Workshop Program

Kirchhoff-Institute of Physics

June 1-2, 2006

Paints and Colors in Molecular Biology: Requirements and Solutions

Organisers:
M. Hausmann and C. Cremer, Heidelberg

Location:
Kirchhoff-Institut für Physik, Im Neuenheimer Feld 227, 69120 Heidelberg
Supported by the Deutsche Forschungsgemeinschaft Priority Program 1128 (SPP)

Exhibition Institute of Desktop Publishing, Mannheim

Program

Donnerstag, 1. Juni

13.00 h Registration

13.30 h Welcome

Greeting
Prof. K. – H. Meier, Dean of the Faculty of Physics and Astronomy

The Power of Colors
Prof. M. Hausmann, Kirchhoff-Institute of Physics, Heidelberg

Scientific lectures – Requirements
Chair: M. Hausmann

14.00 h Clinical Applications and Requirements of Paints and Colors in Human Genetics
Prof. Dr. Ursula Froster, Institute of Human Genetics, Leipzig

14.30 h The human kinetochore studied by live cell imaging
Prof. S. Diekmann; Fritz Lipmann Institute, Jena

15.00 h Functional Cell Microarrays
Dr. H. Erfle; EMBL, Heidelberg

15.30 h Coffeebreak

Scientific Lectures – Sensitive detection
Chair: C. Cremer

16.00 h Light optical Nanoscopy of Fluorescent Labelled Structures
Prof. C. Cremer, Kirchhoff-Institut of Physics, Heidelberg

16.30 h Detection of zeptomole amounts of nuclei acid on DNA-microarrays by use of a self-assembling signal-amplification network structure
Dr. J. Hoheisel, DKFZ Heidelberg

17.00 h Synthesis of double-labelled ribonucleic acids for FRET-studies
A. Nierth, Institute of Pharmacy and Molecular Biotechnology, HD

17.30 h Low dose gene copy number increases of *HER2* are predictive for patient outcome in Barrett's cancer irrespective of *HER2* oncogene amplification, $p185^{HER2}$ overexpression or aneusomy 17
Dr. Roland Weis, Institut für Pathologie, Freiburg

18.00 h Pictures of the Institute of Desktop Publishing, Mannheim

19.00 h Barbecue

Freitag, 2. Juni

Scientific lectures – Dyes
Chair: U. Froster

9.00 h — Toward chromosome FISH in living cells using petide nucleic acid probes
Prof. Dr. Roland Krämer, Anorganisch-Chemisches Institut, HD

9.30 h — Novel Fluorescent Secondary Detection Tools for Flow Cytometry and Immunohistochemistry
Dr. Andrea Glatzel, Invitrogen

10.00 h — ATTO-Labels: Superior Fluorophors for Fluorescence Imaging and Conjugation
Dr. Jutta Arden-Jacob, Attotec

10. 30 h — Coffeebreak

Scientific lectures - Nanocrystals
Chair: R. Krämer

11.00 h — Application of Luminescent Nanocrystals in Bioanalytics
Jürgen Riegler, FMF Freiburg

Scientific lectures – Applications

11.30 h — Focussing Fluorochromophores onto Specific Genomic Regions: A Bioinformatic Problem
Dr. E. Schmitt, Fritz Lipmann Institut, Jena

12.00 h — Discussion - Chair: M. Hausmann

13.00 h — Snack, Departure

The Workshop will be accompanied with an Exhibition to Colors of the Institute of Desktop Publishing (IFPM) (Fachhochschule Mannheim) in the Foyer of the Kirchhoff-Institute of Physics

Abstracts

The Power of Colors

Michael Hausmann, Kirchhoff-Institute of Physics, University of Heidelberg

Colors make live beautiful. Our sensitivity for the environment, for art and life style is mainly determined by colors and their impact on our feeling. From the physical point of view colors are described by complex functions of frequencies of electromagnetic waves. In daily life colors are the result of absorption and reflection of the sun light. The colors of natural as well as artifical light sources are correlated to certain temperatures which are qualitatively closely correlated to our own feeling: blue the cold color of winter, green the warm, dulcet color of spring, and yellow and red the warm to hot colors of summer.

