



**ERA-Instruments WP 4**  
Interaction with Scientists

**Task 2.3**  
User meetings on cutting-edge techniques

**Deliverable D4.6**  
Report on the user meeting on advanced light microscopy

**Task leader**  
Estonia

**November 2009**



## Deliverable D4.6

Report on the user meeting on advanced light microscopy

November 2009

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### ***Authors***

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Christian Renner, DFG, Germany

## 1 Executive summary

The user meeting on “Current and future developments in light microscopy” took place on 24<sup>th</sup> of September 2008 in Bonn and had its focus on successful and efficient use of advanced light microscopy techniques. Pointing out existing problems was a declared aim of the discussion. The following summarizes the recommendations that were given by the participants.

In the beginning a clear distinction between different levels of maturity of the technology was made with specific recommendations for organizing access in each case.

For commercially available and mature technology it was recommended to run such instrumentation in centralized facilities that offer scientific service to the users. The problems such centres will typically face are only to a small degree specific for microscopy, but include many generic facility issues, such as availability of qualified personnel, lack of adequate reward systems for providing service on a high level and problems in funding running and maintenance costs, to name only a few. Moderate user fees were considered useful to encourage efficient use of instrument time by the users. An interesting proposition was to encourage companies to build equipment more robust so that follow-up costs would be reduced in comparison to the initial investment. It was felt that funding of standard equipment is more difficult to obtain than newly developed technology although the broad user community has more need for mature instrumentation. In line with that is the observation that upgrades are more difficult to fund although less costly compared to new investments. It was agreed that after five years an advanced microscope would be outdated. Facility managers reported also that users would request access to the highest level instrumentation available even if the scientific problem can be tackled by standard techniques and instrumentation.

For prototypes and new developments of technology centralized operation was not considered appropriate. Latest cutting edge equipment should be operated by the specialist in the specialists' lab, which however, can be placed close to a central facility. Access to cutting edge technology should be free of charge for the user. Funding for technological developments and for providing to end users technology that is not commercially available was seen insufficient.

The transition of newly developed technology to commercially available equipment was another point of concern. Companies would only develop a product, if there is a market, but it appears unclear who defines the potential market. Some microscopic techniques have even been brought to the market too early, i.e. immature. More transparency in the purchase procedure is desirable and might reduce the time between the funding decision and the point where the instrument is installed and fully operational, which on average seems to exceed the time from application to funding decision.

It was criticised that proprietary hardware and software would lead to reinventing and that patents can potentially even block further scientific exploration.

The availability of adequate software was definitely seen as insufficient. The manifold reasons for that were, however, beyond the scope of the discussion and could only be touched upon.

## **2 Introduction**

It has become increasingly obvious that concepts and strategies for Research Infrastructure (RI) funding should be harmonised and coordinated within the European Union (EU). European Strategy Forum on Research Infrastructures (ESFRI) has determined requirements for European RI funding and has presented a roadmap. Growing attention is paid to life sciences that rely on RIs of a less centralised, but more networked dimension. There is a clear need for action in the interdisciplinary area between physics, chemistry, biology and medical sciences as cutting edge instrumentation becomes increasingly expensive and, yet, indispensable for world-class research.

However, promotion of research policies, apart from the ESFRI projects, has been restricted so far to national efforts without managing these actions with a European view. Funding and research organisations cannot afford to remain at the national stage with worldwide competition for the best scientists and the most promising projects. Frontier research is international since long and funding organisations have to follow scientists to the European level.

ERA-Instruments aims at initiating coordination and a sustainable network of 16 partners including ministries, research councils, funding agencies and charities active in funding of life science RI. This European platform of relevant stakeholders will set up comprehensive tools for adequate treatment of instrumentation related topics enabling conclusions for research policies on both a national and European levels. The ERA-Net will focus on bio-analytical instrumentation (incl. post-genomic highthroughput techniques) such as NMR, mass spectrometry, microscopy, micro-array platforms etc. Midsized equipment has become a strategic essential strength for European countries. Promotion of RI funding in FP7 and support for new member states will also strengthen the position of European research.

## **3 About the deliverable and the work package/task**

### **3.1 Objective of work package and task**

The basic objective of work package (WP) 4 is to engage with scientists and the scientific communities. The scientists' views on RI funding are collected as input for ERA-Instruments' actions. Discussion groups with and within user communities will be encouraged and initiated by topical user meetings. An internet platform will be built that serves for providing tools to the broad scientific public such as best practice guidelines for cutting edge instrumentation or a directory of infrastructure funding in life sciences in non-EU countries. This platform might not only serve for exchange between ERA-Instruments and the scientific communities, but as discussion forum on mid-size RI for scientists.

Task 4.3 is concerned with identifying suitable topics and communities for user meetings. The format of the meeting (part of or attached to larger event vs. stand-alone) and the participants are of importance for organizing a successful event. Different approaches are used to be able to identify specific advantages or disadvantages of a given setting.

### **3.2 Approach**

To promote and ensure the proper operation of newly funded instrumentation user workshops with participation of scientists, manufacturers and administrators can identify (unforeseen) technological problems, facilitate the exchange of experience and expertise gained with the new instrumentation and, thus, promote successful operation of the facilities. The European character of these user meetings will bring together many more experts than national meetings and in this way experience and expertise can be exchanged and pooled to a much larger degree (than for smaller user meetings).

## **4 Results**

### **4.1 Format and participants**

The ERA-Instruments user meeting “Current and future developments in light microscopy” in Bonn on 24.09.2008 was linked to a symposium on new developments in microscopy on 22./23.09.08 “High speed optical sectioning microscopy” organized by Ulrich Kubitscheck, university of Bonn, with support from DFG.

At the user meeting participated eleven scientists, five representatives of funding organisations and two company representatives. All scientists participating were also present at the preceding symposium. Among the scientists were two members of the Scientific Advisory Board of ERA-Instruments (Dietmar Manstein and Tanel Tenson).

A simple questionnaire had been distributed at the beginning of the symposium on 22.9.08 and the 13 returned forms ( 8 from Germany, 5 from other countries) were used as input for the discussion. It is obvious, that the small number of responses does not allow to draw any conclusions by themselves, but they did serve for structuring the discussion.

The organisation of the user meeting was strongly supported by Ulrich Kubitscheck, university of Bonn, and the user meeting itself was co-chaired by Achim Tieftrunk, DFG. ERA-Instruments is indebted to both for their valuable support.

### **4.2 Outcome of the user meeting**

In the following the results of the discussion are bundled according to topics with a potential starter question / response at the beginning (boxed, response in bold).

1. Where should leading-edge microscopes be operated?	
in central facilities	10,5
in the labs of the scientists	8
2. Where are leading-edge microscopes operated?	
in central facilities	7
in the labs of the scientists	10

A clear tendency to centers is visible, which is only partially reflected by the current situation.

Three different steps of maturity of instrumentation were distinguished:

- Commercially available mature microscopy instrumentation that should be run in centers.
- Test instruments and prototypes that belong to the specialist's lab
- Development of new instruments and techniques that can only reasonably be done in the individual labs

#### Centers with mature technology:

- Finding the required qualified personnel with available positions/salaries/conditions is very difficult and hindering the creation of centers.
- Sample preparation is a crucial step for successful microscopy. It should be included in central facilities.
- To be scientifically recognised centers also need to publish. There is no adequate reward system.
- Having any central microscopy facility might be difficult for small universities. In this case labs with microscopy instrumentation should receive some funding in order to provide access to other users.
- A problem is that users are difficult to satisfy with less than the most advanced instrumentation regardless of the needs of the biological question at hand. As a result the average application for a laser scanning microscope is now asking for ~500,000 €.
- Pooling of individual funds for larger investments in a central facility is difficult, but can be done (e.g. Nitschke, Freiburg)

#### Prototypes:

- It was argued that specialized instrumentation and complicated experiments are not possible in centers. However, it was also suggested that specialized labs could be together or next doors with central facilities.
- It was suggested to provide funds for duplicating prototypes within project funding schemes as long as the technology is not commercially available.

#### New technology:

- The question was raised, if developers do have sufficient reason to provide new technology to the biologists.

To provide for the instrumentation needs there should be facilities with standard equipment and specialized labs or centers for the latest cutting-edge technology.

3. How <u>should</u> running costs, maintenance, upgrades for microscopy facilities be paid for?	
increased budget for the central facilities	7
user fees covering some costs, e.g. consumables	5
user fees covering full costs	1
4. How <u>are</u> running costs, maintenance, upgrades for microscopy facilities be paid for?	
increased budget for the central facilities	6
user fees covering some costs, e.g. consumables	6
user fees covering full costs	1

One might be surprised that full costs are not considered an appropriate solution. However, the higher the user fee, the fewer will be the users. Instead scientists will try to obtain their own instruments. even if they do not fully use the microscope and do not have sufficient expertise.

