Scanning luminescence x-ray microscopy exploring the use of quantum dot nanocrystals as high spatial resolution biological labels

A Thesis Presented

by

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to

The Graduate School

in Partial Fulfillment of the Requirements

for the Degree of

Master of Arts

in

Physics

Stony Brook University

May 2006
Stony Brook University
The Graduate School

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Abstract of the Thesis

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The work presented here addresses the question, of whether the advantageous properties of quantum dots as biological labels can be combined with the high spatial resolution of an x-ray microscope, making use of x-ray induced visible light emission of the excited quantum dots. The focus lies on two main subjects. First, optophysical properties of quantum dots that are subject to x-ray excitation are determined using the X1A x-ray microscope at the National Synchrotron Lightsource at Brookhaven National Laboratories. The thesis gives an estimate for bleaching properties and quantum yield of quantum dots,
which are important quantities to evaluate if a certain label can be used in x-ray luminescence experiments. The results indicate that quantum dots are a potential candidate for x-ray luminescence applications in biology.

Second, quantum dot labeling of actin filaments of the cytoskeleton of 3T3 and RCJ cells was performed. The staining protocol was improved; wet and dry samples with well preserved ultrastructure and clearly pronounced actin fibers have been prepared successfully. This data was collected using a visible light fluorescence microscope at the Center for Biotechnology at Stony Brook.

The luminescence experiments with the x-ray microscope required the design of a scanning luminescence detection system consisting of a single photon counting module, a visible light objective, a filter holder, and a mounting plate to incorporate the new detector into the existing setup at the scanning transmission x-ray microscope X1A. The key parameters of detective quantum efficiency and spatial resolution have been determined by using a luminescent sample that has been well characterized for x-ray excitation. The results indicate that the detection system does not yet perform as expected. The thesis suggests different experiments or modifications to existing experiments to overcome this and other experimental shortcomings.
To my family
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Acknowledgements

Before diving into the actual thesis, I want to say thank you to everybody who contributed to this work. Chris Jacobsen, my thesis advisor, gave me the opportunity to work on this interesting field and was also my main contact person for everything thesis related. Helmut Strey, as collaborating professor in the Department of Biomedical Engineering (Stony Brook), helped me tackle the biological and chemical aspects of the project and provided lab facilities as well as helpful discussions concerning the whole project. I want to thank the people from my group, especially Mirna and Holger for helping me out at the beamline even on a Saturday night, Sue for keeping the beamline running, giving me advice and providing all of us with coffee and cookies, and Benjamin for help with the silicon detector.

I would also like to thank Robert Gersch and Jonathan Chiu from the Department of Biomedical Engineering (Stony Brook) for providing me with cells whenever I needed them, Gregory Rudomen from the University Microscopy
Imaging Center (Stony Brook) for fruitful discussion about drying techniques, and last but not least Matthias Riedmann who helped me out in the machine shop.

From a financial point of view I would like to thank the Deutscher Akademischer Austauschdienst (DAAD) and the National Institute of Health (NIH) for support under contract No. R01 EB00479-01A1
Chapter 1

Preparing the Topic

This chapter motivates the subject and provides an introduction by explaining basic physical correlations for x-ray interactions with matter.

1.1 Introductory Motivation

Cell imaging has been developing rapidly over the past years and there are three main techniques with different advantages and disadvantages. Light microscopy offers the possibility of imaging fluorescent labels in living specimens with a resolution of up to 100 nm. Electron microscopy is used for the highest spatial resolution approaching 5 nm of frozen hydrated cell sections or thin peripheral regions of whole cells. The regime of soft X-ray microscopy is just in between visible light and electron microscopy. With a wavelength of < 10 nm and optics with resolution well below 50 nm it improves upon the resolution limit of visible light microscopes. Moreover, the interaction mechanisms with matter are restricted to absorption or refraction without multiple elastic or inelastic scattering complications, which otherwise occur when imaging thick specimens with electron microscopy.

Soft x-ray microscopy provides a means to image thick, wet biological specimens at lower radiation dose than in electron microscopy by working at energies in the so-called water window between the absorption edges of carbon at 290 eV and oxygen at 540 eV. Organic materials show strong absorption contrast in comparison to water and ice at these energies. Fig. 1.1 shows the penetration depth of x-rays and electrons for organic materials containing carbon, relative to water and ice (oxygen). It can be seen that the contrast of organic materials (like proteins) relative to water or ice is much higher upon x-ray illumination than with electron microscopy.

Knowing the spatial distribution of proteins inside and outside cells is cru-
Figure 1.1 Penetration depth of x-rays and electrons for carbon and oxygen in the so-called “water window” between the carbon and oxygen K-absorption edges. At these energies organic materials like proteins show very good contrast relative to ice or water for x-ray illumination, whereas for electron illumination the contrast is poor. Figure reprinted from Kirz et al.[1].
cial for an understanding of molecular processes within cells, which are mainly
driven by protein-protein interaction. Much of the knowledge we have today
about cells, their life circle and their dynamic behaviour in certain environ-
ments stems from detailed structural information of the proteins involved. X-
ray microscopy, electron microscopy and conventional visible light microscopy
have been used extensively to explore subcellular structures. Being able to
mark certain proteins with fluorescing labels has made it easier to obtain good
structural information with ordinary fluorescence microscopy, and the recent
invention of so-called quantum dots as biological labels (see Alivisatos [2])
with optophysical properties superior to other dyes led to a further improve-
ment on cell labeling. However, the complexity and diversity of inner cellular
processes requires fluorescence microscopy with higher spatial resolution, and
a combination of fluorescence and other microscopy forms, such as scanning
transmission x-ray microscopy (STXM).

The present work explores the optophysical properties of quantum dots
upon x-ray excitation and their usability as biological labels for scanning x-ray
luminescence microscopy (SLXM) at high spatial resolution. The thesis starts
with a summary of earlier experiments on luminescence x-ray microscopy. This
is succeeded by a characterization of x-ray interactions with matter followed by
a brief description of the experimental apparatus in general. The experimental
apparatus includes an electron storage ring, an undulator, the X1A beamline,
and a scanning x-ray microscope system. After a more detailed formulation of
the idea of scanning luminescence x-ray microscopy (SLXM) with Quantum
Dots, the experimental setup specific for those kind of experiments, as well as
the experiments themselves, are presented in detail.

1.2 Earlier Experiments on X-ray Lumines-
cence Microscopy

Several different dyes and fluorescent labels have been investigated in earlier
experiments to evaluate their ability to be used in x-ray luminescence experi-
ments. Jacobsen et al. [3] examined bleaching properties and quantum yield
of two proprietary dyes (both Molecular Probes, catalog numbers: L-5181 and
L-5080) by loading polystyrene microspheres with these dyes and recording
fluorescence spectra with a photomultiplier of known detective quantum effi-
ciency. The result showed a quantum yield of \( \approx 7 \) visible photons per absorbed
x-ray. Further experiments have been conducted with sodium salicylate, a well
characterized VUV phosphor. A fluorescence signal was successfully recorded
and the quantum yield was determined to be \( \approx 2 \) visible photons per absorbed x-ray. Several different dyes (ethidium bromide, DAPI, Hoechst 33258, and rhodamine phalloidin) that were also investigated, showed no fluorescence signal upon x-ray excitation.