In biophysical and biological research colors have opened new insights in living systems. Especially specific labelling procedures using fluorescence dyes have become powerful tools in research and diagnostics, although very often dyes are only applied to produce “nice images” of microscopic systems without any consideration of the interaction of dyes and biology. However, the power color and dyes in research Is primarily not to satisfy our feeling of beautiness of images. The power of color is bridging the gap between sensitive physical instrumentation and biological information we wish to obtain. For instance, the power of distance resolution in the range of some ten nanometers using confocal laser scanning microscopy running in the mode of Spectral Precision Distance Microscopy depends on the application of different colors for specific labelling of nanotargets. Moreover, the more the sensitivity of instrumentation is increasing the more the requirements on stability and brightness increase for dyes. For instance, COMBO-FISH, a novel technique to specifically label nanotargets of a few kb DNA only in cell nuclei, works with a few dye molecules only. Therefore, the molecular character of a dye and the dye - target interaction become prominent parameters in the interpretation of the signal detected with highly sophicated, high resolution, sensitive optical instruments like the SMI microscope.

However, not only in cellular systems dyes and colors are determining parameters for the interpretation of function. In miniaturised screening systems as for instance high density CMOS peptide chips the selection of dyes or florescent nanocrystals appears to be significant for their successful application.

Colors make life beautiful; they also act as synergistic elements between scientific disciplines. This must not be the end of the power of colors. So colors may alsoo promote in bridging gaps between natural sciences and visual arts.

Clinical Applications and Requirements of Paints and Colors in Human Genetics

Ursula G. Froster, Institute of Human Genetics, University of Leipzig

Fluorescent in situ hybridization (FISH) was developed in the late 1980s. Since then it developed growing importance for clinical cytogenetics and tumor cytogenetics. It is well established for the detection of microdeletions and in prenatal diagnosis for common aneuploidies. The characterization of marker chromosomes and subtelomere screening are further applications of these methods. The future potential of these methods depends on the specifity of the probes used and on a number of technical details which need further refinement. With a focus on clinical applications the particular requirements of fluorescence molecular techniques are discussed.

The human kinetochore studied by live cell imaging

Stephan Diekmann, Fritz Lipmann Institut, Jena

Cellular functions of central importance are organised and controlled in the nucleus. We try to elucidate the structure and function of the mammalian centromere/kinetochore complex. The centromere is a chromosomal substructure responsible for high fidelity segregation of the genetic material during cell division. Malfunction of this structure leads to aneuploidy. The kinetochore protein complex settles at the centromere and attaches the complex to spindle microtubuli during mitosis. We are studying molecular processes underlying centromere function. For this purpose, we clone all kinetochore proteins as fusions with various tag combinations.

For live cell imaging, we clone N- and C-terminal fusions to EGFP, EYFP, Cerulean and other fluorescent proteins. We study the dynamic behaviour and the neighbourhood relations of the kinetochore proteins and their sub-domains in human living HEp-2 and HeLa cells. We apply FRAP and FCS as well as AB-FRET and FLIM. This combined approach allows us to elucidate the function of single proteins in the complex as well as the architecture of the centromeric chromatin fiber structure.

Functional Cell Microarrays

Holger Erfle, Beate Neumann, Michael Held, Urban Liebel, Phill Rogers, Philippe Bastiaens, Jan Ellenberg, Thomas Walter, Rainer Pepperkok
EMBL, Heidelberg

We have developed an automated pipeline for cell arrays for reverse transfection of tissue culture cells with siRNA and plasmid DNA. The method is suitable for high content screening microscopy at a high spatial and temporal resolution allowing even time-lapse analysis of hundreds of samples in parallel. All the necessary transfection components are mixed prior to robotic spotting on non-coated chambered coverglass tissue culture dishes, which are ideally suited for time-lapse microscopy applications in living cells.

The technique is presently applied to genome-wide loss of function screens using RNAi and automated microscopy of human cultured cells to identify all human genes required for mitosis (within the EU project MitoCheck, www.mitocheck.org), protein secretion and Golgi biogenesis as well as neuronal differentiation and signal transduction cascades (within the National Genome Research Network, SMP-RNAi, SMP-Cell).

