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Ways to the High-Resolution Cytometry Network

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Abstract

This paper concerns fundamental tasks solved in the Laboratory of High-Resolution Cytometry in Brno and present and planned techniques for solving these tasks. The current research is aimed at 2-D and 3-D analysis of FISH-stained interphase nuclei, metaphase spreads and tissues. Both basic research and clinical applications are performed. The research is mostly performed using high-resolution cytometry technique developed in our laboratory. Two instruments capable of automated image acquisition from microscope slides have been developed. The acquired images are analysed by computer. The present image analysis system has some drawbacks. Therefore, development of a new system has started. The new system will be based on the client-server architecture and is called High-Resolution Cytometry Network.

1 Introduction

Cytometry, i.e. performing measurements on cells and their components (cell nuclei, chromosomes, etc.), plays key role in cell research. These measurements can provide both quantitative and qualitative information on the measured components. If images acquired by optical microscopes are used for performing measurements, the observed objects must be visualised by means of one of the available visualisation methods. Most often, fluorescent dyes are used and fluorescence in situ hybridization (FISH) is the most common staining technique for human genome visualisation.

The FISH technique is often used in basic biomedical research. It is used, for example, for the study of chromatin¹ organisation in cell nuclei [2], its depen-

¹Chromatin is the most important part of the cell nucleus where all the genetic information is stored.

dence on the stage of the cell cycle [4] or on the cell type, for the study of the effect of radiation on the chromatin structure [7], or for the identification and quantification of numerical and structural chromosomal aberrations. The FISH technique is also used in clinical applications. This particularly concerns diagnosis and prevention of serious human diseases which are highly correlated to some chromosomal aberrations. For example, the translocation between chromosomes 9 and 22 was observed in chronic myeloid leukemia [10]. However, in many cases a big number of images must be analysed and an automation is required.

The first fully automated system for interphase² cell nuclei analysis using FISH was developed at the Delft University of Technology in the Netherlands by Netten et al. [8, 9]. Another similar fully automated hybridization dot analyser was reported by Ortiz de Solórzano et al. [3]. Both systems work only in 2-D and perform only dot counting. A better cytometry system for FISH-stained interphase nuclei analysis was constructed by Kozubek et al. [5]. The system called High Resolution Cytometry (HRCM) instrument was developed in the Laboratory of HRCM which is a common project of the Faculty of Informatics, Masaryk University and Institute of Biophysics, Academy of Sciences, Czech Republic. The measurements performed using HRCM instrument cannot be performed using any other instrument at comparable speed and comparable quantities of processed cells.

The HRCM instrument performs both 2-D and 3-D analysis of hybridization dots and their spatial studies. However, it has got some serious drawbacks in image analysis. The image analysis part of the HRCM instrument works well only with some cell types of certain properties and is hardly further extendible. Moreover, precise gene-to-membrane distance measurement is not possible because of lack of good 3-D model of observed object boundaries. Therefore, development of a new HRCM system has started.

Section 2 explains how fluorescence images are formed and what repeated FISH means. Section 3 describes high-resolution cyto-acquisition instruments which are used for image acquisition in HRCM systems (Section 3.1), general requirements on image analysis part of HRCM systems (Section 3.2), the HRCM systems which has been developed so far in our laboratory (Section 3.3), and the new HRCM system called HRCM Network (Section 3.4) which is being developed. The last section (Section 4) discusses the future work.

2 Fluorescence In Situ Hybridization

If an optical microscope is used for cell observation, the cells, their components or their parts must be visualised by means of some staining technique. The most often used one is called Fluorescence In Situ Hybridization (FISH). It enables painting of the chosen DNA or RNA sequence by means of fluorochromes. Presently, FISH technique has reached such high sensitivity and multiplicity,

²The stage of the cell cycle in which the cell is resting or preparing for the (next) division.

that several different objects (even individual genes) can be stained in cells simultaneously by different fluorochromes.

If a fluorochrome is excited by the light of a particular wavelength, the light of a different wavelength is emitted. The emitted light goes through an emission filter and is captured by a high sensitivity grey-scale CCD-camera. Each of the used fluorochromes is excited separately. Therefore, a set of grey-scale images is obtained. If three probes are used (most frequent case), a pseudo-colour image can be easily created according to the emission wavelengths of the used fluorochromes. Only one type of object should be observed in a grey-scale channel. This case, however, does not happen in practice (see Figure 1).

