution of 25 nm. The scanning stage achieves a positioning accuracy of 1 μm in x and y at a maximum speed of 70 mm/s. A high resolution CCD camera (JAI, Denmark, 1280 $\times$ 1024 pixels, 12 images/s at full resolution) captures the images. In a first step the counter-stain information is analyzed. Objects are automatically segmented and suitable cells are identified using appropriate shape criteria. Cell clusters are automatically rejected. Then FISH signals are captured in several focal planes to avoid loosing individual spots. When using the 40$\times$ lens typically 5 focus planes separated by approx. 0.7 μm are captured. For FISH spot detection, the individual images are combined to a projection image that includes only in-focus information of the individual focus planes. This is repeated for each colour channel if more than one FISH label is present. The analyzed nuclei are displayed in false colours in an image gallery with their spot counts for on-screen review and interactive correction. In addition to spot counts the system can measure numerous selectable features including shape and texture-related features for cell classification as well as intensity features which yield, e.g., ploidy information. Three-dimensional spot distances are also measurable, e.g., for automatic detection of signal fusions in translocation analysis.

3. Discussion I: Requirements for the laboratory routine

The major areas of clinical application of FISH technology encompass the fields of prenatal diagnostics, hematology and pathology. They work with small numbers of isolated and pre-cultured cells, smears, marrow aspirates, and tissue sections. Commercial kits focus on these so called routine applications. Their protocols do not allow major modifications as these are fixed by FDA approval. The kits are very expensive and the costs are not covered completely by reimbursement from social insurance at least in Germany. The technology requires highly skilled people, expensive equipment, and is time consuming. For this reason routine FISH analysis is established so far mainly at university hospitals only. The samples that are submitted to FISH are highly pre-selected by preceeding tests mainly classical morphology: Giemsa banding of chromosomes in genetics, Pappenheim smears of peripheral blood and bone marrow in hematology, or HE tissue sections in pathology as reviewed by experts. The results are either diagnostic for a specific disease or allow risk assessment for certain tumours. The much less expensive PCR becomes more and more the competing technique, e.g., for BCR-ABL translocation in chronic

myelogenous leukemia or other translocations in lymphomas. As the pressure of cost reduction increases, the possibility for cross compensation from other areas of the laboratory decreases. For this reason laboratories doing routine FISH are still rare and are quite often supported by research money. As a consequence more robust protocols and a higher degree of automation are required. Despite improvements like interphase FISH, painting probes, break apart probes and better software support over recent years, FISH analysis in routine diagnostics still remains so far a field of a few skilled experts.

An additional aspect being outlined by the participants is the interpretation of FISH signals which is limited by many factors, e.g., quality and quantity of cells. In some samples the number of tumour cells is high, as is true especially for certain hematologic malignancies. In others, however, there might only be very few tumour cells, even as few as two or three per high power field. These cells might be obscured by a high number of bystander cells. In these cases the interpretation of FISH signals is difficult and the results might be misleading, especially if signals from tumour cells are considered artificial or neglected because of the use of certain cut-off levels. Due to the sometimes altered morphology and also due to the tumour type, the neoplastic cells might not be identified as malignant during FISH evaluation. Therefore, to overcome such limitations of FISH in some instances, a combination of FISH with other methods has to be considered for special types of tumours and questions. A combination of FISH with immuno-staining (FICTION, Fluorescence Immunophenotyping and Interphase Cytogenetics as a Tool for Investigation of Neoplasms) has proved to be feasible and effective [5–7]. A combination of the two techniques allows the identification of genetic aberrations in even very few tumour cells in a mixture of neoplastic and innocent bystander cells, using the immunophenotype of the cells for identification of the neoplastic cells.