Light Optical Nanoscopy of Fluorescence Labelled Structures

C.Cremer (1, 2)

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For the optical analysis of cellular nanostructures, a basic problem is the conventional light microscopical resolution limited to about 200 nm laterally and 600 nm axially. In recent years, various laseroptical ”nanoscopy” approaches to overcome this impasse have been developed. They are applicable after molecular labelling of the structures to be analysed with appropriate “paints and colors”. In this presentation, the focus will be on the following techniques: (1) Improvement of size resolution by Spatially Modulated Illumination (SMI) Microscopy. This method is based on the creation of a structured, non-focused illumination in the direction of the optical axis of the SMI-microscope and allows a resolution of axial object extension down to few tens of nanometer using 1-Photon visible range continuous light laser excitation. Present application examples are the nanosizing of specific chromatin domains labelled by fluorescence-in-situ-hybridization and of antibody labelled replication factories (collaboration with M.-Cristina Cardoso, MDC Berlin); (2) Improvement of optical resolution of fluorescence labelled cellular structures using a commercial confocal laser scanning 4Pi-microscope. This microscope type (pioneered by S. Hell) is based on highly focused laser illumination. In the version recently established at the Kirchhoff-Institute, it allows the nanoimaging of cellular structures at an optical resolution several times better than the theoretical limit of conventional light microscopy, using 2-Photon near infrared pulsed laser excitation. Examples presented range from cell membrane imaging to nuclear structures.

Detection of zeptomole amounts of nucleic acid on DNA-microarrays by use of a self-assembling signal-amplification network structure

Zheng Li, Anette Jacob and Jörg D. Hoheisel, DKFZ Heidelberg

For many microarray-based assays, sensitivity is an issue of enormous importance, defining not only the mere detection limit but also the dynamics of a measurement. Here, a procedure for the detection of nucleic acid targets on DNA-microarrays is described, which utilises branched oligonucleotide probes for signal amplification. Once the initial hybridisation of the analyte molecules to the microarray has taken place, the branched oligonucleotide probes are applied in a second hybridisation. Upon binding to the target molecules, the fluorophor-labelled probes form a network by self-assembly, thus accumulating fluorescence label at the respective microarray location for an enhanced overall signal intensity. Using labelling with the dyes Cy3 or Cy5, the signal response was found to be linear to the concentration of nucleic acid targets in a range of 10^{-21} to 10^{-16} M. The detection limit – defined as three standard deviations above the background signal – was approximately 4 x 10^{-22} M, equivalent to about 240 molecules. Therefore, this method works at a sensitivity sufficient to detect and quantify DNA or RNA directly at naturally occurring concentrations without the need for applying target amplification schemes. The strategy also avoids any handling of the target samples prior to microarray analysis, such as reverse transcription or labelling, and could thus be well suited for routine application.

(www.dkfz.de/funct_genome)

Synthesis of double-labelled ribonucleic acids for FRET-studies

A. NIERTH, M. HELM and A. JÄSCHKE,
Institute of Pharmacy and Molecular Biotechnology, Heidelberg

A powerful method for studying a broad array of biophysical problems like conformational or structural changes is the time-resolved measurement of fluorescence resonance energy transfer (FRET), which occurs between fluorescent dyes that match in their spectral properties. But introducing two different dyes into the same molecule at site-specific positions requires adequate strategies.

One convenient way of doing so, is by using “splinted ligation”: After choosing proper labelling sites, two fragments of the final molecule are separately synthesized through solid phase synthesis, each carrying one amine modification at the desired position. Uridine for example, can be replaced by 5-C6-amino-2’-deoxythymidine that contains a short C6-linker, assuring sufficient flexibility at the reacting site.

After chemical conjugation of the primary amine with the *N*-hydroxysuccinimide ester (NHS) derivative of the dye molecule, the final fragments carrying the FRET-pair are hybridized to a complementary DNA sequence that acts as a splint molecule. Subsequent incubation with T4-DNA ligase – for which RNA is also a substrate – gives after purification the final, double labelled construct.

In conclusion, this method provides high yields of RNA-constructs suitable for FRET studies, and additionally offers the flexibility for introducing other modifications like mutations or nucleotide analogs.