- The use of high-end equipment should even be totally free of charge.
- Moderate user fees in centers help efficient operation (even at a level of 3 €/ h in the experience of Manstein, Hannover)
- Intermediate fees should apply to users from other universities/institutions.
- Full costs should be charged to companies

For financing running costs and maintenance it was suggested:

- to install contingency funds
- to use part of the overhead on project funds. A sufficient fraction of the overhead must be available to the scientist for this.
- to allow applying for these costs in project proposals to funding organisations.
- to ask manufacturers to build instrumentation more robust

For the situation in Germany it was stated that the commitment of universities in providing funds for running and maintenance costs is not reliable and almost impossible to enforce even if in writing. Funding organisations were asked to follow up those commitments that are given when universities apply for instrumentation or collaborative research centers (that depend on instrumentation).

Contrarily, in other countries the control of commitments is much more stringent and effective.

5. Is the software relevant for your applications adequate in exploiting the possibilities of the technique?	
yes	-/-
no, better proprietary software should be available from companies	7
no, development of open-source software should be encouraged	12

Standardisation and open access to the scientific community, i.e. open source, are considered most important for software tools in microscopy.

The availability of adequate software was definitely seen as insufficient. Reasons are manifold:

- diverse applications make commercialisation difficult
- funding for sustainable software development is difficult to get, especially for ready-to-use software, not only algorithms
- no reward system for open source software
- software depends on companies and their data formats (incl. meta data)
- public domain software is often difficult to use, esp. for biologists, because it is not user-friendly

Suggestions were:

- learn from communities that have established data standards e.g. astronomers or crystallographers (CCP4 initiative)
- impose open format on companies by funding organisations
- strengthen existing "open microscopy" initiative (funded by Wellcome Trust)

The debate was very lively and it was agreed that the issue of software development, especially for image processing, needs to be addressed on a larger scale.

6. In your experience are there time delays for new microscopic techniques from technical development to access for the scientist?

**100% yes**, because:

- costs (of the instruments) are so high
- companies are conservative/hesitant
- long time from prototype to product

The discussion showed that the relation between commercialisation of a new idea and the potential market for the corresponding product is unclear. While companies only develop new products, if there is a market, it is not clear who defines the potential market.

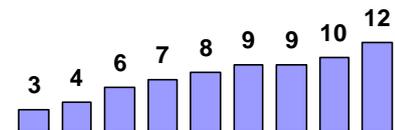
Some new microscopic techniques have been launched too early (immature).

How can the usefulness of a new technique be proven, if there is only one prototype?

It might be useful for some inventions to provide copies to other labs (before commercialisation), but how to get funding for this?

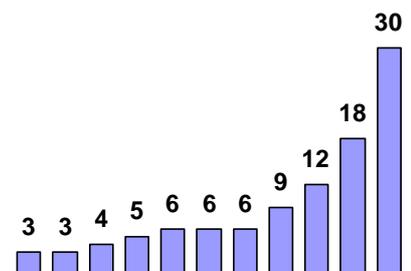
7. For a new microscope how many months does it take from submission of your proposal to decision of funding:

**7.5 ± 3 months**



8. How many months does it take from financial approval to full operation, meeting all specifications as agreed with the company?

**9 ± 8 months**

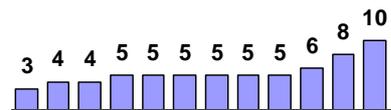


The purchase procedure may be long in some cases, because of lack of experience. Exchange of experience and/or coordination of academic customers might be useful. Making approved investments as well as companies' offers public might allow for more transparency.

Exchange of first experience would be good (what went wrong, what was good, what problems appeared with the new instrument).

9. What is the life-time for state-of-the-art microscopes in your opinion?

**5.4 ± 2 years**



It is generally considered that after approximately five years a state-of-the-art microscope is outdated. This corresponds to the product cycles of companies that are 5 – 10 years according to a company representative (Kukulis, Nikon).

10. How do investment costs, i.e. the price of a microscope, compare with running costs throughout the life-time of the instrument? Please, estimate the ratio “full running costs” / “investment costs”

**10:1, 5:1, 1:1, 1:1, 1:1, 1:3, 1:3, 1:5, 1:5      ?**

There is no agreement on the size of the full running costs of a modern microscope.

This topic was not discussed due to time limitations.

11. Are funding schemes adequate for getting access to new microscopes?

yes **3**

yes, **2** but only:

- instrumentation in centers
- leading-edge instrumentation

no, **8**

because:

- group applications lack appropriate calls
- too expensive for single grants
- wrong techniques are funded
- personnel is missing
- standard equipment is not funded enough

The following problems with current funding schemes were identified:

Funding of instrumentation:

- The funding of standard equipment is difficult although required for a broad user community
- The financing of upgrades is complicated compared to new instrumentation although less costly
- A funding gap from ~ 100,000 € to 500,000 € is felt

Funding of developments:

- Technological developments for biological applications should not always been seen as engineering problem as it is often not accepted in engineering (not “proper” engineering)
- There is a funding gap from prototype to product
- Proprietary software and hardware leads to reinventing: funding should enforce open access of hardware and software
- Scientists are forced to secure IP by patents that can block other scientists.

Suggestions:

- Funding of technological developments should be allowed in biological projects
- Funding schemes for standard equipment and for cutting-edge instrumentation should be separate with even separated budgets
- Used equipment can be inexpensive with no compromise to quality, but it is incompatible with the timing of funding schemes
- Joint applications/proposals should be possible for larger investments (common procedure in UK)

For completeness also the following response to the questionnaire is given, although these topics could not be discussed due to time limitations:

12. In your view what will be the most important development for microscopy in the next years?

<b>high-throughput</b>	<b>4D</b>	<b>add. techniques</b>
<b>software</b>	<b>single molecule</b>	<b>photo toxicity</b>
<b>better fluorophores</b>		
<b>national &amp; European centers with 50% home-made developments</b>		

13. Further comments on funding and operation of leading-edge microscopy instrumentation:

**more funding, more funding**  
**development cannot be done in centers**  
**centers with full support incl. training are needed**  
**list of equipment would be useful**

Finally, it was unanimously confirmed by all attendees that such kind of a user meeting can only be held in combination with a scientific event. This is underlined by the fact that none of the scientists that were invited specifically for the user-meeting, but had not registered for the microscopy symposium, did attend this user-meeting.

## 5 Conclusion

In terms of engagement with the scientific community the user meeting was very successful. The participating scientists ranged from technical developers to end users with biological questions to be solved and from owners of individual microscopes to facility managers. The company representatives were only two, but their comments and input were very valuable. Despite the limited time many issues could be addressed and to many general questions specific answers were found for the field of advanced light microscopy. Notwithstanding a partially controversial discussion several consensus statements emerged, among them a separation between mature instrumentation and development of new techniques. Several shortcomings of current funding schemes were pointed out as well as apparently inadequate pathways from prototype to commercial systems. Microscopy software was a point where the debate became really heated with an enthusiastic vote for open source solutions and common data standards.

Several factors seem to have contributed favourably towards a successful event: The participants were to a large part specifically selected for their potential interest in the more managerial than scientific questions addressed by the user-meeting. The preceding high-profile symposium had attracted outstanding scientists in the field. Notably, only scientists that attended the symposium agreed to participate at the user meeting. Several more were invited specifically for the user meeting, but all of them declined unless they came to the symposium as well. The questionnaire did not have a significant response rate, but pre-defined questions addressing potential problems or inquiring scientists' preferences were very well-suited to provide a structuring frame for the discussion.

A shortcoming of the user meeting was that there was an unwarranted bias towards participants from Germany and the situation in Germany. This originated from the fact that the symposium to which the user meeting was attached was also dominated by German participants. Future user meetings might try to find a more balanced distribution of participants if the topical focus is not to be mingled with a national one. On the other hand, research in these areas is international long since and differences between European countries might well be smaller than between different areas of Life Sciences.

### **Acknowledgement:**

The organisation of the user meeting was strongly supported by Ulrich Kubitscheck, university of Bonn, and the user meeting itself was co-chaired by Achim Tieftrunk, DFG.

ERA-Instruments is indebted to both for their valuable support.