4. Lectures: Novel techniques and instrumentation

The principle of Fluorescence *In Situ* Hybridization (FISH) with COMBinatorial Oligo (COMBO) probes was presented by Michael Hausmann as a new approach that permits specific labeling of any given genomic sites for all species with an established genome data base [3]. COMBO-FISH takes advantage of homopurine/homopyrimidine oligo-nucleotides that form triple helices with intact duplex genomic DNA without the need for prior thermal or chemical denaturation of the target sequence, usually applied for probe binding in standard FISH protocols. An analysis of human and mouse genome data bases has shown that homopurine/homopyrimidine sequences longer than 14 DNA bases are nearly homogeneously distributed over the genome and that they represent about 1–2% of the entire genome. Considering that the minimum observation volume in a confocal laser scanning microscope equipped with a high numerical aperture lens corresponds on average to a ∼250 kb chromatin domain in a normal mammalian cell nucleus (e.g., lymphocyte), this volume should typically contain 150–200 homopurine/homopyrimidine stretches. Using DNA data base information, a set of distinct, uniformly labelled oligo-nucleotide hybridization probes can be configured from these stretches. This set is expected to exclusively co-localize within a 250 kb chromatin domain, although some of the oligo-nucleotides have additional binding sites somewhere else in the genome. Due to the diffraction limited resolution of a microscope, the fluorescence signals of the joined oligo probe set merge into a typical, nearly homogeneous FISH "spot". Using a set of 32 homopyrimidine probes, experiments in the ABL region of human chromosome 9 were performed as a very first "proof of principle" of COMBO-FISH. The technique was applied to human peripheral blood lymphocytes and routine bone marrow smears (Fig. 2). The protocol offers the advantage of gentle specimen treatment in contrast to standard protocols.

Roland Krämer showed how interdisciplinary work between biophysics, chemistry and biology can improve the methods and tune probes by chemical modifications. He demonstrated that it is possible to synthesize novel probes tailored to certain microscopic devices or specimen conditions. This may overcome the limitation on the commercially available probes designed by standard molecular cytogenetic techniques. Besides DNA oligo-nucleotides new PNAs, "Smart Probes", and surrogates can contribute to an improvement in labelling quality, sequence specificity, FISH hybridization efficiency, and photo-stability by means of specific variations in the dye components or structure modifications of the sequence. Novel chemical reactions can contribute to a reduction of the background fluorescence in such a way that the fluorochrome is only switched on if the probe is specifically bound to its complementary target. These switch-on/off effects can be achieved by appropriate metal ions.

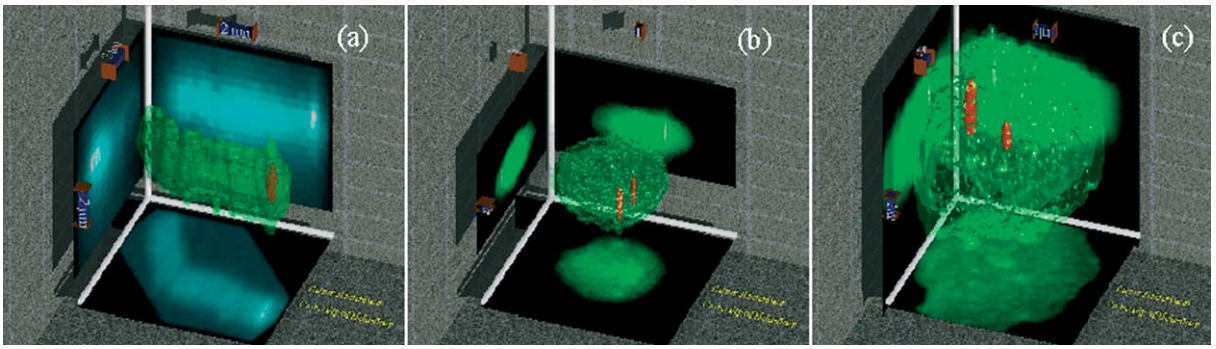

Fig. 2. 3D-image of a metaphase chromosome 9 (a) and cell nuclei obtained from a lymphocyte preparation of peripheral blood (b) and a bone marrow smear (c) after specific labelling of the abl gene (9q34) by COMBO-FISH using a combination of 32 pyrimidine oligo-nucleotides labelled with two dye-molecules Alexa 514 each. The oligo-probes co-localise only at the abl gene shown in red false colour. The FISH procedure was performed without thermal denaturation of the target DNA. For image acquisition a laser scanning microscope was used. The 3D-image was obtained from an image stack of optical sections. The bottom and the back show the maximum projections of the 3D-image in each direction.