Low dose gene copy number increases of HER2 *are predictive for patient outcome in Barrett's cancer irrespective of* HER2 *oncogene amplification, p185^{HER2} overexpression or aneusomy 17*

Dr. Roland Weis, Institut für Pathologie, Freiburg

PURPOSE: To determine the prognostic significance of *HER2* (17q21) gene copy number changes, p185^{HER2} protein expression, and chromosome 17 aneusomy in Barrett's cancer (BCA).
MATERIALS AND METHODS: Tissue microarray sections of resected BCAs (n = 124) were investigated with 3D-fluorescence *in situ* hybridization of complete nuclei using locus-specific and centromeric probes for *HER2* and chromosome 17. Centromeric probes for chromosomes 1, 8 and 14 were used as controls to confirm ploidy levels. Expression of *HER2* gene product p185^{HER2} protein was assessed by immunohistochemistry (DAKO HercepTest). Correlations of experimental findings with clinical and follow-up data (mean follow-up, 30.6 months; range, 0 to 163.2 months) were analized.
RESULTS: The *HER2*-to-chromosome 17 ratios varied from 0.19 to 20.00. Values of 1.0 - 2.0 indicate low copy number gain and were most frequently found 55.6% (66 cases). Increased gene copy number of *HER2*, irrespective of numbers of further additional genes was predictive of disease-free survival (DFS, p = 0.0084) and overall survival (p = 0.024) on univariate and multivariate analysis (p = 0.03). In contrast, no prognostic significance was found for chromosome 17 aneusomy as well as chromosomes 1, 8 and 14, excluding aneusomy 17 or ploidy as a source of increased *HER2* gene copy number. As well, p185^{HER2} overexpression was not a predictor of prognosis.
CONCLUSION: Low dose gene copy number changes of *HER2* are predictive for adverse patient outcome in Barrett's cancer irrespective of further additional gene amplification. In contrast, no prognostic influence on patient outcome was found for p185^{HER2} overexpression, chromosomal aneusomy 17 or aneuploidy. Thus, thorough evaluation of *HER2* low copy number changes by 3D-FISH offers a sophisticated tool for the detection of such subtle alterations. This may also lead to new opportunities for patient stratification in clinical trials and possibly enables novel therapeutical approaches.

Toward chromosome FISH in living cells using petide nucleic acid probes

R. Krämer, Universität Heidelberg

A convenient method for the selective in situ labelling of nuclear DNA sequences in live cells would be extremely valuable for analyzing nuclear organizsation and native chromosome structure and dynamics. Peptide nucleic acids (PNAs), DNA analogs that have N-(2-aminoethyl) glycine linkages rather than a phosphodiester backbone, posses properties that make them leading candidates for this task, including sequence recognition in ds-DNA by strand invasion and resistance to degradation by nucleases. To realize the potential of PNA for chromosome fluorescence in situ hybridization (FISH) in live cells, several challenges have to be addressed such as effective cellular and nuclear uptake, background fluorescence and target binding in vivo. This lecture is focused on:

- Cellular/nuclear accumulation of usually impermeable PNA probes by small-molecule chemical modifications [1].
- Fluorogenic PNA probes whith hybridization dependent fluorescence and variable fluorophores, emission ranging from 450 to 800 nm.[1]
- Application of the PNA probes to the selective labelling of repetitive sequences of human chromosomes in fixed cells under nondenaturating conditions, and preliminary results with living cells [2].

[1] A. Füssl, A. Schleifenbaum, M. Göritz, A. Riddell, C. Schultz, R. Krämer, *J. Am. Chem. Soc*. **2006**, *128*, 5986-5987.
[2] Z. Kaja et al., submitted.

Novel Fluorescent Secondary Detection Tools for Flow Cytometry and Immunohistochemistry

Advantages of the Alexa Fluor® dyes in Molecular Imaging

Dr. Andrea Glatzel, Technical Sales Specialist – Imaging & Microscopy.

The introduction of fluorescence-based detection technologies has greatly contributed to our understanding of biological process. However during the last years it became more and more obvious that the usage of traditional dyes such as FITC, R-PE or ABC, Cyano-dyes is limited. Common issues in fluorescent based technologies are stability, intensity and the spectral overlap of the dyes in multiplexing applications.