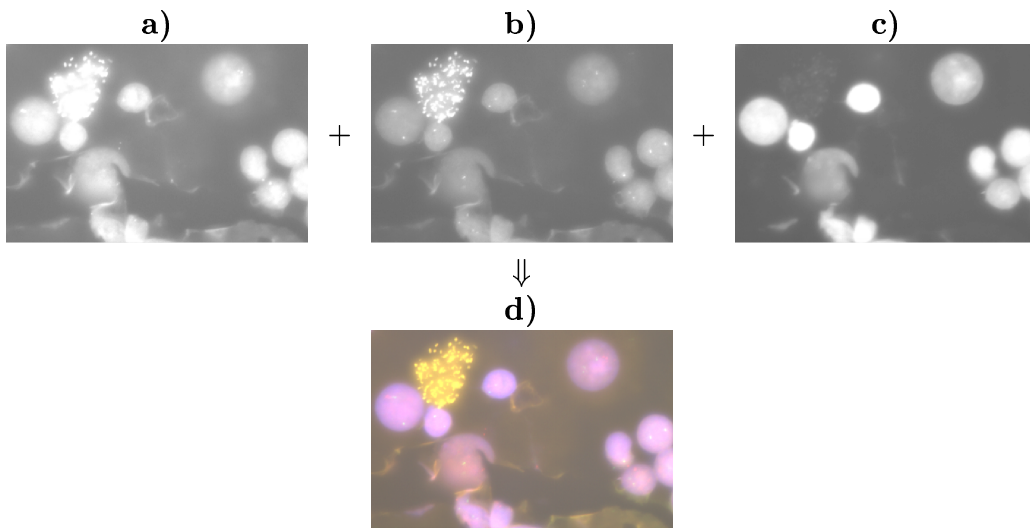


Figure 1: Composition of a pseudo-coloured FISH image. Three types of objects were stained: ABL gene (a), BCR gene (b), and chromatin (\approx nucleus)(c), by fluorochromes Rhodamin (emits red light), FITC (emits green light), and DAPI (emits blue light), respectively. Each component was captured by a high-sensitivity grey-scale CCD camera and the resulting image (d) was composed appropriately. The images can contain some dust (the upper left corner of (a) and (b)). Washing the fluorochromes out is more difficult inside the nuclei than outside them. Therefore, the nuclei are also visible in both (a) and (b) in addition to the genes (small dots).

Overlapping of the excitation and the emission wavelengths is undesirable (it leads to a poor quality of the acquired images). Therefore, at most four (but usually three) fluorochromes are used in practice for visualisation purposes on a single slide. If some application requires visualisation of more objects a repeated FISH is used, i. e. a microscope slide is rehybridized in the laboratory (some probes are removed and some other added), see Figure 2.