Other experiments by Irtel von Brenndorff et al. [4] and Moronne [5] addressed x-ray luminescence characteristics of lanthanide-based fluorescent probes. The results for a terbium antibody conjugate show a quantum yield of 0.2 visible light photons per absorbed x-ray. Strong luminescence signals could be recorded with doses exceeding \( 10^8 \) Gy.

### 1.3 X-ray Interactions with Matter

As already mentioned in the introductory section, one advantage of sub-10 keV x-rays is that the dominant interaction mechanisms with matter are absorption and coherent scattering. Contributions from incoherent scattering can be neglected because of its small cross section at these energies, as noted by Hubbell et al. [6] and Michette [7]. In addition, multiple scattering is unlikely since the cross section \( \sigma_{\text{coh}} \) for coherent scattering is much smaller than that for photoelectric absorption \( \sigma_{\text{abs}} \). With these assumptions, the theoretical treatment of x-ray interactions with matter is rather simple. Following an approach by Henke et al. [8], the relevant cross sections can be expressed as

\[
\sigma_{\text{coh}} = \frac{8}{3} \pi r_e^2 (f_1^2 + f_2^2) \quad \text{and} \quad (1.1)
\]

\[
\sigma_{\text{abs}} = 2 r_e \lambda f_2, \quad (1.2)
\]

where \( r_e \) is the classical electron radius, and \( f(\omega) = f_1(\omega) - i \cdot f_2(\omega) \) is the complex atomic scattering factor; \( f_1 \) can be associated with refraction and \( f_2 \) with absorption. Likewise, the complex index of refraction expressed in terms of the complex atomic scattering factor \( f(\omega) \) becomes

\[
n = 1 - \delta - i\beta = 1 - \frac{n_a r_e \lambda^2}{2 \pi} (f_1 + i \cdot f_2), \quad (1.3)
\]

where \( n_a \) is the number density of atoms and \( \delta \) and \( \beta \) describe the variation in phase shift and absorption respectively. They can be expressed in terms of
\[ \delta = \frac{n_a \lambda^2 r_e}{2} f_1 \quad \text{and} \quad (1.4) \]

\[ \beta = \frac{n_a \lambda^2 r_e}{2 \pi} f_2. \quad (1.5) \]

A wave \( \Psi_0(x) = A e^{-ikx} \) traveling through a homogeneous medium with refractive index \( n \) can then be described as experiencing an attenuational phase advance of

\[ \Psi(x) = \Psi_0(x) \ e^{i\delta kx} e^{-\beta kx}. \quad (1.6) \]

After traveling a distance \( t \) through the medium, the intensity becomes

\[ I(t) = I_0 e^{-2\beta kt}. \quad (1.7) \]

For absorption contrast images, where the thickness of the specimen is not known, the optical density \( D \) is given by

\[ D = -\log \left( \frac{I}{I_0} \right) = 2 \cdot \beta kt. \quad (1.8) \]

It is convenient to define the attenuation coefficient \( \mu = 2 \cdot \beta k \) which leads to a simple equation for the optical density

\[ D = \mu t. \quad (1.9) \]

The attenuation coefficient which characterizes the absorptivity of the material depends on the imaginary part of the atomic scattering factor \( f_2 \), which in turn depends on the frequency of the incident light and the chemical element exposed, with its distinct resonance frequencies. A very detailed derivation as well as theoretical calculations for \( f_1 \) and \( f_2 \) for elements with \( Z = 1 \) – 92 and \( E = 50 \) – 30,000 can be found in Henke et al. [9]. Accordingly, in the soft x-ray regime (200 - 800 eV), the absorption cross section for promoting an electron to a higher niveau or the vacuum level is very high for inner shell electrons (K- or L-shell) and certain elements, e.g. C, Ca, N, O, Mn and Fe. The energies of the respective absorption edges of these elements are included in Fig. 2.2.
Electronic Transitions - Fluorescence

If the process of absorption leaves the atom in an excited state, there are several ways for the atom to return to its ground state. They can be illustrated by a so-called Jablonski diagram, which shows the possible decay mechanisms, by taking into account the specific electronic structure of the element or molecule of interest. Fig. 1.2 shows a typical Jablonski diagram for a molecule with singlet $S_i$ and triplet $T_i$ states, as well as vibrational states. The principal de-excitation mechanisms shown are fluorescence, phosphorescence, internal conversion, and intersystem crossing. The latter two mechanisms are non-radiative. Fluorescence is the radiative transition between two states with the same spin, where the energy difference between the initial and the final state ($E_i$ and $E_f$, respectively) is converted into a photon of the same energy

$$h\nu_{\text{photon}} = E_i - E_f.$$  \hspace{1cm} (1.10)

The probability (or the transition rate) for each of these transitions is given by quantum mechanics as being proportional to the square of the matrix element between the initial and the final state, $\Psi_i$ and $\Psi_f$ respectively, depending on the type of transition. For an electric dipole transition (like fluorescence), the
matrix element $p_{fi}$ can be written as (Eisberg and Resnick [10])

$$p_{fi} \equiv \left| \int \psi_i^* \cdot e\vec{r} \cdot \psi_i \, d\vec{r} \right|,$$

(1.11)

where $\psi_{i/f}$ denotes the spatial part of the wavefunction and $e\vec{r}$ is the dipole moment for a one-electron atom. This equation shows that in order to make predictions for the probability of a certain transition, the wavefunctions of the initial and the final state have to be known. For a one-electron atom this can be achieved analytically by solving the Schrödinger equation, thus retrieving the eigenfunctions of the atom.

For multi-electron atoms, the Schrödinger equation can only be solved numerically and by applying some approximations. A common approach is called the Hartree-Fock method, which was developed in 1928. In this approach, the electrons are assumed to be moving independently, electron-electron interactions are treated by a self-consistent net potential, relativistic effects are ignored, and the Born-Oppenheimer approximation is assumed. Starting out with hydrogenic one-electron orbitals as initial guess, the Hamiltonian of that system is estimated as a sum of kinetic energies plus a net potential (containing electron-nucleur attraction and electron-electron repulsion) for each electron. This Hamiltonian is then inserted in a new Schrödinger equation which is solved and which leads to new guesses for the electron orbitals, which in turn are used to construct a new Hamiltonian, and so on. There are several refinements that lead to better results for systems which are not well characterized by the Hartree-Fock method, i.e. where theoretical predictions are not in agreement with experimental results; for a discussion, see e.g. Eisberg and Resnick [10].

Going towards more complex systems like molecules and solids, a theoretical calculation of the eigenfunctions is cumbersome or simply not doable. Therefore probabilities for certain transitions of a specific system are mostly determined experimentally, relying on knowledge of the eigenvalues (i.e. the electronic structure) that has been acquired either experimentally or theoretically. For instance, a theoretical approach to calculating the electronic structure of solids (applicable to quantum dots) is outlined in Section 5.1.2.