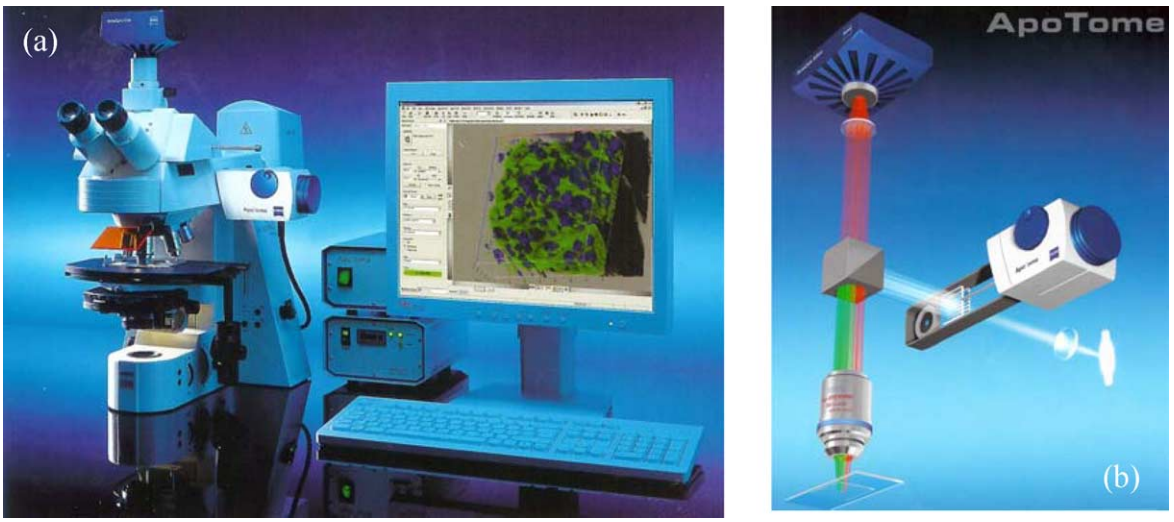

Fig. 3. Axioplan microscope with APOTOME (a) and the illumination and detection light path of the system (b).

Karl-Heinz Körtje from Leica gave an overview about the hard- and software presently available from his company for FISH analysis.

Jochen Tham from Carl Zeiss Microscopy introduced the APOTOME as a new method for the easy generation of 3D-image stacks in fluorescence microscopy. In addition to the established confocal laser scan microscopy and the deconvolution Carl Zeiss has recently introduced the APOTOME on the market of 3D-imaging systems (Fig. 3). Based on structured illumination in the wide-field fluorescence beam-path, this new system is able to capture and display "confocal" images online and in high image quality. A peltier cooled AxioCam MRm camera is providing the advantages of CCD sensors as sensitive and fast imaging devices. Using the beam-path of an Axioplan imaging microscope the system creates multi-channel images using the standard fluorescence filter sets. Even DAPI images are possible without the need for expensive uv-laser equipment.

The handling of the software module based on AxioVision allows an easy acquisition of 2D- and 3D-images even in routine applications. Optical sectioning and the elimination of off-focus plane light is the basic method to locate fluorescence signals in cell nuclei precisely. Additionally, these image data with their exact x–y–z information for every pixel (z-position is automatically read out from the motorized microscope stand) are opening up the possibilities of rendering and three-dimensional projection. For the aspect

of FISH, this technique provides the chance for a reproducible detection of the precise localisation of each signal.

5. Discussion II: New developments – FISH, quo vadis

There were two different aspects of the application of FISH: The routine application in clinical practice, i.e., in pathology, haematology, prenatal diagnosis, etc., areas in which commercially available kits and probes are available and have reached a certain standard. However, limits in optimisation are very often given by the patent situation which for instance limits the availability of probes with any given dye.

Nevertheless, it appears to be a useful step to optimise practical conditions to define platforms for protocol and experience exchange. Such steps in protocol improvements are to season the specimen, to amplify weak signals, to apply RNase or pepsin, or to find the best combination of fixation and FISH modifications.

On the other hand, in fundamental research, FISH as it exists is not enough to solve questions related to nuclear organisation, to elementary nuclear compartments and to gene expression. Basically we must consider FISH as a methodology to target DNA in morphological structures: cells and tissues are able to generate much more information at a supra-molecular level than chips. In consequence, topology and dynamics of specific sequences in the nucleus become major points of interest, however, considering the aspect at which scale such phenomena should be investigated (?). Classical photonic microscopy using typical fluorochromes is restricted by diffraction limits of Abbe resolution. So new approaches, like SPDM, 4pi, SMI, two photons microscopy, and the generation of second harmonics [4], potentially allow the analysis at the nano scale level. Reporter molecules must to be revisited: if the idea is to work at a nano-scale dimension, we ought to use also nano-particles as reporter molecules. One base pair has a dimension which can be calculated at 0.4 nm. Theoretically, we can target few base-pairs using tiny particles. Finally detection of single molecules and the orientation in space allowing a supra-molecular research in fundamental nuclear organisation is a major goal of the future development on FISH technology.

In his closing remarks Michael Hausmann summarized some major aspect of this workshop and expressed his feeling that although FISH has become routine, it is still a challenging subject of research and development which can be supported by the exchange of experience and ideas.

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