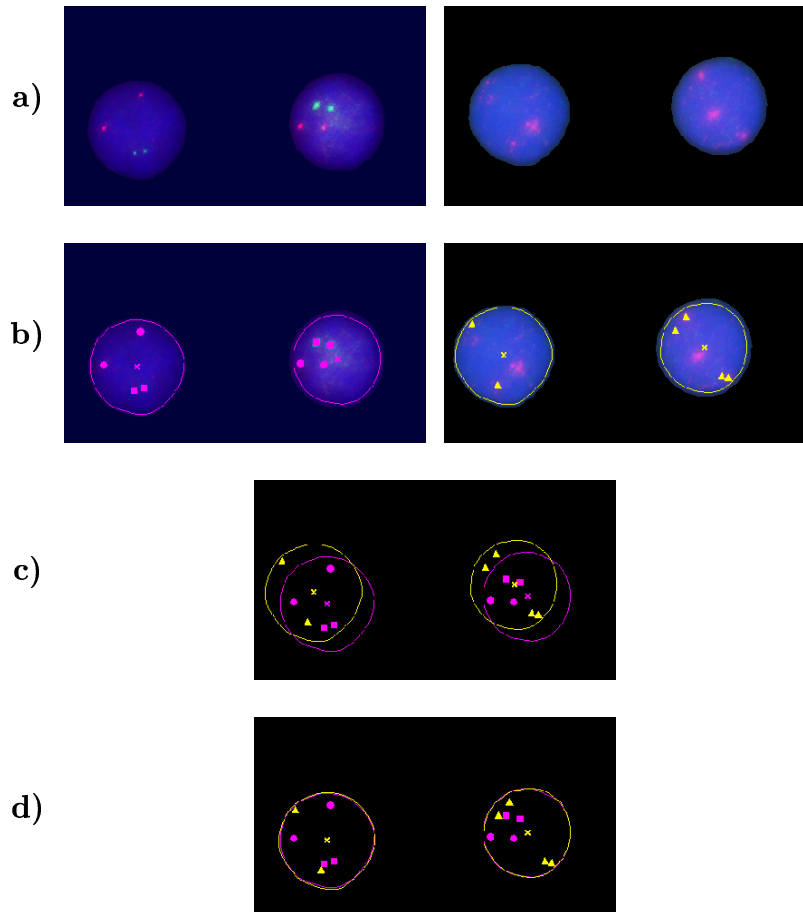


Figure 2: Repeated FISH. During the first hybridization (left column of (a)), three objects were stained: ABL gene (red component), BCR gene (green), and chromatin (blue). During the second hybridization (right column of (a)), genes ABL and BCR were unstained and gene CMYC stained by Rhodamin (red). The images (a) were analysed (b). The microscope slide must be removed from the stage and put back again between the hybridizations. It can cause a shift which is visible on the overlaid results (c). The shift has to be compensated for. The centres of nuclei were used for compensation in this example (d)

3 High-Resolution Cytometry systems

The current research in the Laboratory of HRCM is aimed at 2-D and 3-D analysis of FISH-stained interphase nuclei and metaphase³ spreads of different types of cells. Our basic research tries to contribute to the understanding of chromatin organisation in human cell nuclei. Besides, some clinical applications are carried out in collaboration with several medical centres in Brno.

The research is mostly performed using a HRCM technique developed in our group [5]. The heart of this technique is a high-resolution cytometer (also called HRCM instrument) which does some measurements on the observed cells or their parts. The high-resolution cytometer can be divided into two main parts: the HRCA (High-Resolution Cyto-Acquisition) instrument used for image acquisition (Section 3.1) and software for the detailed image analysis of acquired images. The requirements for image analysis are described in Section 3.2.

So far, two HRCM systems have been developed in the laboratory: the 1st generation cytometer and the 2nd generation cytometer. They are shortly described in Section 3.3. Image analysis in the current HRCM systems does not satisfy all the requirements stated in Section 3.2 and implementing new features into the current software is difficult. Therefore, development of a new HRCM system has started. Some information about the new system called HRCM Network is presented in Section 3.4.

3.1 HRCA instruments

The HRCA instrument consists of a computer-controlled microscope, a computer-controlled CCD camera, and a special software which enables automatic meander scanning of the microscope slides, automatic focusing of each field of view, automatic acquisition of each image (2-D) or a stack of images using axial scanning (3-D), and simple on-line analysis⁴ of the acquired images.

The modality and the type of acquired images can be modified by additional devices connected to the HRCA instruments (e.g. a confocal unit or a 2π -tilting device). The confocal unit enables HRCA instruments to perform image acquisition not only in the conventional mode but also in the confocal mode. In the confocal mode, out-of-focus information is removed from the images (see Figure 3). The confocal mode is required for proper 3-D studies, especially studies of cells in tissues. However, the acquisition time is much longer in the confocal mode than in the conventional mode because of the lower light efficiency.

The 2π -tilting device⁵ [1] enables observing of the same cell nuclei from many sides (see Figure 4) and thus acquiring of images viewed from different angles. See Figure 5 for summary of image types that HRCA instruments can

³The stage of the cell division in which chromatin is condensed to chromosomes and the nuclear membrane has disappeared.

⁴E.g. only interesting fields of views (with objects) are stored to a hard disk.

⁵The device is protected by a patent. At the present time, the Laboratory of HRCM has borrowed the device from the Ruprecht-Carls University of Heidelberg.