From experimental evidence, the probability of a fluorescent transition is typically much higher than that of a classically forbidden phosphorescent transition. Rates are typically on the order of $10^{-8}$s for fluorescent transitions and up to minutes for phosphorescent transitions (that is because their transition probability is very low), but these times are strongly dependent on the actual system of interest.
In chapter 5 of this thesis, quantum dots will be described as fluorescent labels. Quantum dots employ one principal radiative transition with high probability and a lifetime on the order of 10 nsec.
Chapter 2

The X1A soft-x-ray microscope at BNL

This chapter describes the experimental setup used at Brookhaven National Laboratory: The storage ring, the undulator, the beamline and the microscope. Scanning procedures are described, along with the microscope’s spatial and temporal resolution.

2.1 The NSLS Electron Storage Ring and the X1 Undulator

The NSLS is a second generation electron storage ring fed by a 750 MeV booster synchrotron, which in turn is fed by a 120 MeV linac. The storage ring operates at 2.8 GeV and is usually filled to a current of 300 mA. Construction started in 1979 and first stored beam was obtained in September 1982. Since then, the ring as well as the insertion devices have been upgraded several times. Modifications include the implementation of a global and a local orbit feedback system (the latter one stabilizes the orbit for insertion device beamlines such as X1A) as well as a reduction in vertical emittance yielding an order of magnitude increase in brightness. The ring is designed according to the eight superperiod Green-Chasman lattice. A schematic overview of the NSLS (which also has a VUV storage ring in operation), the attached beamlines and the insertion devices is given in Fig. 2.1. The x-rays for X1A are produced by the X1 soft x-ray undulator, a permanent magnet/steel hybrid device based on the Halbach scheme. Some of its characteristics are given in Tab. 2.1. The wavelength of the first fundamental can be adjusted by changing the undulator gap

\[ \lambda \approx \frac{\lambda_0}{2\gamma^2} \left( 1 + \frac{K^2}{2} \right), \quad (2.1) \]
where $\lambda_0$ is the undulator's magnetic period and $K$ is given by

$$K = \frac{e}{2\pi mc}B_0\lambda_0, \quad \text{with}$$

$$B_0 \approx 0.534/\sinh\left(\frac{\pi \times \text{Gap}[cm]}{\lambda_0[cm]}\right).$$

A plot of the calculated X1 undulator intensity as a function of energy for two different gap sizes is shown in Fig. 2.2 next to a comparison of different nowadays lightsources in terms of brightness. The undulator has been described in detail in the literature; see for example Jacobsen et al. [11], Rarback et al. [12], and Buckley et al. [13].
2.2 The X1A Beamline

The X1A beamline as it exists now profited to a big extent from its predecessors. The first successful attempt to perform scanning X-ray microscopy using a synchrotron source was achieved by Horowitz and Howell in 1972 [14]. The first X-ray microscope in the line of ancestors of X1A, U15 (named after the bending magnet it was attached to), was designed by Kirz, Rarback, and Kenney. It was in operation from 1983 till 1988 and offered scanning microscopy with a 300 nm spot size, a ∆E of approximately 1 eV and a flux of $10^4$ photons/sec. It also had a holography setup with 2 µm resolution. For a more detailed explanation, see e.g. Rarback et al. [15].

The next step by Rarback and Kirz took place in 1988, see Rarback et al. [16]. The completely new built microscope profited from a new undulator X17t (which was also the eponym of the microscope) and improved beamline design, thus offering scanning microscopy with 80 nm spot (0.5 eV ∆E, $10^5$ photons/sec) and holography with 50 nm resolution (see e.g. Jacobsen et al. [17])
Table 2.1 Undulator source parameters. The last four entries describe the properties of the electron beam at the symmetry center of the straight section of X1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period (cm)</td>
<td>8</td>
</tr>
<tr>
<td>Number of periods</td>
<td>35</td>
</tr>
<tr>
<td>Gap (mm)</td>
<td>32 - 98</td>
</tr>
<tr>
<td>Horizontal size (µm)</td>
<td>637</td>
</tr>
<tr>
<td>Horizontal divergence (µrad)</td>
<td>456</td>
</tr>
<tr>
<td>Vertical size (µm)</td>
<td>14</td>
</tr>
<tr>
<td>Vertical divergence (µrad)</td>
<td>40</td>
</tr>
</tbody>
</table>

The first incarnation of X1A was commissioned in 1990. Both the microscope itself as well as its new x-ray source, the undulator X1, and the beamline design have been described in the literature (see Rarback et al. [12], [18]). Spatial resolution for scanning microscopy improved to 45 nm (0.3 eV ∆E, 10⁶ photons/sec) and to 40 nm for Holography.

2.2.1 Beamline Components

The current beamline (see Winn et al. [19]) was designed to provide two independently operating x-ray microscopes, using zone plates to focus the source onto the sample and using the beam that is emitted by the undulator X1. The overall layout of the beamline is illustrated in Fig. 2.3.

The beam is first split by a water-cooled plane scraping mirror, intercepting ≈ 35 % of the central cone (the undeflected part feeds another beamline X1B). It also absorbs all the radiation above 2 keV and reduces the power sent downstream to 40 W or less. The beam then is collimated by a water-cooled Cu-mask and afterwards split again by a scraping toroid mirror (outboard toroid), deflecting 50 % of the beam towards outboard. It also focuses this beam horizontally onto the entrance slit of the monochromator chamber (outboard SGM) and vertically onto its exit slit. The undeflected part intercepts a similar toroid (inboard toroid) with the same functions as for outboard.

The water-cooled entrance slits of the monochromator chambers (inboard/outboard SGM) consist each of a set of fixed slits with width of 10, 25, 40, 70, 120, 200 µm and fully open. The slits can be scanned under computer control.
to measure the beam profile or to center the chosen slit on the beam, using aluminum photodiodes mounted just downstream of the entrance slits to measure the intensity. The gratings are horizontally dispersing. They are mounted on precision spindles with a sine arm mechanism and can be controlled by a stepper motor. Calculated grating efficiencies are shown in Fig. 2.4. Further downstream is another set of slits, identical to the entrance slits, that define the vertical extent of the beam, followed by a continuously adjustable vertical slit, that defines the horizontal extent of the beam. Again, the intensity can be monitored by aluminum photodiodes, mounted just downstream of the exit slits.

Both branches offer the possibility to insert order sorting mirrors (OSM), consisting of two parallel grazing-incidence plane mirrors. Especially for Outboard, where quantitative chemical state mapping using XANES peaks is performed, it is important to prevent higher-order spectral contamination. Thus, the incident angle is chosen so that $> 99\%$ of the second order (at 570 eV) is rejected at 285 eV.

Finally Fresnel zone plates with central stop followed by an order-sorting aperture, a pinhole, are used to focus the x-ray beam onto the sample and to further reduce the amount of second order radiation that reaches the specimen. Fresnel zone plates are circular diffraction gratings, leading to constructive interference for pathlength differences of adjacent zones of $\lambda$ times the diffraction order $m$. The lateral resolution of a zone plate with numerical aperture N.A. and an outermost zone width $\delta_{\text{rX}}$ is (see Michette [7])

$$\delta_t = 0.61 \frac{\lambda}{m \cdot \text{N.A.}} = 1.22 \delta_{\text{rX}} / m. \quad (2.4)$$
Figure 2.4 Calculated grating efficiencies. The Outboard grating is optimized for operation near the C and N K-absorption edge. Inboard is optimized for higher energies.