## 6 Annexes

### 6.1 Annex 1 – Agenda and participants

#### ERA-Instruments' first user-meeting:

#### “Current and future developments in light microscopy” in Bonn, Germany on 24<sup>th</sup> of September

Venue: “Festsaal”, University of Bonn, Main Building, Regina-Pacis-Weg 3

#### **Programme:**

8:30 Opening

Introduction to ERA-Instruments

9:00 Discussion I: What is the current situation, what are current problems?  
Results of the questionnaire

10:30 Coffee break

11:00 Discussion II: Round table

13:00 Closing

#### **Participants:**

##### Scientists:

Cristina	Cardoso	Berlin
Rainer	Heintzmann	London
Zvi	Kam	Rehovot, Israel
Ulrich	Kubitschek	Bonn
Erik	Manders	Amsterdam
<u>Dietmar</u>	<u>Manstein</u>	<u>Hannover (SAB)</u>
Roland	Nitschke	Freiburg
Raimund	Ober	Dallas, USA
John W.	Sedat	San Francisco
Ernst H.K.	Stelzer	Heidelberg
<u>Tanel</u>	<u>Tenson</u>	<u>Tartu (SAB)</u>

##### Funding organisations:

David	McAllister	BBSRC
Marie-Denise	Breton	CNRS
Benoît	Dardelet	CNRS
Christian	Renner	DFG
Achim R.	Tieftrunk	DFG

##### Company representatives

Jörg	Kukulies	Nikon
Heinrich	Spiecker	LaVision

The user meeting was linked to a symposium on new developments in microscopy on 22./23.09.08 “High speed optical sectioning microscopy” organized by Ulrich Kubitschek, university of Bonn, with support from DFG.

All scientists participating were also present at the preceding symposium.

## **6.2 Annex 2 – Questionnaire**

The following simple questionnaire was distributed at the beginning of the symposium on 22.9.08 and the 13 returned forms ( 8 from Germany, 5 from other countries) were used as input for the discussion.



## What is your view?

For the discussion on Wednesday, 24<sup>th</sup>, on microscopy infrastructure we would like to know your opinion on the issues addressed below.

Please take a moment to give your comments.

In which country are you currently working: \_\_\_\_\_

1. Where should leading-edge microscopes be operated?

- in central facilities
- in the labs of the scientists
- \_\_\_\_\_

2. Where are these microscopes situated at your institution?

- in central facilities
- in the labs of the scientists

3. How should running costs, maintenance, upgrades for microscopy facilities be paid for?

- increased budget for the central facilities
- user fees covering full costs
- user fees covering some costs, e.g. consumables
- \_\_\_\_\_

4. How are running costs paid for at your place (in case there is a microscopy facility)?

- no user fees
- user fees covering some costs, e.g. consumables
- user fees covering full costs

5. Is the software relevant for your applications adequate in exploiting the possibilities of the technique?

- yes
- no, better proprietary software should be available from companies
- no, development of open-source software should be encouraged
- \_\_\_\_\_

6. In your experience are there time delays for new microscopic techniques from technical development to access for the scientist? If yes, what do you think is the major obstacle?

- yes, because \_\_\_\_\_
- no

7. For a new microscope how many months does it take from submission of your proposal to decision of funding:

\_\_\_\_\_ month

8. How many months does it take from financial approval to full operation, meeting all specifications as agreed with the company?

\_\_\_\_\_ month

9. What is the life-time for state-of-the-art microscopes in your opinion? \_\_\_\_\_ years

10. How do investment costs, i.e. the price of a microscope, compare with running costs throughout the life-time of the instrument? Please, estimate the ratio “full running costs” / “investment costs”

\_\_\_\_\_

11. Are funding schemes adequate for getting access to new microscopes?

- yes
- no, because

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

12. In your view what will be the most important development for microscopy in the next years?

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

13. Further comments on funding and operation of leading-edge microscopy instrumentation:

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

Please return this form to the box at the registration desk (until Tuesday 15.00 h).

**Thank you for your comments!**

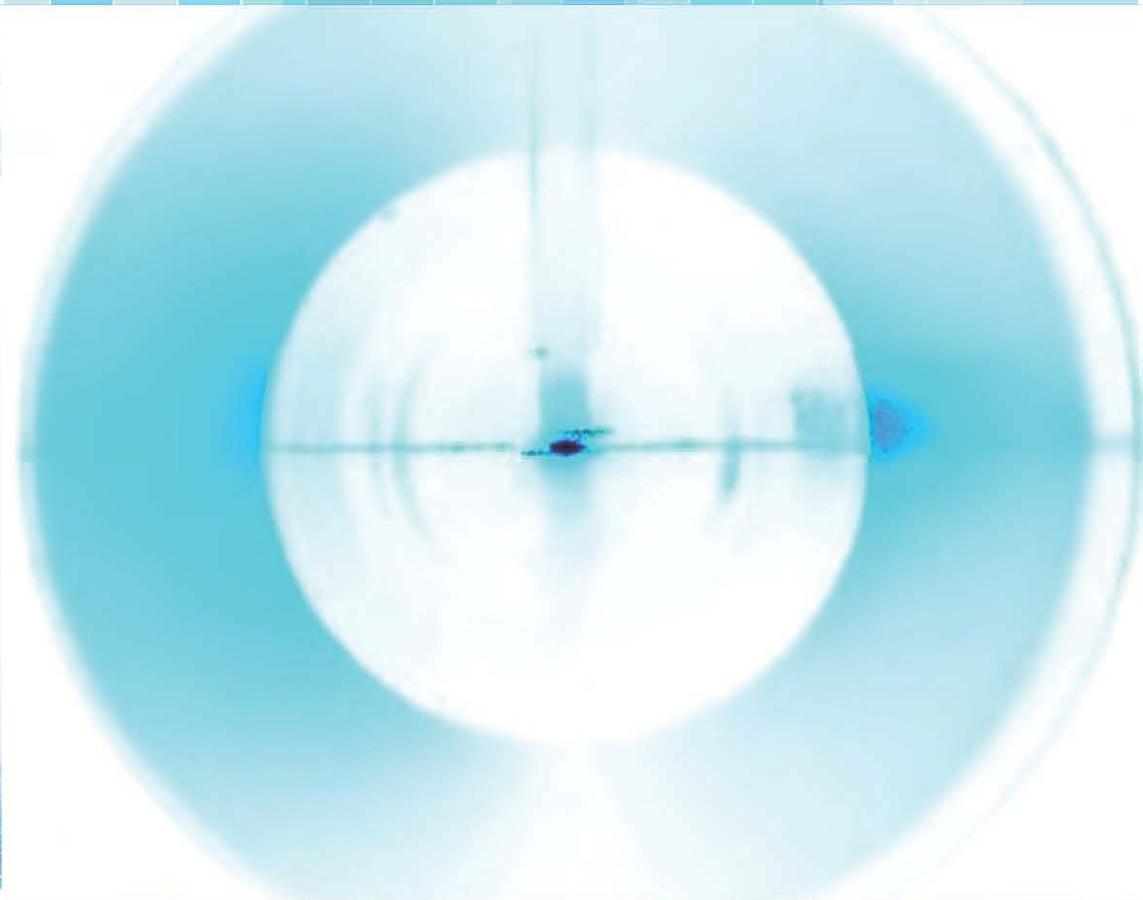
### **6.3 Annex 3 – Programme of the symposium preceding the user meeting**

The user meeting was linked to a symposium on new developments in microscopy on 22./23.09.08 “High speed optical sectioning microscopy” organized by Ulrich Kubitscheck, university of Bonn, with support from DFG.

In the following the programme of the symposium is reproduced.

# High Speed Optical Sectioning Microscopy

**Sept. 22nd - 23rd, 2008**



[www.thch.uni-bonn.de/microscopy2008](http://www.thch.uni-bonn.de/microscopy2008)

[microscopy2008@pc.uni-bonn.de](mailto:microscopy2008@pc.uni-bonn.de)

**Supported by  
the DFG  
Highlight 2004**



Organizer Ulrich Kubitscheck

Institute for Physical and Theoretical Chemistry

# High Speed Optical Sectioning Microscopy 2008

September 22-23, 2008

53115 Bonn, Nussallee 10, Hörsaal B des Instituts für Anatomie

Monday, 22 September, 2008

12:00 – 13:20 [Mixer / Registration](#)

13:20 Welcome & Introduction:

**Ulrich Kubitscheck, Achim R. Tieftrunk, Johannes Janssen**

Lectures: Hörsaal B

Chair: Ulrich Kubitscheck

13:30 – 14:05 **Dietmar Manstein**

Regulation of Myosin Motor Activity and Processivity by Changes in Free  $Mg^{2+}$ -Ion Concentration

14:05 – 14:40 **Martin Kahms (AG Reiner Peters)**

4Pi Microscopy of the Nuclear Pore Complex

14:40 – 15:15 **Thorsten Lang**

Optical Sectioning Microscopy for Studying Anatomy, Dynamics and Function of Supramolecular Membrane Protein Clusters

15:15 – 15:50 **Jörg Bewersdorf**

Recent Advances in 4Pi Microscopy and Photoactivation Localization Microscopy (FPALM)