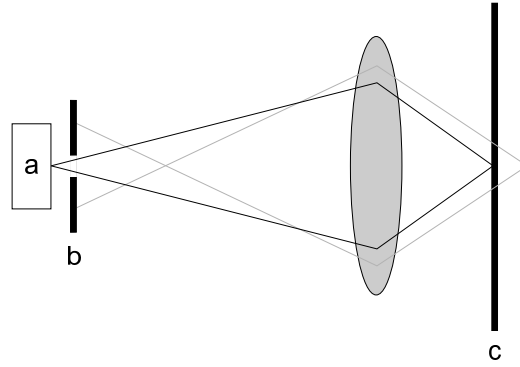


Figure 3: Confocal effect. The confocal effect can be easily obtained by putting a plate with a small pinhole (b) in front of the detector (a). Only rays from focal plane (c) can go through the pinhole. The smaller the pinhole, the stronger is the confocal effect. However, the lower light efficiency comes as a drawback.

produce using the z -movement and the 2π -tilting device. Two or more 2-D images viewed from different angles can be used for 3-D model construction. However, the stack of (parallel) images is more suitable for 3-D analysis. The lateral resolution of microscopes is always better than their axial resolution. Therefore, the advantage of the tilting device lies in 3-D-resolution improvement.

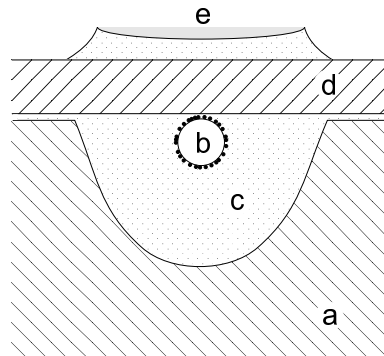


Figure 4: 2π -tilting device. A groove is made into the microscope slide (a). The observed objects (the cells) are fixed on a thin fibre (b). The fibre with objects is put into the groove, the surrounding space is filled with the immersion oil (c) and everything is covered with the cover slip (d). Manual or automatic rotation of the fibre is possible. Thus, the objects can be observed through the objective (e) from various directions.

Two HRCA instruments have been developed so far in the Laboratory of HRCM. The first one (HRCA1) is based on Leica DMRXA microscope, Micromax CCD camera, and a control computer with two Intel Pentium II processors. The second one (HRCA2) consists of Zeiss Axiovert 100 microscope, Quantix CCD camera, CARV confocal unit, Atto Instruments, and

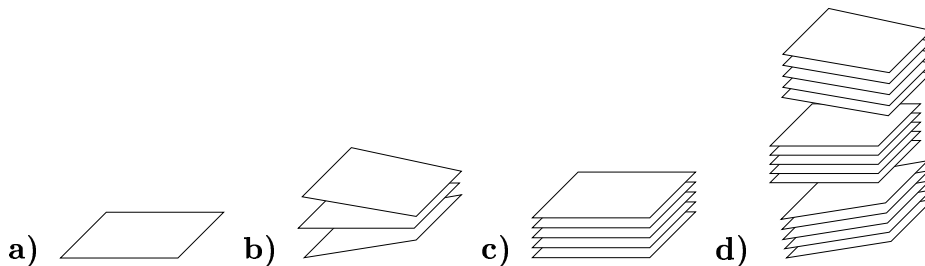


Figure 5: Image types. (a) 2-D image; (b) 2-D images viewed from different angles, 2π -tilting device must be used; (c) the stack of 2-D images i.e. 3-D image, the z -movement of the stage or the objective is needed; (d) 3-D images viewed from different angles, the 2π -tilting device and the z -movement must be used.

a control computer with two Intel Pentium II processors as well. The first processor controls image acquisition and the second one performs on-line image analysis (for more detailed description of the hardware see page: <http://www.fi.muni.cz/lom/hardware.html>).

The software used in this system is called FISH 2.0 and was developed in our laboratory. It allows automatic acquisition of (usually 10 000) 2-D or (usually 1 000) 3-D images from the slide. Beside the images, also lateral stage positions are recorded for each image so that the instruments can re-allocate the objects of interest with high accuracy even after removing the slide from the stage and placing it back again. This enables acquiring of images of the same slide repeatedly after repeated hybridization. Furthermore in the case of HRCA2, an automatic alternation between the conventional and the confocal mode is possible. The image acquisition using the 2π -tilting device is being implemented.