Thus, the best achievable spatial resolution is on the same order of magnitude as the outermost zone width, which in our case is 30 nm.

Spatial and Temporal Resolution  The design was driven by the goal to achieve high spatial and temporal coherence at a high photon flux. For zone plates, the resulting spot profile is a convolution of the demagnified source size and the point spread function of the optic. Fig. 2.5 shows the convolved spot profile as a function of a parameter $p$ equal to the source size multiplied by the full angle accepted divided by the wavelength $\lambda$ (one can think of $p$ as a wavelength-normalized phase space area, whereas the phase space area is just the product of the source size times the solid angle of radiation that is accepted). The source size can be adjusted by adding a pinhole aperture of variable size between the source and the objective. From Fig. 2.5 it is obvious that $p$ has to be $\leq 1$ to preserve full spatial resolution. Actually, there is full spatial coherence for $p \leq 0.5$ and almost diffraction limited imaging for $p \approx 1$ (see Jacobsen et al. [20]). Notice that the coherence criterion is clearly wavelength dependent and is easier satisfied for longer wavelengths. The phase
Figure 2.5 *Surface rendering of the convolved spot profile as a function of the coherence parameter $p$ and the radial coordinate normalized to the Rayleigh resolution. Full spatial resolution is achieved for $p \leq 1$.*

The phase space area of the undulator source is given by the convolution between the phase space area of the radiation emitted by one electron and the phase space area of the electron beam. The actual parameters of the undulator source, $\epsilon_x = 296 \text{ nm-rad}$, which is $\approx 75 \times 4 \text{ nm}$, and $\epsilon_y = 2.5 \text{ nm-rad}$, which is smaller than 4 nm, indicate that the constraints from above are satisfied by using the full vertical intensity and accepting $\approx 15\%$ of the horizontal output. By moving to smaller entrance and exit slits we can reduce the coherence parameter $p$ (e.g. at shorter wavelengths) and thus increase the spatial resolution (but we also lose flux!) and vice versa. The same effect works for the temporal coherence: the size of the entrance slits affects the resolving power of the monochromators. A calculation leads to the result from Fig. 2.6. Accordingly, a high energy resolution together with high spatial resolution requires smallest slit sizes and thus reduces the intensity (the intensity goes with the square of the slit size reduction factor relative to the slit size that accepts 100\% ) that actually reaches the sample. However, the beamline has been designed in such a way that the user can regain some of the flux, if the experiment requires only moderate energy resolving power (by opening the entrance slits). Thus, the beamline can be used for both x-ray absorption near edge spectroscopy (XANES) requiring high energy resolution, and conventional microscopy requiring high spatial resolution and a fair amount of flux.
Figure 2.6 Resolving power and energy resolution of both the X1A1 (outboard) and the X1A2 (inboard) monochromator as a function of energy and entrance slit size for a 80 µm diameter zone plate and at 1.2 m from the exit slit with coherence parameter $p = 1$.

2.2.2 The Inboard Experimental Chamber

Both beamlines end in similar endstations. The inboard branch is optimized to work at the O K-absorption edge and the outboard branch for the C K-absorption edge. All luminescence experiments were conducted at the inboard microscope. The chamber itself is mounted on Newport air support legs for seismic isolation. A schematic of the endstation is illustrated in Fig. 2.7. The x-ray beam coming from the right hand side leaves the evacuated beamline through an 100 nm thick SiN window and is then focused by the zone plate onto the sample. The chamber itself is at atmospheric pressure, but to prevent high absorption of x-rays by air oxygen, the chamber is first evacuated and then vented with helium. Downstream from the sample holder is the detector stage that supports 3 different detectors: a visible light microscope (VLM) to prefocus on the sample and to select interesting regions, a segmented silicon detector (SIDET) for x-ray absorption and a single photon counting module for luminescence experiments (SPCM). The detector stage is fully motorized; exchange of detector or changes in detector alignment can be done under computer control (see below).
Figure 2.7 Schematic of the microscope chamber. The picture shows the detector stage (orange), the silicon detector (red), the sample stage (green) and the interferometer (black). The soft x-ray CCD in the middle of the detector stage (blue) has been replaced by a visible light microscope. The fluorescence detector is not shown, it sits right above the visible light microscope.
Figure 2.8 Schematic of the STXM geometry. The x-rays coming from the undulator are focused onto the sample by a zone plate. Different detectors sitting behind the sample record the transmission contrast or the fluorescence. To record a 2 dimensional image, the sample is moved line by line, perpendicular to the beam in the x-y-plane, through the zone plate focus.

2.2.3 Collecting Data

Scanning the Sample Scanning the sample usually involves several steps, slightly varying depending on the sample and the recording mode (absorption contrast or fluorescence). For scanning transmission x-ray microscopy (STXM) images the sample is prefocused with the visible light microscope (VLM) (which in the same step also preselects the region of interest) and afterwards brought in x-ray focus using the segmented Silicon detector. For fluorescence applications it is somehow trickier as will become obvious in the experimental section. The sample then is ready to be scanned which means that it is moved perpendicular to the beam direction (see Fig. 2.8) through the zone plate focus while simultaneously counting the transmitted x-rays. In doing so a 2 dimensional image is recorded line by line. This process can be repeated for different energies thus creating a 3 dimensional data cube (x, y, E), containing point spectra for each pixel. Recording 2 dimensional images over an energy range is called recording a “stack” of a sample.

The STXM 5 Software All scan parameters (like dwell time, pixel size, number of pixels, energy and more) can be modified by the user via a graphical user interface which is part of the STXM 5 software. The software also keeps
track of all the motor positions during the scan, which makes it possible to move to and zoom in regions of interest by just clicking in the picture and setting up a new scan. This also holds for focal scans (scanning one line over and over again while modifying the sample-zone plate distance). Moreover the graphical user interface (GUI) lets you change detectors in between scans and, once the sample was recorded in focus for any detector, the software takes this into account to move any newly inserted detector close to its actual focus position. A screenshot of the GUI is shown in Fig. 2.9. Since all the microscope functions involved during a stack - except for exchanging the specimen - are controlled by the software, the chamber maintains its helium atmosphere during the whole process.
Figure 2.9 Graphical user interface used to remotely control the functions of the microscope, including focusing, scanning, changing the energy, setting up stacks and more. The picture is part of a stack on a 3T3 rat skull cell that was run across the carbon K-absorption edge at the Outboard microscope.
Chapter 3

The Idea of Using Quantum Dots as a SLXM Label

This chapter discusses immunolabeling with Quantum Dots, including biological mechanisms and staining procedures for conventional visible light microscopy and x-ray microscopy.

3.1 Cytoskeleton of an Eukaryotic Cell

Since all labeling procedures presented in this thesis target the cytoskeleton of eukaryotic cells, this section gives a brief overview of the chemical composition and biological function of the cytoskeleton. It also motivates again the investigation of this crucial part of the cell. The information from this section can be found in more detail in e.g. Alberts et al. [21].