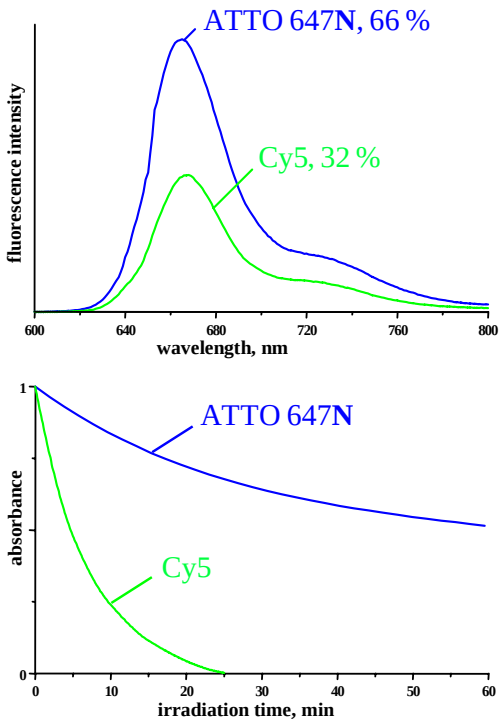
Invitrogen's **Alexa Fluor® Dyes** are very bright and photostable chemical dyes available for conjugation in both Flow Cytometry and Immunohistochemistry. The fluorescence of these dyes is very intense, long lasting and pH independent. This presentation will give an overview of the advantages of using the **Alexa Fluor® Dyes** with respect to more traditional dyes and will show their use in secondary detection technologies.
It will also introduce a novel technique called **Zenon Technology** that provides unsurpassed diversity, speed and multiplex ability in secondary detection. It is a very economical technique that enables fluorescent multicolor labelings to be carried out in a matter of minutes.
Low expressed antigens are normally not easy to detect. The **Tyramide Signal Amplification System (TSA**) is a technology which allowed enhancing the signal intensity in a very easy and specific way.
Furthermore, the **Qdot® nanocrystals** will be introduced. Qdot® nanocrystals are tiny semiconductor crystals (quantum dots) that emit brilliant light in colors from ultraviolet to infrared. The promise of Qdot® nanocrystals extends across a diverse array of environments. From beads to tissues, gels to cells, titer plates to test strips, and Cytometers to Microscopes. The focus will be on their use in secondary detection.

ATTO-Labels: Superior Fluorophors for Fluorescence Imaging and Conjugation

Dr. Jutta Arden-Jacob, ATTO-TEC GmbH

ATTO-TEC offers a new generation of patented fluorescent markers whose spectral properties span the range from the ultraviolet to the near infrared. Newest among these dyes are ATTO 488, ATTO 532, ATTO 550, ATTO 620 and ATTO 647N with absorption and emission spectra very similar to those of the frequently used Cy3, Cy5 and AlexaFluor 647.

ATTO-TEC's dye philosophy is to offer highly rigidized dye structures. The chromophoric systems of ATTO-dyes do not exhibit the well-known disadvantage of cis-trans isomerisation which is a common feature of all cyanine dyes. The cis-trans isomerisation leads to absorption shifts and fluorescence quenching. Moreover the degree of cis-trans isomerisation depends strongly on solvent and temperature. Due to their rigid structure the new ATTO-labels do not form equilibria with various isomers. Characteristic features of ATTO-dyes are strong absorption, little triplet formation and a high fluorescence quantum yield nearly independent of temperature and solvent environment. Furthermore many ATTO-dyes show excellent water solubility and a very low tendency for aggregation, which often causes fluorescence quenching in aqueous solution. Another important feature of ATTO-labels is their outstanding photostability. Low photostability decreases sensitivity and quality of imaging. ATTO-dyes with rigid structures are the most photostable labels available. ATTO 647N is the superior fluorescent label in the red spectral region. With a fluorescence quantum yield of 66 % in water, twice as high as the quantum yield of Cy5, and an unmatched photostability it should be useful for all kinds of fluorescence labelling and detection applications.



Nowadays ATTO-labels find applications in fluorescence microscopy, flow cytometry, DNA sequencing and amplification (PCR), time-resolved spectroscopy, fluorescence resonance energy transfer (FRET) and single-molecule detection. Some ATTO-dyes have proven very useful in recent developments like STED-microscopy as well.

Application of Luminescent Nanocrystals in Bioanalytics.

Jürgen Riegler*, Thomas Nann, Materials Research Center of Freiburg

In the last few years, the unique properties of nanosized, crystalline materials (nano-crystals, (NCs)) gained an increasing interest. This properties can be advantageous for many applications, especially in the field of biological and diagnostic imaging [1,2]. For instance, luminescent, semiconductor NCs (Quantum Dots (QDs)) display a bright, narrow, and tuneable fluorescence with nearly no fading effects. The figure depicts A) a TEM picture of CdSe-QDs, B) emission-spectra of differently sized QDs, C) an absorption spectrum and the corresponding emission spectrum of a QD solution.