3.2 Image Analysis Requirements

The input of image analysis are images acquired by the HRCA instruments and some knowledge (for example, knowledge about chromatic aberrations of microscope can improve the precision of small distance measurement up to 10 times [6], or genes can be looked for only in the area belonging to nuclei and therefore the whole image need not be scanned). The goal of image analysis is to create representations suitable for performing measurements required by biologists. The most often studied cell parts in the Laboratory of HRCM are: nuclei, chromosome territories, and genes.

Chromatin is the most important part of the nucleus. It contains all the genetic material of the cell together with proteins. It occupies nearly the whole nucleus in interphase. Thus, chromatin staining is often used for visualisation of interphase nuclei (see e.g. the blue objects in Figure 6a). In metaphase, chromatin is condensed to chromosomes (see Figure 6d). In interphase nuclei, the genetic material of chromosomes is stored in distinct chromosome territories (see Figure 6f). Smaller objects than chromosomes can be stained in nuclei (e.g.

centromeres or genes). These objects are observable as so-called hybridization dots (see e.g. ABL and BCR genes (red and green dots) in Figure 6a)

The shape of nuclei is usually very close to a sphere (see Figure 6a). However, nuclei of some cell types have more complex shapes (see Figure 6e). Nevertheless, all nuclei are more or less smooth and compact. Nuclei of some cells are usually isolated (see Figure 6a) but can create clusters (see Figure 6b). Nuclei in tissues are very close to each other and can form some structures (see Figure 6c).

Biologists require to perform the following measurements as quick and as precise as possible.

- the number of objects in the superior object (e.g. the number of appearances of a gene in a nucleus — in the studies of numerical chromosomal aberrations),
- the mutual position of objects in the superior object (e.g. the mutual position of some genes in a nucleus — in the studies of translocations⁶),
- the distribution of the distance between an object and the superior object border (e.g. the distribution of some gene in a cell nucleus (the gene-to-membrane distance needed) — in spatial chromatin studies),
- the average intensity of an object, and the volume of an object (e.g. the studies of chromosome territories).

Obviously, a model of individual objects and relations between them (called comprehensive model) is the suitable representation for performing the stated measurements. The only required relation is “is part of” (or “contains”). The model of an the given object depends on the modelled object. A point (with some characteristics computed from image data — e.g. position and intensity) is usually sufficient for modelling of hybridization dots. More complex models must be used for modelling the other objects (e.g. chromosome territories and nuclei). The model of the object boundary is the most important part of these models.

The construction of a comprehensive model is an image analysis task. Specification of the image analysis process (i.e. selection of the correct algorithms and their parameters) so that it is successful on a particular image data is mostly performed by biologists (i.e. experts in biology, not in image analysis), thus the algorithm and parameter selection must be easy and preferably in their language. Moreover, the comprehensive model must be easily editable to enable the biologists to correct the image analysis results and to create the model manually in case of an automatic analysis failure. In order to support the correction of the image analysis results, visualisation of both the image data and the computed models is required.