The cytoskeleton is a system of filaments that controls the shape of the cell (its boundaries as well as its internal structure), which also means that it can change the shape of the cell. It also contributes to cell robustness. The cytoskeleton consists of three main types of filaments, each of them based on a different family of proteins and therefore each of them with distinct properties. Intermediate filaments are ropelike fibers with a diameter of about 10 nm. Their primary function is to give mechanical strength and resistance to shear stress to the cell. Another type of filament is the so-called microtubules. They are responsible for intercellular transport and for the positioning of membrane-enclosed cell organelles. Their shape is that of a long hollow cylinder with a relatively large diameter of 25 nm. Finally, the last group of filaments is of actin filaments. They appear in the form of two-stranded helical polymers winding around each other. Their organization as linear bundles or three-dimensional gels makes them flexible. They are responsible for the shape of the cell boundaries and cell locomotion. In addition to that there are sev-
eral accessory proteins, which link the cytoskeleton to other cell components. Fig. 3.1 shows a RCJ cell, whose actin cytoskeleton has been labeled with quantum dots. The fluorescence picture was recorded with a Zeiss microscope (model Axiovert 200M). For more information on cell labeling with quantum dots in general see Section 3.3. Information about the specific staining procedures, leading to this picture, can be found in Section 5.2.3.

The key element of all these proteins is their assembly from smaller subunits (individual protein molecules, a few nanometer in size) thus creating a very dynamic system, which is able to grow, shrink or even completely disassemble or assemble on the spot. Before the growing mechanism starts, some subunits have to form an initial aggregate (nucleation phase) that withstands thermal energy. The cell can therefore control when and where a specific polymer is to be synthetized by the use of special proteins that catalyze nucleation.

The importance of the cytoskeleton for many cell processes cannot be overemphasized. Fig. 3.2, for instance, illustrates the role of microtubules during cell division. Together with kinetochores (red), they form the mitotic spindle that holds the chromosomes and pulls them towards the poles during anaphase. This is simply achieved by gradually depolymerizing (shrinking) of the microtubules. The figure has been reprinted from Desai [22]. The following explanations restrict themselves to the actin filaments, since they were the focus of all labeling efforts presented in this thesis. The actin monomer is a single globular polypeptide chain with a binding site for the nucleotide ATP.
Figure 3.2 Role of microtubules (green) during cell division (here metaphase). The depolymerization of the microtubules attached to the chromosomes (blue) during the anaphase pulls the daughter chromosomes towards the poles. Reprinted from Desai [22].

(or ADP) in a deep cleft in the center of the molecule. The hydrolysis of ATP at this site to produce ADP is important for the dynamics of the filament.

3.2 Immunolabeling Techniques

An introduction to fluorescence in terms of the underlying physics has been given in Section 1.4. This section intends to shed some light on the biological processes that are exploited for immunolabeling and discuss labeling techniques for visible light fluorescence microscopy using conventional dyes.

There are two major restrictions for visible light microscopy as far as the investigation of biological systems is concerned. First it only offers limited spatial resolution. For an optically thick medium like oil with a refractive index $n_{\text{oil}}$ of around 1.5 and purple light ($\lambda = 0.4 \mu m$) the spatial resolution can be pushed to just below 200 nm. Many intracellular features, like for instance filaments of the cytoskeleton, are thus detectable but not resolved if close together. Improvements in resolution can be achieved by going to electron microscopy or imaging techniques with shorter wavelengths like x-rays (see Section 3.3).

Another way to enhance the perception of objects smaller than the resolution also addresses the second problem that compromises visible light mi-
croscopy in biology: since cells consist to over 70% of water, which is transparent to visible light, and the remaining 30% of cellular structures have low contrast for visible light illumination, it is hard to obtain structural information. To work around this, one has been using dyes to stain more or less selectively certain features within the cells to enhance their contrast. While there are many kinds of dyes, the explanations of this section are restricted to fluorescent dyes that are conjugated to antibody molecules. Such dyes are the direct competitors of quantum dots in terms of labeling and detection mechanisms.

Conjugation of a fluorescent dye to an antibody leads to highly specific staining, because the underlying process, the basic principle of antigen-antibody reaction of the immune system of a cell, is highly specific, as indicated by the terms immunolabeling or immunocytochemistry for this technique. A very detailed description of the whole process from production of antibodies to the actual staining procedures can be found in Polak and Noorden [23]. Once the desired target-antigen is chosen, there are several ways to produce the corresponding antibodies. A common approach is the infection of a rabbit or similar animal with the antigen and the subsequent harvesting of the antiserum. Another way is the artificial fusion of antibody producing B-lymphocytes from infected mice with cultured myeloma cells from mice of the same strain that do not produce the antibody. This procedure leads to so-called monoclonal antibodies (meaning all cell lines derived from a single cell) with very high specificity, which is not necessarily always an advantage since it might result in a weak staining signal or complete loss of signal if the corresponding antigen is only slightly modified.

The quality of a certain antibody therefore strongly depends on experimental details and is usually determined empirically. Criteria for a good antibody are high affinity for its antigen, high avidity and stickiness (in other words high sensitivity, high binding rate and therefore resulting in a strong and stable signal). The signal can be amplified further by using a primary non-fluorescent antibody that binds to the desired antigen and a fluorescent secondary antibody that targets the primary antibody. The experiments described in Section 5.2.3 involved biotin -XX phalloidin as primary antibody and streptavidin, with which the quantum dots were coated, as secondary antibody (see also Section 3.3). The process of attaching the antibody to the fluorophore (bioconjugation) is not discussed; the reader is referred to the literature (see e.g. Niemeyer [24].

The remaining question now is the choice of dye that will work best with the antibody and the experimental parameters. The field is way too vast to go into more detail, but the most general considerations (from a physicists
Figure 3.3 Schematic of a typical fluorescence microscope. The excitation filter accepts only a small wavelength range of the broadband source. The stained specimen is excited with this wavelength and emits light of a different (longer) wavelength. The purpose of the emission filter (and also of the beamsplitter) is to separate exciting from emitted light.

Point of view) would be quantum yield, photostability, emission and absorption spectra and size of the particles, plus of course chemical and biological aspects like e.g. toxicity and solubility. Emission and absorption spectra are especially important for multistaining applications, where different antigens are labeled with different dyes for simultaneous imaging. Ideally one would like to have no overlap in the emission wavelengths, to be able to distinguish between different labels, and almost matching absorption wavelengths, to be able to use one excitation wavelength for all labels. An overview of many commercially available dyes, classified by their target proteins and with further references can be found in Molecular Probes [25], which is also available online.

A decisive factor in the above considerations is the experimental apparatus that is used to investigate the labeled samples: the fluorescence microscope. Fig. 3.3 shows a schematic of a typical fluorescence microscope. The light-source delivers intense light over a long wavelength range. The excitation filter selects a narrow band of frequencies which has to be adjusted according to the absorption spectrum of the fluorophore that has been used to stain the specimen (sometimes the choice of fluorophores depends on the excitation filters that one has available). The labeled specimen is then excited with the
filtered light and emits light of a different (longer) wavelength. This light is collected by the objective and sent back to the dichroic beamsplitter, which only transmits a small frequency band centered at the main emission wavelength of the fluorophore. Together with the emission filter, it separates the exciting light (as well as unwanted autofluorescence) from the light emitted by the fluorophore.

### 3.3 Immunolabeling with Quantum Dots

This section addresses the use of quantum dots as fluorescent labels in biology with a focus on their performance in comparison to conventional dyes. For a description of the chemical synthesis of quantum dots see Section 5.1.1. For a description of electronical and optical properties, please refer to Section 5.1.2.