15:50 – 16:25 **Rainer Heintzmann**

High Resolution Microscopy: Structured Illumination and Pointillism

16:25 – 16:45 [Coffee Break / Foyer](#)

Lectures: Hörsaal B

Chair: Thorsten Lang

- 16:45 – 17:20 **Petra Schwille**  
Fluorescence Correlation Spectroscopy in Developmental Biology
- 17:20 – 17:55 **Stefan Hell**  
Far-field Optical Nanoscopy
- 17:55 – 18:30 **Zvi Kam**  
Screening Microscopy - Combining Detailed Cellular Information with Throughput
- 18:30 – 19:05 **Roland Nitschke**  
High Speed Imaging Using the LSM 5 Live
- 19:05 – 19:40 **Erik Manders**  
Controlled Light Exposure Microscopy (CLEM) for Prolonged Live-Cell Imaging
- 20:30 [Conference dinner: Restaurant „Zur Lese“](#)  
[Adenauerallee 37, 53113 Bonn, Telefon: 0228 / 22 33 22](#)

## **Tuesday, 23 September, 2008**

Lectures: Hörsaal B

Chair: Rainer Heintzmann

- 8:30 – 9:05 **Ernst Stelzer**  
Light Sheet Based Fluorescence Microscopes (LSFM, SPIM, DSLM) Reduce Phototoxic Effects by Several Orders of Magnitude
- 9:05 – 9:40 **Raimund Ober**  
3D Single Particle Tracking in Live Cells with High Spatial and Temporal Resolution: Endocytosis and Exocytosis
- 9:40 – 10:15 **John W. Sedat**  
New Directions for Live 4-Dimensional Imaging Using OMX, a Novel Imaging Platform
- 10:15 – 10:30 [Coffee Break: Foyer](#)

Lectures: Hörsaal B

Chair: Erik Manders

- 10:30 – 11:05 **Andreas Zumbusch**  
Coherent Anti-Stokes Raman Scattering (CARS) Microscopy in Living Cells
- 11:05 – 11:40 **Don C. Lamb**  
Single Particle Tracking: Developments and Application to HIV Assembly and Release
- 11:40 – 12:15 **Donna Arndt-Jovin**  
From the Molecule to the Neurosurgeon: Quantum-Dot and Expression-Probe Based Sensing of Signal Transduction in the Research Lab and the Operating Room

12:15 – 13:15 [Lunch Break: Foyer](#)

Lectures: Hörsaal B

Chair: Don C. Lamb

- 13:15 – 13:50 **M. Cristina Cardoso**  
A Single Molecule View of the Mammalian Cell Nucleus
- 13:50 – 14:25 **Heinrich Leonhardt**  
Subdiffraction Multicolor Imaging of the Nuclear Periphery with 3D Structured Illumination Microscopy
- 14:25 – 15:00 **Tom Jovin**  
PAM: Programmable Array Microscope/Microscopy/Module
- 15:00 – 15:10 Closing Remarks  
**Ulrich Kubitscheck / Achim R. Tieftrunk**

15:10 – 15:30 [Coffee Break: Foyer](#)

Discussions: Institute of Physical and Theoretical Chemistry  
& Institute of Physiological Chemistry

15:30 – 17:15 Round-table discussions with company representatives

# High Speed Optical Sectioning Microscopy 2008. Bonn

## City Map of Bonn



- |  |   |
|--|---|
| <p><b>A</b> Venue<br/>Hörsaal A, Institute for Anatomy<br/>Nussallee 10, 53115 Bonn</p>  | <p><b>5</b> Günnewig Hotel Residenz<br/>Kaiserplatz 11, 53113 Bonn<br/><a href="http://www.guennewig.de/bnreside">www.guennewig.de/bnreside</a><br/>Tel. +49 (0) 228-2697-0</p>   |
| <p><b>B</b> Institut für Physikalische und Theoretische Chemie<br/>Wegelerstr. 12, 53115 Bonn<br/><a href="http://www.thch.uni-bonn.de">www.thch.uni-bonn.de</a></p>   | <p><b>6</b> Günnewig Bristol Bonn<br/>Prinz-Albert-Str 2, 53113 Bonn<br/><a href="http://www.guennewig.de/bnbristo">www.guennewig.de/bnbristo</a><br/>Tel. +49 (0) 228-2698-0</p> |
| <p><b>1</b> Best Western Premier Hotel Domicil<br/>Thomas-Mann-Str. 24-26, 53115 Bonn<br/><a href="http://www.domicil-bonn.bestwestern.de">www.domicil-bonn.bestwestern.de</a><br/>Tel. +49 (0) 228-729090</p> | <p><b>7</b> President Hotel<br/>Clemens-August-Str. 32-36, 53115 Bonn<br/><a href="http://www.presidenthotel.de">www.presidenthotel.de</a><br/>Tel. +49 (0) 228-7250-0</p>        |
| <p><b>2</b> Hotel Mozart<br/>Mozartstr.1, 53115 Bonn<br/><a href="http://www.hotel-mozart-bonn.de">www.hotel-mozart-bonn.de</a><br/>Tel. +49 (0) 228-659071</p>  | <p><b>R</b> Restaurant Zur Lese<br/>Adenauerallee 37, 53115 Bonn<br/><a href="http://www.zurlese.de">www.zurlese.de</a><br/>Tel. +49 (0) 228-223322</p>                           |
| <p><b>3</b> Hotel Villa Esplanade<br/>Colmantstr. 47, 53115 Bonn<br/><a href="http://www.hotel-villa-esplanade.de">www.hotel-villa-esplanade.de</a><br/>Tel. +49 (0) 228-98380-0</p>                           | <p><b>FS</b> University of Bonn, Main Building<br/>Festsaal<br/>Regina-Pacis-Weg 3, 53113 Bonn</p>  |
| <p><b>4</b> Hotel Kurfürstenhof<br/>Baumschulallee 20, 53115 Bonn<br/><a href="http://www.kurfuerstenhof-bonn.de">www.kurfuerstenhof-bonn.de</a><br/>Tel. +49(0) 228-98505-0</p>                               |   |

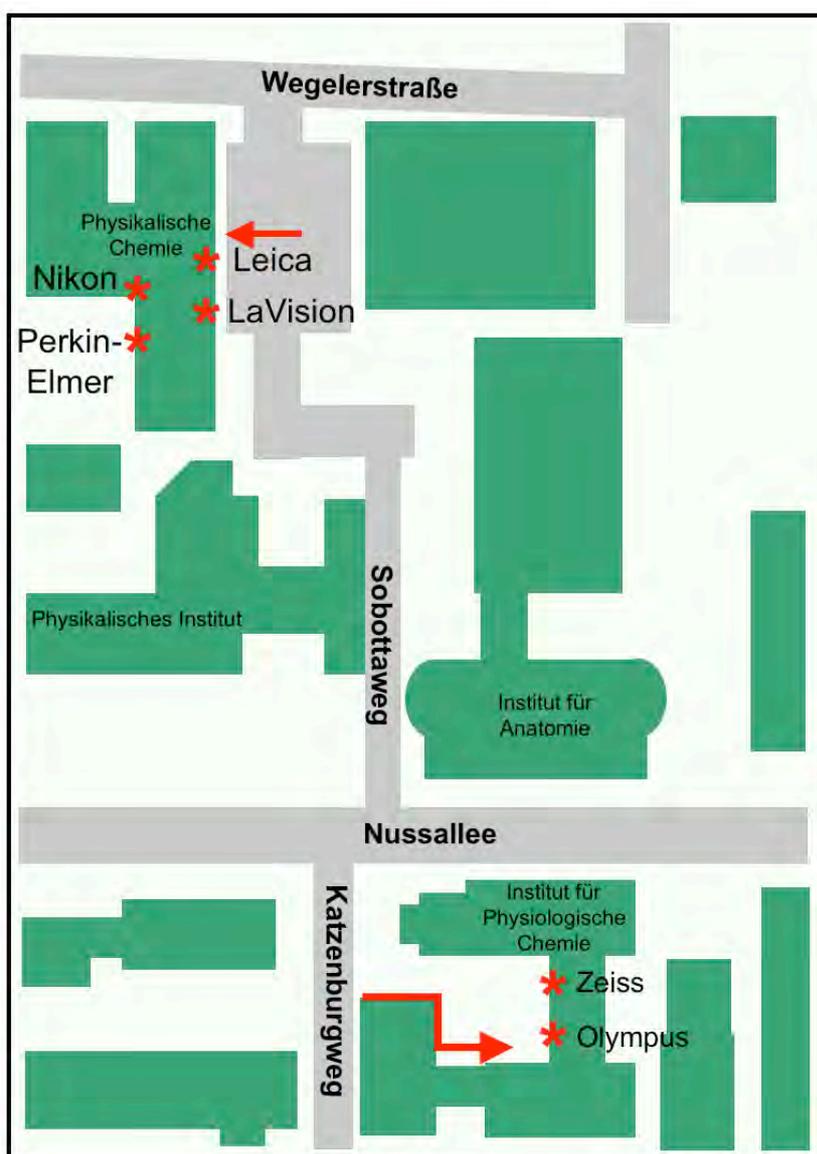
## Round-Table Discussions with Company Representatives