⁶Exchange of genetic material

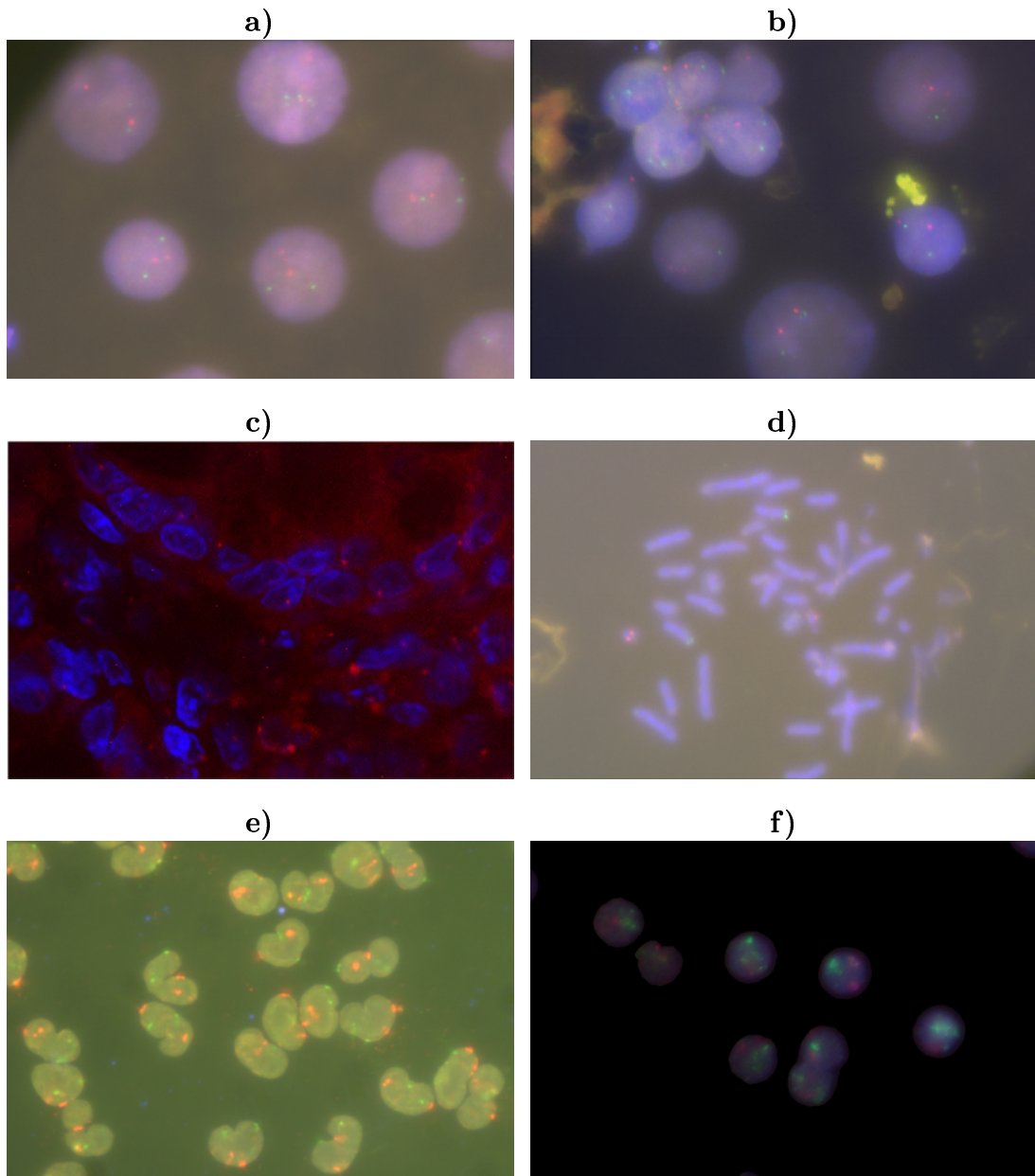


Figure 6: Examples of input images. (a) interphase nuclei (blue component) of HL-60 cells (blood cells), the small green and red dots are ABL and BCR genes (b) cluster of interphase nuclei of HL-60 cells (c) tissue of intestinal cells (d) cell in metaphase (the condensed chromosomes are well visible) (e) granulocytes (shape of their nuclei is not a sphere) (f) chromosome 9 territories (green) and chromosome 22 territories (red) in the interphase nuclei (blue)

3.3 Current HRCM systems

Presently, two HRCM systems are used in the Laboratory of HRCM: the 1st generation cytometer and the 2nd generation cytometer. The first one is based on the HRCA1 instrument and the second one on the HRCA2 instrument. Both instruments use the FISH 2.0 system⁷ for creation of models on which measurements are performed.

The image analysis part of the FISH 2.0 system represents hybridization dots as points and only 2-D boundaries of larger objects can be represented. Therefore, the 2-D studies of chromatin organisation and the 3-D studies of the mutual position of hybridization dots are possible, but the 3-D measurements of the gene-to-membrane distance cannot be performed. Furthermore, the present segmentation algorithms give satisfactory results only for isolated or slightly connected nuclei (simple clusters). Thus, manual segmentation is used in the more complex cases (e.g. tissues).

The FISH 2.0 system uses no general 3-D model and thus full 3-D studies are not possible. It has been designed only for two dimensions and has not been properly modularised from the beginning. Therefore, it is hard to extend at present. Moreover, more complex computations are difficult because the system is fastened to the MS Windows operating systems. These are the most important reasons for the design and implementation of the new image analysis system.