Quantum dots as semiconductor nanocrystals have been subject of research since the 1980’s; see numerous publications by E. Matijevic for instance. Back then, they were interesting catalysts because of their high surface-to-volume ratio. The discovery of their special optical properties due to quantum confinement effects (see e.g. Alivisatos [2] and Section 5.1.2) established them in many other fields of contemporary science, like molecular biology.

Quantum dots have some outstanding advantages mostly because they can be adapted to any kind of situation due to their modularity: the wavelength can be chosen by adjusting the size of the particles. They can be coated with different kinds of biological interfaces not only to make them water soluble or biocompatible but also to let them target different molecules, to give them membrane-crossing capabilities or an enzymatic function (see e.g. Michalet et al. [27]). Since their surface is relatively large and allows for 10 to 100 potential surface attachments, they can even be equipped with different functionalities at the same time.

In addition to that, they have superior optophysical properties compared to conventional dyes. Fig. 3.4 shows absorption and emission spectra for fluorescein and a typical quantum dot (reprinted from Bruchez et al. [26]). The emission spectrum of fluorescein stretches out to longer wavelengths. This is a typical characteristic of conventional dyes and makes their discrimination in multi-color experiments difficult. The quantum dot emission spectrum does not show the red tail and is also narrower. At the same time the absorption spectrum of the quantum dot stretches out to the UV (and beyond), which makes it possible to excite quantum dots of all different sizes with one wavelength. As can be seen, this is not the case for the fluorescein.

Also, unlike conventional dyes, quantum dots are more photostable; they
Figure 3.4 Absorption (dashed) and emission spectra (solid) of fluorescein (left) and a quantum dot (right). Full width at half max is 45 nm for fluorescein and 32 nm for the QD. The emission spectra of fluorescein shows a red tail, which is not the case for the QD. Also, the absorption spectrum for the QD extends to the UV. Reprinted from Bruchez et al. [26].

hardly suffer from bleaching effects. It is possible for instance to image quantum dots in a biological system over a longer period of time (minutes to hours) or to use them in confocal microscopy (again see Michalet et al. [27]).

The use of soft x-rays in biology has already been motivated in Section 1.1. The combination of quantum dots and soft x-ray fluorescence offers more than the sheer improvement of spatial resolution in the fluorescence pictures. Scanning luminescence x-ray microscopy (SLXM) images can be directly compared to x-ray transmission images of the same sample, to gain more information about the interaction of labeled proteins with other structures of the cell. There is also a potential impact on x-ray spectroscopy: recording XANES spectra from labeled proteins of known chemical composition would lead to a simplification of the analysis of spectra from unknown structures. Besides biological and chemical applications, there are also interesting questions regarding the optophysical properties of quantum dots alone when excited with x-rays, like quantum yield, bleaching properties, absorption spectra for higher energies, and so on. Part of these questions have been adressed by experiments that are described in Section 5.2.
Chapter 4

Characteristics of the Detection System

This chapter offers information about the detection system as well as physical background for some of the experiments which in turn are discussed in the second part.

4.1 Parameters of the Optical System

4.1.1 SLXM Geometry

Scanning luminescence x-ray microscopy at X1A has already been performed in the early 90’s (see Jacobsen et al. [28]). Experiments with P31 phosphor grains and fluorescent dyes have been conducted to characterize the detective quantum efficiency (DQE) and the spatial resolution capabilities of the detection system and to test the usability of x-rays for immunocytochemistry. The experiments with P31 indicate that a spatial resolution of 50-75 nm can be achieved, restricted by the dark current of the detection system. The detective quantum efficiency of the whole system (DQE) has been estimated to be \( \leq (0.045 \times 0.27) \), depending on the wavelength of the fluorescence and on the detector model used. The first factor corresponds to the finite collection solid angle of the detection geometry (the fluorescence is assumed to be isotropical) and the second reflects the DQE of the photomultiplier used. A basic schematic of their experimental setup is shown in Fig. 4.1.

The new SLXM system is based on the same basic layout (Fig. 4.1), but has been completely rebuilt to fit the STXM 5 geometry and to satisfy the requirements for quantum dot fluorescence labels. The actual detection unit is shown in Fig. 4.2. It consists of a 20x Plan objective (Edmund Optics), a tube with a filter holder, and a single photon counting module (SPCM-PQ 100 from
Figure 4.1 *Schematic of the experimental SLXM setup.* The x-ray beam coming from the source propagates through a 100 nm thick SiN window and is focused onto the sample by a zone plate. The sample can be moved perpendicular to the beam direction and at the same time the emitted fluorescence-photons are collected by an objective and focused onto the photomultiplier tube (PMT).

former EG&G Canada, now Perkin Elmer). The objective has been chosen to offer high numerical aperture (0.4) at a reasonably short working distance (3.3 mm). The tube (tube-length is 16 cm, corresponding to the objective’s specifications) consists of three parts where the middle part can hold optical filters (from Chroma) that match the emission profile of the quantum dots used to minimize the autofluorescence signal.

4.1.2 Detection Mechanism of an Avalanche Photodiode

The single photon counting module is a passively quenched silicon avalanche photodiode. A detailed description of its performance can be found in Dravins et al. [29]. An incident photon which has at least a certain minimum energy creates an electron-hole pair in the photodiode. The minimum energy depends on the band gap of the material used. For silicon with a band gap of 1.12 eV at room temperature, the photodiode becomes sensitive to wavelengths of less than 1100 nm. Electron-hole pairs that are generated in the depletion zone of the photodiode are accelerated by the electric field that results from the reverse voltage applied to the PN-junction. If the field exceeds a certain strength \(2 \cdot 10^5 \text{ V/cm}^2\) for silicon) the electrons and holes gain so much energy that they create additional electrons and holes when they collide with the crystal
lattice (see e.g. Hamamatsu Tech Report [30]). An incident photon thus triggers an electronic avalanche that then is passively quenched by a resistor R in series with the photodiode, thus creating an RC-circuit. The dead time is determined by the time it takes to recharge the detector’s capacitance. It usually ranges from several ns up to 1 µs. The detective quantum efficiency depends on the absorption efficiency of the active material and the triggering probability. The DQE for this specific detector has been estimated to be ≈ 43% at 643 nm (see Dravins et al. [29]).

4.1.3 Estimations of Dark Current

The dark current for avalanche photodiodes results mainly from thermally excited electrons which are also able to trigger an electronic avalanche. Thus, even for no incident photons present, the detector measures “photons” at a certain count rate. This so-called dark current depends on the material used for the photodiode (semiconductors with higher bandgap show less dark current), the temperature of the sensitive area, and the applied bias voltage (the higher the voltage, the higher the dark current). Since the bias voltage also affects the detection probability for a real photon event, a trade-off between detection probability and dark current has to be made. A second but much less significant contributing factor to the dark current arises from crystal im-
purities that potentially can trap an avalanche electron, which in turn can
trigger a new avalanche if it is thermally excited after a time longer than the
dead time. The dark current for the passively quenched SPCM-100 used in the
experiments described below has been measured to be around 133 Hz (Dravins
et al. [29]).