Tuesday, September 23<sup>rd</sup>, 2008

Begin 15:30h

Locations:

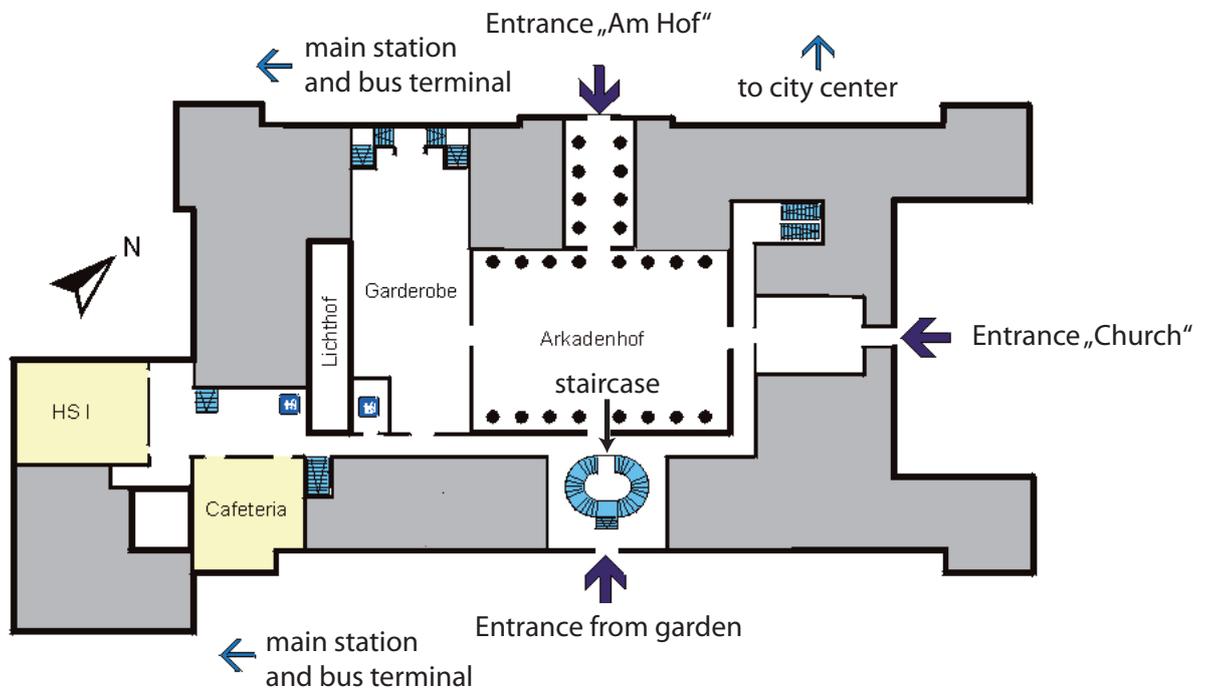
Company	Room	Institute
LaVision	1.016	Physical Chemistry
Leica	S1 (1.010)	Physical Chemistry
Nikon	Bib (1.013)	Physical Chemistry
Olympus	041	Physiological Chemistry
PerkinElmer	S2 – 1.017	Physical Chemistry
Zeiss	043	Physiological Chemistry



# Overview of the Bonn University Main Building

## Location of ERA-Instruments Meeting Sept. 24th 2008

### ground floor



### 1st floor



# **Abstracts**

# From the Molecule to the Neurosurgeon: Quantum-Dot and Expression-Probe Based Sensing of Signal Transduction in the Research Lab and the Operating Room

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Quantum dots (QDs) are colloidal inorganic semiconductor nanocrystals composed typically of a CdSe, CdS or CdTe core and a ZnS shell. There are many advantages in the use of QDs as fluorophores: they can be excited over a broad spectral range and they have narrow emission bands that can be tuned from ultraviolet to infrared by adjusting size and composition. Their bright emission fluorescence and resistance to photobleaching make QDs ideal for single-particle detection and permit imaging over prolonged time periods. Because of these advantages, QDs are finding increasing use in *in vivo* and *in vitro* studies, particularly in combination with expression probes and advanced microscopy techniques. Activation of the erbB receptor tyrosine kinases (erbB1-4) induced by the extracellular binding of peptide ligands triggers signaling cascades responsible for cellular motility, cell division, and differentiation. We have genetically tagged the ErbB proteins with fluorescent proteins and/or the acyl carrier protein (ACP) sequence. QDs have been targeted to receptors on the external cell surface through the growth factor receptor, EGF, or by covalently linking to the ACP tag allowing the visualization in living cells of individual receptors, the diffusion of which has been determined on different cell types. We have also used them to detect dimerization and activation of the transmembrane erbB proteins upon ligand binding. These reagents have revealed a new mode of retrograde transport of the activated receptor from cellular extensions (filopodia) to the surface of the cell [1,2; unpub. data]. The process is linked to treadmilling of actin filaments, which may occur on the cell body as well as on the filopodia. Recently, we have extended such studies by the use of magnetic nanoparticles.

Results from basic research studies of erbB tyrosine kinase receptors have led to the application of QD probes in delineating glioblastoma tumors, a collaboration with neurosurgeons in Göttingen. The objective of these studies is to facilitate the localization of tumor margins during surgery, thereby facilitating the accurate resection of the tumor (including of small cellular foci) with minimal loss of normal brain tissue [3]. Similar probes have also helped to elucidate a new role for the amyloid precursor protein (APP) in the maintenance and progression of malignant melanoma [4].

The detection and tracking of single molecules, and the elucidation of fast, short-lived physiological processes, such as dimerization of proteins on the surface of live cells, require rapid and sensitive optical sectioning microscopy. For that purpose, we have devised new spectroscopic techniques (e.g. FRET-based) as well as an instrument denoted as the Programmable Array Microscope (PAM) [5,6]. The PAM utilizes a spatial light modulator (SLM) placed at the primary image plane to construct patterns of conjugate (as well as non-conjugate) illumination and detection. By integrating over a number of such patterns (points, lines, psuedorandom), an optically sectioned image is generated in the short periods required for live cell studies.

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# Recent Advances in 4Pi Microscopy and Photoactivation Localization Microscopy (FPALM)

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Fluorescence microscopy is an irreplaceable and central method of modern biological and biophysical research. Its power lies in the ability to directly observe sub-cellular structures, interactions and functions and provide context for biochemical data. This power is, however, limited fundamentally by diffraction. Recent advances over the last decade have shown that the diffraction limit can be overcome enabling far-field microscopy at the sub-100 nm scale.

This presentation will outline recent advances in super-resolution fluorescence microscopy and their application from our lab. 4Pi-Microscopy of the cell nucleus and in thick tissue sections will be highlighted to demonstrate the potential of super-resolution 3D microscopy in chromatin research and in imaging of thick samples.

Additionally, the newly developed Biplane FPALM will be presented. Biplane FPALM generates images with currently 30 x 30 x 75 nm resolution over a depth of several micrometers by combining a double-plane detection scheme with fluorescence photoactivation localization microscopy (FPALM) without compromising speed or sensitivity.

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# A Single Molecule View of the Mammalian Cell Nucleus

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\* Equal contribution

Genome activity and nuclear metabolism clearly depend on accessibility but it is not known whether and to what extent nuclear structures limit the mobility and access of individual molecules.

We used fluorescently labeled streptavidin with a nuclear localization signal as an average-sized, inert protein to probe the nuclear environment. The protein was injected in the cytoplasm of mouse cells and single molecules were tracked in the nucleus with high-speed fluorescence microscopy. We analyzed and compared the mobility of single streptavidin molecules in structurally and functionally distinct nuclear compartments of living cells.

Our results indicated that all nuclear subcompartments were easily and similarly accessible for such an average-sized protein and even condensed heterochromatin did neither exclude single molecules nor impede their passage. The only significant difference was a higher frequency of transient trappings in heterochromatin lasting though, only tens of milliseconds. The streptavidin molecules, however, did not accumulate in heterochromatin suggesting comparatively less free volume. Interestingly, the nucleolus seemed to exclude the streptavidin like many other nuclear proteins, when visualized by conventional fluorescence microscopy. The tracking of single molecules, nonetheless, showed no evidence for repulsion at the border but rather unimpeded passage through the nucleolus.