3.4 HRCM Network

Development of the new HRCM system has started a year ago in the Laboratory of HRCM. The system is called High-Resolution Cytometry Network. The central part of the new system will be a high-performance UNIX server. Clients and HRCA instruments will be connected to the server over the network. (see Figure 7).

The software equipment of the HRCM Network called FISH 3.0 will consist of three parts: server, client, and HRCA part. The first part will run on a two-processor server connected to a cluster of PCs. Therefore, parallel computing will be possible on the server side. The most important server task will be image analysis i.e. construction of a comprehensive model of observed objects. Presently, the key issues are: how to represent the model and how to create it automatically.

The HRCA part of FISH 3.0 will be responsible for image acquisition (the process of image acquisition is described in Section 3.1). The acquired images together with some information about the acquisition process (which acquisition modes were used, the acquisition time values, the positions on the slide, ...) will be sent to the server. This information is useful especially for repeated hybridizations and for performing measurements more precisely (e.g. for the suppression of known defects).

⁷The system has been already mentioned in Section 3.1. It is used for the hardware control and the simple on-line image analysis on the HRCA instruments.

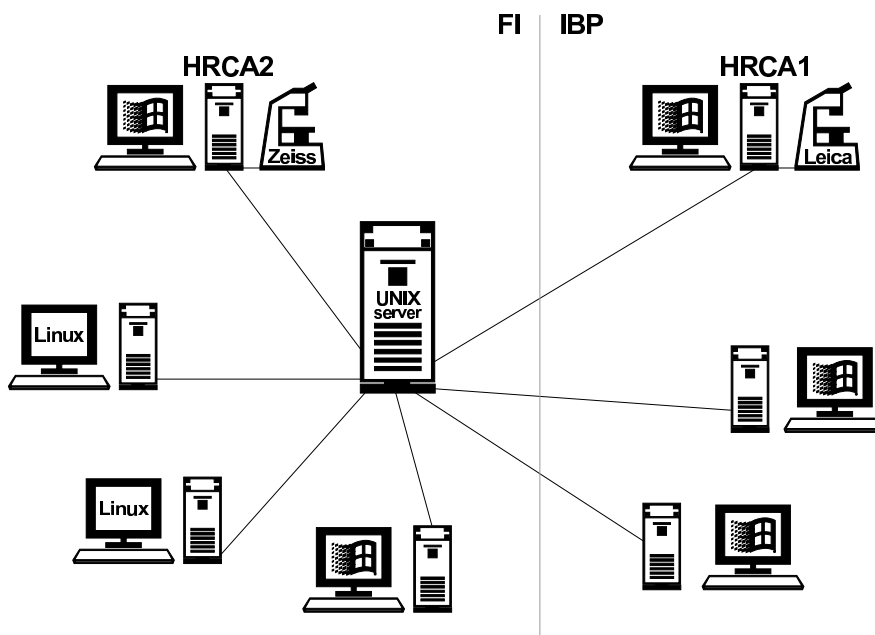


Figure 7: HRCM Network. The new HRCM system will be built on the client-server architecture. Both HRCA instruments will be connected to the server over the network and will send acquired images to it. Image analysis will be performed on the server. Clients will run the user interface and present image analysis results. One part of the system will be located at the Faculty of Informatics (FI) and the second one at the Institute of Biophysics (IBP)

The client part of the software will run on PCs with MS Windows or Linux operating systems. Its main task will be interaction with a user, particularly adjustment of image analysis parameters (usually on small subset of acquired images) and presentation of image analysis results (for the whole data set) to the user. Moreover, it will enable modifying or creating of the model manually (if image analysis does not give satisfactory results on some data).

4 Conclusions and future plans

Two HRCM instruments have been developed in the Laboratory of HRCM. These systems enable acquiring of a large number of images from a microscope slide using fluorescence microscope and performing some measurements on objects observed in the acquired images. The systems are very quick and precise for 2-D biological studies and for some 3-D studies. Unfortunately, fully 3-D studies and automatic analysis of some cell types are not possible. Especially the image analysis part of the systems is hard to extend at present.