4.1.4 Detective Quantum Efficiency

The detective quantum efficiency (DQE) of a detector indicates how much
noise is added to the signal during the detection process. The following theo-
retical approach can be found in more detail in Feser [31].

According to the definition above, the DQE can be defined as the ra-
tio of the signal-to-noise ratio after detection (SNR\textsubscript{out}) and before detection (SNR\textsubscript{in})

\[ \text{DQE} = \left( \frac{\text{SNR}_{\text{out}}}{\text{SNR}_{\text{in}}} \right)^2. \]  \hfill (4.1)

For an average of \( \bar{N} \) photons incident on the detector, one would expect that
the actual number of photons for a measurement will fluctuate from mea-
surement to measurement according to the Poisson distribution, which can be
replaced with a Gaussian distribution for \( N > 10 \), with a mean deviation of
\( \sqrt{\bar{N}} \). Thus the signal-to-noise ratio before detection is given by

\[ \text{SNR}_{\text{in}} = \frac{\bar{N}}{\sqrt{\bar{N}}}. \]  \hfill (4.2)

For a photon counter which detects a percentage \( x \) of the incident photons,
that is it will only detect \( x \cdot \bar{N} \) photons on average, the signal-to-noise ratio
will be

\[ \text{SNR}_{\text{out}} = \frac{x \cdot \bar{N}}{\sqrt{x \cdot \bar{N}}}. \]  \hfill (4.3)

All in all, this leads to a detective quantum efficiency of just \( x \).

This result was derived under the assumptions that the detection unit
responds linearly to the number of incident photons and that contributions
from dark current can be neglected. In the case of the SPCM-100 detector,
linearity holds for count rates \(< 1 \) MHz (this is true for the experiments to
be described below). For count rates \(> 1 \) MHz, linearity cannot be assumed,
because the average time between two photons which is inversely related to
the count rate is on the order of the dead time of the detector. Contributions
from dark current can be neglected if the number of dark current “photons”
Figure 4.3 Schematic of the geometrical dimensions of the imaging process with a zone plate.

\[ N \ll \bar{N} \] which again is the case for the experiments conducted in order to estimate the detective quantum efficiency.

### 4.1.5 Spatial Resolution Revisited

Section 2.2.1 discussed the effect of the source size and the phase-space parameter on the spatial resolution. For a given source size which depends on the specific slit settings (the horizontal and vertical exit slits after the SGM chamber define the size of the beam) and known beamline dimensions, the expected maximum spatial resolution can be estimated from a calculated point spread function, where point spread function in that case means the far field diffraction pattern that the optical system (i.e. zone plate) creates in its image plane if the initial disturbance comes from a point source \( \propto \exp[ikz/z] \).

According to Michette [7], the point spread function (PSF) of a zone plate can be approximated by that of a lens for a zone plate whose number of zones \( N \geq 100 \). The optical system can then be treated as a circular aperture, where the fact that the aperture actually is filled with the zone plate is taken into account by applying the lens law where appropriate (see Goodman [32]):

\[
\frac{1}{g} + \frac{1}{b} = \frac{1}{f}, \tag{4.4}
\]

where \( g \) is the distance from the source to the zone plate, \( b \) is the distance from the zone plate to the image plane and \( f \) is the focal length of the zone plate (see Fig. 4.3). In general, the field \( U_b(x_b, y_b) \) that is created in the image plane can be calculated from the initial disturbance \( U_g(x_g, y_g) \) and the impulse
response function \( h(x_b, y_b; x_g, y_g) \) of the optic (i.e. the PSF) as

\[
U_b(x_b, y_b) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} h(x_b, y_b; x_g, y_g) U_g(x_g, y_g) \, dx_g \, dy_g. \tag{4.5}
\]

As mentioned earlier, \( h(x_b, y_b; x_g, y_g) \) describes the effects of the optical system on a point source emitting a spherical wave. The field \( U_l(x, y) \) incident on the zone plate, and the field \( U'_l(x, y) \) right after the zone plate, can each be written as

\[
U_l(x, y) = \frac{1}{i\lambda g} \exp \left[ i \frac{k}{2g} \left( (x - x_g)^2 + (y - y_g)^2 \right) \right]
\] and \( \tag{4.6} \)

\[
U'_l(x, y) = U_l(x, y) P(x, y) \exp \left[ -i \frac{k}{2f} (x^2 + y^2) \right], \tag{4.7}
\]

where the last factor in the second equation describes the phase shifting effects of the lens, and \( P(x, y) \) is the pupil function of the lens that takes into account its properties as a circular aperture of finite radius. The field in the image plane which essentially is the point spread function (PSF) of the system can now be calculated as

\[
\begin{align*}
\int_{-\infty}^{\infty} U'_l(x, y) \exp \left[ i \frac{k}{2b} \left( (x_b - x)^2 + (y_b - y)^2 \right) \right] \, dx \, dy.
\end{align*}
\]

Combining the above equations, one obtains a result for the point spread function that, if put in the correct form, immediately reveals its connection to the lens law from Eq. (4.4):

\[
\begin{align*}
& h(x_b, y_b; x_g, y_g) = \\
& \frac{1}{\lambda^2 g b} \exp \left[ i \frac{k}{2b} (x_b^2 + y_b^2) \right] \exp \left[ i \frac{k}{2g} (x_g^2 + y_g^2) \right] \times \\
& \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} P(x, y) \exp \left[ \frac{i}{2} \left( \frac{1}{g} + \frac{1}{b} - \frac{1}{f} \right) (x^2 + y^2) \right] \times \\
& \exp \left[ -i \frac{k}{2} \left( \frac{x_g}{g} + \frac{x_b}{b} \right) x + \left( \frac{y_g}{g} + \frac{y_b}{b} \right) y \right] \, dx \, dy. \tag{4.9}
\end{align*}
\]
The first exponential under the integral yields one if the lens law is applied. To make things easier it can also be shown that the first two exponentials can be neglected (see Goodman [32]). Furthermore, the coordinates for the point source are chosen to be the origin of the coordinate system \((x_g, y_g) \to (0, 0)\). The resulting impulse response function is then

\[
h(x_b, y_b; 0, 0) \approx \frac{1}{\lambda^2 g b} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} P(x, y) \exp \left[ -\frac{i k}{b} (x_b x + y_b y) \right] \, dx \, dy. \tag{4.10}
\]

Finally, going to polar coordinates with

\[
x = r \cos (\theta) \\
y = r \sin (\theta)
\]

in the plane of the lens and

\[
x_b = \rho \cos (\phi) \\
y_b = \rho \sin (\phi)
\]
in the image plane, the point spread function becomes

\[ h(x_b, y_b; 0, 0) = \frac{1}{\lambda^2 b} \int_0^a \int_0^{2\pi} e^{\frac{ik}{b} r \rho \cos(\theta - \phi)} \cdot r \, d\phi \, dr. \] (4.11)