These results clearly show that single molecule tracking can provide novel insights into mobility of proteins in the nucleus that cannot be obtained by conventional fluorescence microscopy. Our results suggest that nuclear processes may not be regulated at the level of physical accessibility but rather by local concentration of reactants and availability of binding sites.

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# High Resolution Microscopy: Structured Illumination and Pointillism

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In structured illumination [1] the sample is illuminated with a number of different patterns of light. In our case this is a series of sinusoidal grids at different grid positions and orientations generated by a programmable spatial light modulator. Experimental datasets acquired under these conditions and reconstructed results from these data, demonstrating a resolution improvement of up to a factor of two over standard widefield microscopy are presented.

The non-linear approach [2] of saturating optical transitions (for structured illumination as well as beam-scanning approaches) has a great potential especially in combination with photo-switchable dyes [3] such as the recently released DRONPA protein by Atsushi Miyawaki's group or the Cy3-Alexa647 system used in Xiaowei Zhuang's group.

A further approach to high resolution imaging is based on the localization of multiple particles in an image. This approach was named Pointillism [4]. Experimental data with particle separation based on independent component analysis will be presented.

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# Far-Field Optical Nanoscopy

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The resolution of a far-field optical microscope is usually limited to  $d = \lambda / (2 n \sin \alpha) > 200$  nm, with  $n \sin \alpha$  denoting the numerical aperture of the lens and  $\lambda$  the wavelength of light. While the diffraction barrier has prompted the invention of electron, scanning probe, and x-ray microscopy, the 3D-imaging of the interior of (living) cells requires the use of focused visible light. Here, I will discuss lens-based fluorescence microscopy concepts that feature a resolving power on the nanoscale. All these concepts share a common basis: exploiting selected (pairs of) states and transitions of the fluorescent marker to neutralize the limiting role of diffraction<sup>1,2</sup>. The first viable concept of this kind was Stimulated Emission Depletion (STED) microscopy<sup>3</sup> where the diameter of the focal spot of fluorescence follows

$d \approx \lambda / (2 n \sin \alpha \sqrt{1 + I/I_s})$ .  $I$  is the intensity that drives a fluorophore from the bright fluorescent state to the dark ground state by stimulated emission.  $I_s$  depends (inversely) on the lifetime of the states. For  $I/I_s \rightarrow \infty$ , it follows that  $d \rightarrow 0$ , meaning that the resolution can be molecular.

Although it is an ensemble method *per se*, STED microscopy allows the detection of single molecules with improved spatial definition<sup>4</sup>. As an example, we showed the detection and the characterization of the dynamics of individual lipid molecules in the membrane of a living cell<sup>5</sup>. Unlike confocal spots, the nanosized spots created by STED directly revealed substantial differences between the diffusion of phospho- and sphingolipids. Single sphingolipids, but not phospholipids, are transiently (< 10 ms) and locally (< 20 nm) trapped in the plasma membrane, mediated by cholesterol. Thus STED revealed inhomogeneities of lipid diffusion in the plasma membrane on a single molecule basis. By the same token, we show that STED augments the power of fluorescence correlation spectroscopy<sup>6</sup>.

The idea underlying STED microscopy can be expanded by employing other transitions that shuffle the molecule between a dark and a bright state, such as (i) shelving the fluorophore in a dark (triplet) state<sup>7</sup>, and (ii) photoswitching between a 'fluorescence activated' and a 'fluorescence deactivated' conformational state<sup>1,8,9</sup>. Examples for the latter include photochromic organic compounds, and switchable fluorescent proteins which undergo a cis-trans photoisomerization. Due to the long lifetimes of the states involved, photoswitching provides nanoscale resolution at ultralow light levels<sup>1,9</sup>.

Switching can overcome the diffraction barrier in molecular ensembles<sup>10</sup>, but recent nanoscopy schemes in which individual molecules are switched to a state that emits a bunch of  $m \gg 1$  detectable photons are also very effective<sup>11</sup>. The calculation of the position of these molecules with high precision allows one to assemble an image. These concepts greatly complement the ensemble schemes. By providing molecular markers with the appropriate states and transitions, synthetic organic chemistry and protein biotechnology obviously play a key role in all these concepts<sup>9</sup>.

Altogether, far-field optical 'nanoscopy' is a fascinating development in applied physics with high relevance to the many areas of sciences, in particular the life and the colloidal sciences. Since it has already been a key to answering important questions in biology, and owing to its simplicity and commercial availability, I expect far-field fluorescence 'nanoscopes' to enter most cell biology laboratories in the near future.

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# PAM: Programmable Array Microscope/Microscopy/Module

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The detection and tracking of single molecules, and the elucidation of fast, short-lived physiological processes, such as dimerization of proteins on the surface of live cells, require rapid and sensitive optical sectioning microscopy. For that purpose, we have devised new spectroscopic techniques as well as an instrument denoted as the Programmable Array Microscope (PAM). The PAM is a high throughput system (microscope module) enabling widefield optically sectioned, multi-dimensional fluorescence microscopy. The distinctive feature is the placement of a spatial light modulator (SLM) in an image plane of a microscope to generate programmable *structured illumination* (excitation) and *structured detection* (conjugate  $\approx$  "in-focus, confocal" and non-conjugate  $\approx$  "out-of-focus", rejected in conventional CLSM and Nipkow disk designs). By integrating over a number of patterns (points, lines, pseudorandom), an optically sectioned image is generated in the short periods required for live cell studies. Optical sectioning can be achieved with up to a 50% excitation duty cycle and at rates limited ultimately only by the detector(s).

## *General features*

- add-on module to (any) fluorescence microscope for sensitive, rapid, live-cell imaging
- full-field optical sectioning with programmable and adaptable resolution
- structured illumination with SLM at image plane;
- Spatial Light Modulator: reflective (DMD, LCoS [1,2]), transmissive (LCD)
- lamp, LED, laser, luminescent light sources; single or multiphoton (pulsed, cw)
- low to moderate cost: "bare-bones"  $\rightarrow$  high-end; modularity

## *Advantages*

- dual detected images: *conjugate* ( $\approx$  in-focus) + *nonconjugate* ( $\approx$  out-of-focus):
- no emission rejected, leading to superior image reconstruction
- multiple virtual apertures and arbitrary regions-of-interest
- illumination duty cycles of up to 50% using pseudorandom sequences
- 10-100-fold increase in speed relative to CLSM and Nipkow disk systems
- emCCD detector(s): > 3-10-fold increase in sensitivity relative to CLSM
- no (macro) moving parts required for imaging

## *Multiple operational modes(some achieved, others prospective)*

- spectroscopic (sectioning) modes: spectra (excitation, emission)
- other fluorescence spectroscopy modalities: FRET; fluorescence lifetime; polarization patterning on excitation (3 axis?) and emission; non-linear regimes (depletion, saturation, transients)
- patterned/adaptive control: light dose, photobleaching, photoactivation, photoconversion, photodecaging, correlations (spatial, temporal)
- spatial superresolution
- medical: diagnostics; neurosurgery; microendoscopy
- other: field use; semiconductor; high throughput screening (HTS)

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# 4Pi Microscopy of the Nuclear Pore Complex

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In 4Pi microscopy, the coherent addition of two wavefronts originating from two opposing high-aperture lenses provides an increase in resolution along the optical axis by a factor of 5-7 compared to standard confocal microscopy (1). This improved resolution enables a more detailed visualisation of cellular structures but also precise localization of fluorophore labelled components in large protein complexes as well as dynamic studies using photobleaching and correlation techniques with increased spatial resolution.

In this presentation recent results are presented obtained with a commercial 4Pi microscope type A. Besides 3D-imaging of cellular structures, 4Pi microscopy was applied to map topographic features of single cellular protein complexes like the nuclear pore complex (NPC). Using glycerol immersion lenses, the distances between immune-labelled epitopes at the NPC in fixed specimens could be determined with a standard deviation down to 15 nm (2) and the results obtained were in good agreement with former electron microscopy estimates. While the employment of glycerol lenses restricted 4Pi analysis to fixed specimens, application of water immersion lenses opened the way to 4Pi analysis under physiological conditions. Using the latter configuration, binding site distributions of proteins involved in nuclear-cytoplasmatic transport at the NPC could be analyzed under equilibrium conditions and were compared with single molecule studies using a highly sensitive widefield microscope setup.

Additionally, we tried to benefit from the increased resolution in mobility analysis. Therefore the theoretical and computational framework for Continuous Fluorescence Microphotolysis (CFM) and Fluorescence Correlation Spectroscopy (FCS) in combination with 4Pi microscopy has been developed and validated in simple model systems (3). Current studies focus on mobility analysis of proteins involved in cyto-nucleoplasmatic transport using 4Pi-CFM in living yeast cells, where spatial resolution is a major issue due to small specimen size.