Development of the new system called HRCM Network which should overcome drawbacks of the current systems has started. A part of the current HRCM systems used for image acquisition called HRCA instrument will still be used in the new system. The biggest changes has to be made in the image analysis part of the system. This part will be moved to the server and possibly parallelised and its new and the most important feature will be a general 3-D model of observed objects. The key issue is how to represent the model.

The development of the new system should be done in the following steps:

- design and implementation of communication between the three main parts of the system (i.e. the server, the clients, and the HRCA instruments),
- incorporation of a general 3-D model of observed objects into the system and enabling its manual creation,
- at the same time, development of the kernel of an automatic image analysis system (in order to provide the possibility of specification of image analysis algorithms used in the current systems so that they could create the new model),
- insertion of the most important image analysis algorithms so that the basic automated image analysis would be possible,
- reimplementing of image acquisition into the new system.

After these steps the new system should replace the current systems.

When the old systems are replaced by the new one, some other issues could be discussed. For example: automatic adjustment of image analysis algorithms (e.g. using knowledge about performed corrections by biologists on the image analysis results); simplification of selection of the suitable image analysis algorithms for the given data (e.g. using a knowledge-based system); enhancement

of visualisation algorithms; or possibilities of the use of uncertainty (e.g. decisions “it is hybridization dot” or “it is noise” are uncertain) and fuzziness (e.g. nucleus borders are fuzzy).

References

- [1] J. Bradl, M. Hausmann, B. Rinke, and C. Cremer. A versatile 2π -tilting device for fluorescence microscopes. *Journal of Microscopy*, 176:211–221, 1994.
- [2] T. Cremer, A. Kurz, R. Zirbel, S. Dietzel, B. Rinke, E. Schröck, M. R. Speicher, U. Mathieu, A. Jauch, P. Emmerich, H. Scherthan, T. Reid, C. Cremer, and P. Lichter. Role of chromosome territories in the functional compartmentalization of the cell nucleus. *Cold Spring Harbor Symp Quantitative Biology*, 58:777–792, 1993.
- [3] C. O. de Solórzano, A. Santos, I. Vallcorba, J. M. García-Sagredo, and F. del Pozo. Automated FISH spot counting in interphase nuclei: Statistical validation and data correction. *Cytometry*, 31:93–99, October 1998.
- [4] R. Hulspas, A. B. Houtsmuller, P. J. Krijtenburg, J. G. J. Bauman, and N. Nanninga. The nuclear position of pericentromeric DNA of chromosome 11 appears to be random in G_0 and non-random in G_1 human lymphocytes. *Chromosoma*, 103:286–292, 1994.
- [5] M. Kozubek, S. Kozubek, E. Lukášová, A. Marečková, E. Bártová, M. Skalníková, and A. Jergová. High-resolution cytometry of FISH dots in interphase cell nuclei. *Cytometry*, 36:279–293, 1999.
- [6] M. Kozubek and Petr Matula. An efficient algorithm for measurement and correction of chromatic aberrations in fluorescence microscopy. *Accepted to Journal of Microscopy in July 2000*.
- [7] S. Kozubek, E. Lukasova, L. Ryznar, M. Kozubek, A. Liskova, and V. Kroha. Chromatin structure and its cell cycle kinetics in intact and irradiated cell nuclei. In G. Horneck, editor, *Fundamentals for the assessment of risks from environmental radiation*. NATO ASI Series, Kluwer Academic Publishers B. V., 1998.
- [8] H. Netten, L. J. Van Vliet, H. Vrolijk, W. C. R. Sloos, H. J. Tanke, and I. T. Young. Fluorescent dot counting in interphase cell nuclei. *Bioimaging*, 4:93–106, 1996.
- [9] H. Netten, I. T. Young, L. J. Van Vliet, H. J. Tanke, H. Vrolijk, and W. C. R. Sloos. FISH and chips: automation of fluorescent dot counting in interphase cell nuclei. *Cytometry*, 28:1–10, 1997.

- [10] D. Tkatchuk, C. Westbrook, M. Andreef, T. A. Donlon, M. L. Cleary, K. Suryanarayan, M. Homge, A. Redner, J. W. Gray, and D. Pinkel. Detection of BCR-ABL fusion in chronic myelogeneous leukemia by two colour fluorescence in situ hybridization. *Science*, 220:559–562, 1990.

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