There are three main concepts to functionalize NCs biocompatible (Figure D). A: NCs are enclosed into micelles, B: the surface of the NCs is designed by means of ligand-exchange, C: single NCs are enclosed into a polymerized network (e.g. silica-shell) [3].

In a proof of principle experiment biocompatible QDs were coupled to tubulin and the self-assembly of microtubules was monitored. The process was visualized over a period of 2 hours under continuous excitation with a fluorescence microscope. Figure E and F show filamentous as well as globular microtubule formed during this process. This was the first time that a dynamic biological process was visualized for such a long period of time under continuous excitation using fluorescent techniques [4].

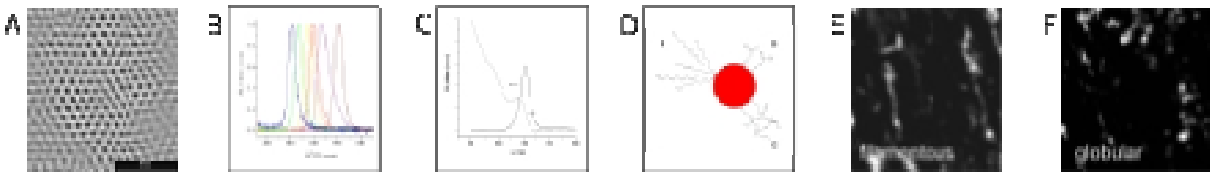


Figure: A) TEM-picture of QDs, B) emission-spectra of various QDs, C) absorption- and emission spectrum of QDs, D) schematic view of surface treatment, E, F) filamentous and globular QD-tagged microtubules

REFERENCES

[1] W. Chan, S. Nie, *Science*, (Quantum Dot Bioconjugates for Ultrasensitive Nonisotopic detection), **281** (1998) 2016-2018.
[2] J. Bruchez, M. Moronne, P. Gin, S. Weiss, A. Alivisatos, *Science*, (Semiconductor Nanocrystals as Fluorescent Biological Label), **281** (1998) 2013-2016.
[3] J. Riegler, T. Nann, Anal. Bioanal. Chem., (Application of Luminescent Nanocrystals as Labels for Biological Molecules), **379**, (2004), 913–919.
[4] J. Riegler, P. Nick, U. Kielmann, T. Nann, *J. Nanosci. Nanotech.*, (Visualizing the Self Assembly of Tubulin with Luminescent Nanorods), **3** (2003) 380-385.

Focussing Fluorochromophores onto Specific Genomic Regions: A Bioinformatic Problem

Eberhard Schmitt, Fritz Lipmann Institut, Jena

Labelling specific genomic regions has become a standard tool for design and execution of scientific experiments and diagnostic protocols. Closely inspected however, some of these techniques, e.g. standard FISH protocols based on ready to use kits, turn out to be rather unspecific and useless for the investigation of detailed questions about the nano-structure or the nano-combinatorics of the genome. In particular, when using molecular biologically Bac- or Yac-clone derived probe sets, complete genes including large parts of their neighbourhood are labelled by one signal.

In our approach, a set of 10 to 30 sequences of length 15-30 nucleotides each are designed in such a way that they bind to parts of the region to be labelled. The total set can be detected by standard biochemical and optical methods spotting fluorescent tags etc. As these short sequences hybridize also to other loci within the genome (in general to about 100 to 400 additional loci per sequence), special care is taken to avoid colocalizing except for the specific region to be labelled. This is achieved by special algorithmic investigation of the total sequenced genome of the corresponding organism. It is necessary to scan the whole genome for all occurrences of the respective probes and to reduce the set of candidates whenever a colocalization of more than six probes within a certain range is found. This is an iterative process, which id NP-hard.

The method has been worked out for homopurine and hompyrimidine sequences as targets. Using only homopurines (or homo-Y) has several advantages. They can be used for double as well as triple helical binding (via Hoogsteen triplets) and allow therefore also vital labellings which have been successfully protocolled. Their binding energetics is well described. The search algorithms for non-colocalizing sequence sets can be implemented extremely efficiently. On the other hand, there are limitations on the targets effected by the spare distribution of such sequences. So, labelling of regions comprising less than 100 kb becomes a problem.

We therefore extend the method to arbitrary sequence combinatorics. In addition to the more complicated search for appropriate sets, the hard problems encountered here include the investigation of binding affinity and dynamics and their parameters (by experiment, molcular modelling, and combinatorial algorithms) which are needed to set up secure experimental protocols.