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## **SCREENING MICROSCOPY:**

Combining detailed cellular information with throughput

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Light microscopy offers a detailed look into live specimens at multiple dimensions: space, time, “color” and various manipulations. The emphasis in this meeting is on high-speed 4D data, yet, even the fastest methods cannot effectively capture 4D data for a large number of specimens. Our developments during the last few years are directed towards systems biological research, aiming to increase the throughput in microscopy of multiple cellular samples. Screening of cells, typically cultured in multi-well plates, and treated by drug or siRNA libraries, have become an important tool in biology. However, increasing the number of samples inevitably compromises image information. Degradation of the recorded details in sub-cellular structures, classically used in cell biological microscopy, limits the capability to relate the responses of cells to perturbations with mechanisms.

We shall describe the screening microscope we developed, with the goal of optimized speed of image acquisition at the highest magnification, and the pipe-line we assembled for the analysis and exploration of terabytes of image data produced in large screens.

# Single Particle Tracking: Developments and Application to HIV Assembly and Release

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In the last decade, a revolution has occurred in fluorescence microscopy making it possible to follow individual molecules and particles in real time. Imagine tracking a tourist in Rome using GPS. From the resulting trajectory, you can gather information over what mode of transportation this person prefers, what their daily routine is and what interests he or she has. We can gather similar information by following individual biomolecules within living cells using single particle tracking (SPT).

With the aid of SPT, we have investigated the assembly of HIV viruses. The main structural protein (GAG) has been fluorescently labeled with GFP. As a virus assembles, GAG proteins are delivered to the budding site. We have investigated the dynamics of the virus assembly process, visualizing whether GAG is delivered from the membrane or directly from the cytosol and determined the size of the budding sites. By monitoring the mobility of the budding sites, we have also been able to visualize virus release.

We have developed a novel 3-D tracking microscope where the laser beam is orbited about the particle and its position determined (in two dimensions) from the phase and modulation of the fluorescence signal. For the third dimension, two confocal planes, immediately below and above the particle, are monitored. The position of the tracked particle is determined in 3-D in real time and the orbit of the laser beam is recentered on the tracked particle using a feedback loop. In addition to the orbital tracking, a wide-field detection system is incorporated into the microscope to allow visualization of the environment about the tracked particle.

# Optical Sectioning Microscopy for Studying Anatomy, Dynamics and Function of Supramolecular Membrane Protein Clusters

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The morphology of the plasma membrane is unique as it expands laterally over many  $\mu\text{m}^2$  but has a thickness of only several nm. As its two-dimensional shape fits perfectly well in the focal plane of an objective lens, the basal plasma membranes of substrate-adhered cells represent an ideal object for optical sectioning microscopy. In order to remove out-of focus light, the unfocussed cellular part can be removed mechanically or a confocal pinhole can be used.

Applying such methods, including the plasma membrane sheet technology, the FRAP (fluorescence recovery after photobleaching)-method and STED (stimulated emission depletion)-microscopy, we have studied the anatomy, dynamics and function of a supramolecular membrane protein cluster.

Using the SNARE fusion protein syntaxin 1 as example, we showed that the protein forms clusters in the plasma membrane at which secretory vesicles dock and fuse. Syntaxin clustering can be explained by self-organization depending on weak homophilic protein-protein interactions involving the SNARE motif of syntaxin. On average, syntaxin clusters exhibit a diameter of 50-60 nm and contain 75 densely crowded syntaxins that dynamically exchange with freely diffusing molecules. The function of syntaxin clustering is most likely to control the activity of the protein and to prevent the formation of unproductive SNARE-complexes with other SNAREs involved in vesicle fusion.

As at the eukaryotic plasma membrane an extraordinarily high number of cellular activities take place, including e.g. membrane trafficking, signaling or adhesion, these methods are suitable for studying many aspects of membrane biology.

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# Subdiffraction Multicolor Imaging of the Nuclear Periphery with 3D Structured Illumination Microscopy

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Fluorescence light microscopy allows multicolor visualization of cellular components with high specificity, but its utility has until recently been constrained by the intrinsic limit of spatial resolution. We applied three-dimensional structured illumination microscopy (3D-SIM) to circumvent this limit and to study the mammalian nucleus. By simultaneously imaging chromatin, nuclear lamina, and the nuclear pore complex (NPC), we observed several features that escape detection by conventional microscopy. We could resolve single NPCs that colocalized with channels in the lamin network and peripheral heterochromatin. We could differentially localize distinct NPC components and detect double-layered invaginations of the nuclear envelope in prophase as previously seen only by electron microscopy. Multicolor 3D-SIM opens new and facile possibilities to analyze subcellular structures beyond the diffraction limit of the emitted light.

# Controlled Light Exposure Microscopy (CLEM) for Prolonged Live-Cell Imaging

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Controlled Light Exposure Microscopy is a novel and simple technology that strongly reduces phototoxicity and photobleaching in live-cell imaging without compromising image quality [1,2]. This technology is based on a non-uniform illumination of the fluorescent sample that allows tuning the light dose for every individual pixel. Results show that CLEM reduces photobleaching and phototoxicity by a factor of 5 to 10.

The reduction of phototoxicity and photobleaching is quantified by a CLEM-factor. The value of this CLEM-factor depends on the properties of the sample, microscope and imaging parameters and settings of CLEM-electronics. By computer simulation we have investigated the influence of these parameters on the CLEM-factor and image quality [3].

We will present applications of CLEM in cell biology. For example, we will show how we monitor the dynamics of telomeres in human cells for prolonged imaging periods. Application of CLEM in this research leads to biological results that cannot be obtained with non-CLEM (conventional imaging).

Finally, we will discuss quantitative imaging with CLEM, photobleaching and noise properties of CLEM and how CLEM can be applied in wide-field microscopy.

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# Regulation of Myosin Motor Activity and Processivity by Changes in Free $Mg^{2+}$ -Ion Concentration

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Several members of the myosin-5 family are characterized by a high duty ratio, which enables them to move as single molecules over long distances along actin filaments without dissociating. We investigated the functional role and kinetic properties of several myosins from *Dictyostelium discoideum* and found that the motile activity and tension bearing ability of several *Dictyostelium* myosins is strongly affected by changes in the concentration of free  $Mg^{2+}$ -ions<sup>1,2</sup>. Additionally, our results show that myosin-5b can switch between processive and non-processive movement under physiological conditions<sup>3</sup>. In *Dictyostelium* cells myosin-5b is enriched at highly dynamic vesico-tubular structures in the cell periphery corresponding to the contractile vacuole (CV) system. The cellular localization of the CV system and the tight association of myosin-5b with the CV system facilitate the direct observation of changes in myosin-5b motor activity and their correlation with changes in the concentration of free  $Mg^{2+}$ -ions by TIRF microscopy. To investigate the functional importance of myosin-5b at the CV system, we have exposed *Dictyostelium* cells expressing wild-type and a dominant negative mutant variant of myosin-5b to high and low osmotic pressure and show that myosin-5b is directly involved in the active contraction of the CV system. Our results from kinetic analyses and *in vitro* motility assays demonstrate that the processive properties of myosin-5b depend on physiological changes in the concentration of free  $Mg^{2+}$ -ions. Free  $Mg^{2+}$ -ions strongly affect the release of hydrolysis products thereby influencing the fraction of time the motor spends in the strong actin binding states. To dissect the physiological relevance of the observed  $Mg^{2+}$ -dependent functional changes of myosin-5b, we followed the spatial and temporal changes in free  $Mg^{2+}$ -ion distribution in *Dictyostelium* cells. Using the  $Mg^{2+}$ -sensitive fluorescent dye KMG-104AM<sup>4</sup>, we observed fluctuations in the concentration of free  $Mg^{2+}$ -ions that take place in the millisecond to second range and thus within a time domain where the consequences for myosin motor activity are of physiological relevance.

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# Applications and Experiences Using the Zeiss LSM 5 Live

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Confocal imaging requiring higher frame rates than 4 - 6 Hz (at a typical image size of 512x 512 pixels) is often at the speed performance limit of conventional point scanning confocal microscopes. This is the classical field for faster confocal systems like spinning disk, line scanning- or other multi-point systems.

I report about different applications and questions, which have been worked out on a Zeiss LSM510 Live Duo. Beside typical biological applications including live cell imaging in single cells as well as model organisms like Zebrafish or Arabidopsis other experiments were performed aiming for the evaluation of the properties of microfluidic devices for life science applications like cell sorting.

Using simultaneously the second scanner of the LSM 5 Live for fast photo-conversion or photo-activation of fluorescent proteins (FP) like PA-GFP or dronpa2 we examined the diffusion of the FPs as well as the possibility to overcome the omnipresent bleaching problems related to all high resolution imaging applications. Resolving the distribution process after FP-activation or conversion required acquisition rates of 50 Hz and higher. Using a special low-intensity activation scheme for dronpa2 we achieved the recording of more than 60000 images from a single cell at high sub-cellular optical resolution. The suitability of fast scanning systems for high resolution large area and high dynamic range imaging will be discussed, which is more and more demanded in neuro-and developmental biology applications. I will present a software tool (XUV-tool) for real 3D-stitching and results of high-dynamic range imaging combining multiple recordings at a single sample location to overcome the insufficient dynamic range of confocal detectors, which is most obvious in thick and/or heavily stained samples.

# 3D Single Particle Tracking in Live Cells with High Spatial and Temporal Resolution: Endocytosis and Exocytosis

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Single particle tracking in three dimensions (3D) in a live cell environment promises to reveal important new insights into cell biological mechanisms. However, such experiments are currently severely limited by a lack of appropriate methodology that allows for tracking in 3D with high temporal and spatial resolution. As a result, important biological processes remain largely unexplored. For example, there is a lack of data concerning intracellular trafficking pathways in 3D of exocytosing receptors prior to exocytosis and the pathways of endocytosed receptors or ligands post endocytosis. In [1] we introduced a novel imaging modality, multifocal plane microscopy (MUM) for the study of subcellular dynamics. Here we show that MUM provides a powerful approach with which single molecules can be tracked in 3D in live cells. MUM allows for the simultaneous imaging at different focal planes, thereby ensuring that trajectories can be imaged continuously at high temporal resolution. Importantly, this approach overcomes the very limited resolution of a conventional microscope along the optical axis and opens the way for high resolution 3D single molecule tracking within a live cell environment. In the current study, we use MUM to reveal complex intracellular pathways that could not be imaged with classical approaches. In particular we track quantum dot labeled immunoglobulin (IgG) molecules from the sorting endosome to exocytosis [2] and from the plasma membrane, through endocytosis to a sorting endosome. We will also discuss in detail the multifocal plane microscopy localization algorithm (MUMLA) with which z-estimates can be obtained with very high accuracy.

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# Fluorescence Correlation Spectroscopy in Developmental Biology

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Cell and developmental biology are immensely complex and rapidly growing fields that are particularly in need of quantitative methods to determine their key processes. With all the data known about protein interactions and interaction networks from biochemical analysis, there still remains the important task of *in situ* proteomics, i.e. determining the thermodynamic and kinetic parameters of certain reactions in the cellular environment. Further, to understand how cells polarize and develop into organisms, we need quantitative methods to determine concentration gradients and diffusion coefficients of key factors such as morphogens. In conjunction with two-photon excitation and spectrally resolved detection, Fluorescence Correlation Spectroscopy (FCS) is a powerful means for the study of concentrations, translocation processes, molecular association or enzymatic turnovers. It is fair to state that this technique raises strong hopes for the possibility of *in situ* proteomics, but also for a more quantitative access to developmental processes. During the past years, we applied FCS to a variety of cell-associated phenomena, among them protein-protein binding, enzymatic reactions, endocytosis, and gene delivery. To study processes on cell membranes, and to elucidate the delicate interplay between membrane proteins and the surrounding lipids, we devised cell-like model membrane systems mimicking the formation of membrane domains whose cellular counterparts are potentially active as recruitment platforms for signalling proteins. We established one- and two-photon scanning FCS for processes on membranes which are too slow for standard FCS observation with a fixed beam. Performing circular scanning FCS on developing embryos of *C. elegans*, we show how the motion of labelled proteins is non-uniformly distributed in the cortex during cell polarization. Additionally, scanning FCS overcomes the problems of photobleaching and low statistical accuracy commonly encountered in FCS with fixed measurement volume, when applied to slowly moving molecules. By using two-photon excitation one additionally benefits from the possibility of long measurement times without disturbing the embryo development.

## **New Directions for Live 4-Dimensional Imaging Using OMX, a Novel Imaging Platform**

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Live imaging requires conditions that do not perturb the biological processes being studied. Even with the high sensitivity of wide-field microscopy, the excitation photons frequently photo-damage the biology. Using OMX, a novel radical imaging platform, it has been possible to reduce the excitation intensity by at least four orders-of-magnitude, thereby essentially negating photo-toxicity and recover the image information from the noise through the use of denoising algorithms. Other interesting directions, including data collection at a very rapid ten 3-dimensional images per second at 4 simultaneous wavelengths, follow, and will be discussed.

OMX allows facile modifications for PALM imaging, with the first results on chromosomes and nuclei forthcoming.

# Light Sheet Based Fluorescence Microscopes (LSFM, SPIM, DSLM) Reduce Phototoxic Effects by Several Orders of Magnitude

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Most optical technologies (microscopy, optical tweezers [1], laser nanoscalpel [2]) are applied to two-dimensional cellular systems, i.e. they are used in a cellular context that is defined by hard and flat surfaces. However, physiological meaningful information relies on the morphology, the mechanical properties, the media flux and the biochemistry of a cell's context found in live tissue [3, 4]. A physiological context is certainly not found in single cells cultivated on cover slips. It requires the complex three-dimensional relationship of cells cultivated e.g. in an ECM-based gel or in naturally developing small embryos of flies or embryos and, of course, in tissue sections [4]. However, the observation as well as the optical manipulation of extended biological specimens suffers from at least two severe problems. 1) The specimens are optically dense, i.e. they scatter and absorb light. Thus, the delivery of the probing light and the collection of the signal light tend to become inefficient. 2) Many biochemical compounds apart from fluorophores also absorb light and suffer degradation of some sort (photo-toxicity), which induces malfunction or death of a specimen [4]. The situation is particularly dramatic in conventional and confocal fluorescence microscopy. Even though only a single plane is observed, the entire specimen is illuminated. Recording stacks of images along the optical z-axis thus illuminates the entire specimen once for each plane. Hence cells are illuminated 10-20 and fish embryos even 100-300 times more often than they are observed. Surprisingly, this can be avoided by a simple change of the optical arrangement. The basic idea is to use light sheets, which are fed into the specimen from the side and which overlap with the focal plane of a wide-field fluorescence microscope [5]. Thus, in contrast to an epi-fluorescence arrangement, which uses the same lens, our azimuthal arrangement uses two independently operated lenses for illumination and detection. Optical sectioning and no photo-toxic damage outside a small volume around the focal plane are its intrinsic properties. Light sheet based fluorescence microscopes (LSFM) take advantage of modern camera technologies, which provide them with a signal to noise ratio that is at least thirty times better than that of a confocal microscope [6]. LSFM can be combined with essentially every contrast and specimen manipulation tool to operate in a truly three-dimensional fashion. In a current application, they are used to record early zebrafish (*Danio rerio*) development *in vivo* and *in toto* from the early 32-cell stage until late neurulation with sub-cellular resolution and very short sampling periods (60-90 sec/stack) [7]. The recording speed is more than 30 Million voxels/sec or more than five very large frames/sec with a dynamic range of 12-14 bit. We follow the cell movements during gastrulation and reveal its development during the cell migration processes. We can show that an LSFM exposes an embryo to 200 times less energy than a conventional, 5,000-6,000 times less than a confocal and about one million times less than a two-photon fluorescence microscope. Based on this outstanding performance, we claim that our novel, truly three-dimensional approach will have a dramatic impact on developmental and cell biology as well as on biophysics [8].

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# Coherent Anti-Stokes Raman Scattering (CARS) Microscopy in Living Cells

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During the last decade, ultrasensitive microscopy has become one of the most important tools in biophysics. Most prominent among the various techniques is confocal fluorescence microscopy. It is a very widespread technique with which sensitivities down to the single molecule detection limit can be achieved. In applications of fluorescence microscopy, however, one faces two difficult problems: i) The samples need to be labelled with a fluorophore which can be difficult and can change the properties of the sample. ii) All fluorophores are prone to photobleaching which severely limits the maximum achievable observation times. We therefore developed Coherent Anti-Stokes Raman Scattering (CARS) microscopy as a non-linear optical technique suited for live cell microscopy. [1] It generates contrast on basis of the vibrational spectra of the sample molecules and can thus be used for microscopical imaging without the need to use external staining. [2] Because of the low scattering of the near-IR excitation wavelengths used, CARS microscopy also holds promise for whole tissue investigations. After a brief introduction into the basic principles of CARS microscopy, recent technical developments as well as first applications to imaging of live cells and organisms will be presented. [3]

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