Because the aperture is circular with diameter 2a, the pupil function \( P(x, y) \) is 1 for \( r \leq a \) and 0 for \( r > a \). This equation is recognized to be the integral representation of the Bessel functions (see e.g. Born and Wolf [33]). With that, the point spread function of the zone plate is given by an Airy pattern and the field in the image plane becomes

\[ h(\rho; 0, 0) = \frac{a^2 \pi}{\lambda^2 b} \left[ \frac{2J_1(ka\rho/b)}{ka\rho/b} \right], \] (4.12)

where \( J_1 \) is the first order Bessel function. The left part of Fig. 4.4 shows the 2-dimensional field distribution in the focal plane of a zone plate illuminated by a point source. Recalling Eq. (4.5), the field in the image plane is just a convolution of the point spread function and the demagnified source, which is given by

\[ w' = \frac{w \cdot f}{g}, \] (4.13)

where \( w' \) is the demagnified source size and \( w \) is the initial source size. This means that the maximal experimental resolution which is determined by the spot size of the focused beam, can be calculated if the source size is determined. Since the source size is determined by the exit slits of the monochromator chamber, they can be seen as rectangular pupil function, as is illustrated in the right part of Fig. 4.4. The mathematical operations to perform the convolution were done with Mathematica. With a slit size of 40 \( \mu \)m in the horizontal and 40 \( \mu \)m in the vertical (which corresponds to a demagnified source size of 20 nm in each direction), the theoretical calculated intensity distribution in the image plane has a FWHM of

\[ \text{FWHM} = 33.5 \text{ nm} \] (4.14)

and the distance to the first minimum, which according to the Rayleigh criterion determines the spatial resolution is estimated to be

\[ \delta_{\text{min}} \approx 39.5 \text{ nm}. \] (4.15)

On the experimental side there are various tools available to estimate the spatial resolution of an image that has already been recorded. A common tactic is to look at the power spectral density of the image and estimate the
contributions from noise and signal, respectively. The corresponding concepts are best understood by looking at an example of Fourier domain filtering that involves signal and noise estimation by means of the PSF of an image or signal. This technique has been developed in 1949 by Norbert Wiener \cite{34} and is called the Fourier-domain minimum mean squared error filtering, or (as it is now called) Wiener filtering. Following an approach by Press \textit{et al.} \cite{35}, a measured signal \( s(t) \) can be regarded as a convolution of an initial signal \( i(x) \) and an optics response function \( r(x) \) plus uncorrelated noise \( n(x) \):

\[
    s(x) = c(x) + n(x), \quad \text{with}
\]

\[
    c(x) = \int_{-\infty}^{\infty} r(x - x') i(x') \, dx' \iff C(k) = R(k) \, I(k), \quad (4.17)
\]

where \( C(k), R(k) \) and \( I(k) \) are the respective Fourier transforms. The right-hand-side of Eq. (4.17) implies, that - in the absence of noise - the initial signal \( i(k) \) can be retrieved in the Fourier domain by simply dividing the Fourier transform of the measured signal by the Fourier transform of the response function:

\[
    I(k) = \frac{S(k)}{R(k)}. \quad (4.18)
\]

In the presence of noise, the initial signal cannot be retrieved in the same way. Instead, a filter function \( \xi(k) \) is applied to the measured signal \( S(k) \) in the Fourier domain, which is then divided by the response function \( R(k) \):

\[
    I'(k) = \frac{S(k) \xi(k)}{R(k)}. \quad (4.19)
\]

The filter function is chosen such that it minimizes the mean squared deviation of \( I'(k) \) from \( I(k) \):

\[
    \int_{-\infty}^{\infty} \left| I'(k) - I(k) \right|^2 \, dk = \int_{-\infty}^{\infty} \left| \frac{(C(k) + N(k)) \xi(k)}{R(k)} - \frac{C(k)}{R(k)} \right|^2 \, df \quad (4.20)
\]

Evaluating the integrand (products containing \( S(k) \) and \( N(k) \) are zero, when integrated over all frequencies), differentiating it with respect to \( \xi(k) \), and setting the result equal to zero leads to an equation for the filter function \( \xi(k) \)
of
\[ \xi(k) = \frac{|C(k)|^2}{|C(k)|^2 + |N(k)|^2}. \] (4.21)

This equation suggests that the optimal filter can be determined if one is able to distinguish the convolved signal \( C(k) \) from noise \( N(k) \).

An estimate for this is given by the power spectral density (PSD) of the signal:
\[ PSD = |S(k)|^2 \approx |C(k)|^2 + |N(k)|^2. \] (4.22)

A double-logarithmic plot of the PSD of a typical measured signal against the frequency is usually high in the lower frequencies, where the contribution of the actual signal \( C(k) \) is strongest, until - at a certain frequency - it rolls off to a more or less steady noise floor. It is important to mention, that this behaviour only occurs for noise which is independent of the spatial frequency (shot noise), such as noise from photon statistics which is the case of interest. Such a plot is shown in Fig. 4.5 (also compare with Fig. 4.7, which shows the calculated PSD of the scan on the right side in Fig. 4.6). Extrapolating the signal down towards the frequency axis yields the crossing frequency \( f_c \) of the signal with the noise. The spatial resolution corresponding to \( f_c \) however is not useful for practical applications. Instead, according to the Rose criterion, one defines the spatial resolution as proportional to that inverse frequency \( f_{SR}^{-1} \), where the signal-to-noise ratio SNR is \( \geq 5 \).

### 4.2 Determining Detector Parameters

#### 4.2.1 Implementing and Aligning the Detector

The detector is mounted on the detector stage next to the visible light microscope (VLM) and the segmented silicon detector (SIDET). As power supply a Condor board (model HTAA-16W-A+) is being used. It connects to the unit over a DB9 feedthrough. For each counted photon, the detector puts out a TTL pulse that leaves the experimental chamber via a BNC feedthrough and goes into a multifunctional board in the computer that includes a pulse counter. The counts are then processed by the STXM 5 software (see Section 2.2.3) and displayed for immediate evaluation. After mounting the detector, it has been aligned using a sample of P31 on glass, which provides good fluorescence (see below for details on sample preparation). First, the z-position was adjusted by eye to be around 3 mm, which is the working distance of the objective. Then, the chamber was closed, wrapped up in aluminum (see below...
Figure 4.5 Double logarithmic plot of the power spectral density of a typical signal. The signal is contributing at lower spatial frequencies. For higher spatial frequencies, the curve rolls off to a steady noise floor. Extrapolating the signal down towards the frequency axis gives a justified estimate for the spatial resolution. From the frequency $f_c$ where signal and noise coincide, one can calculate the spatial resolution that fulfills the Rose criterion which is proportional to $f_{SR}^{-1}$.

for details), and a helium atmosphere was established. After prefocusing with the VLM, the detector was moved in the XY-plane perpendicular to the beam direction to find the correct XY position; that is, when the detector is on the optical axis. Then the sample was brought in both x-ray and detector focus by consecutively scanning one line on the sample against the sample-zone plate distance or the sample-detector distance. After focusing, the detector positions were recorded and stored in the alignment file.

### 4.2.2 Sample Preparation

The first experiments were conducted in order to characterize the detection system in terms of its detective quantum efficiency (DQE) and its spatial resolution. Therefore samples made of a phosphor P31 with well known quantum yield were prepared in the following way. Some of the P31 powder (produced
by USR Optonix Inc.) was suspended in distilled water and further diluted by hand to a concentration of $< 100,000 \mu l^{-1}$ in order to be able to look at isolated grains if a drop of this suspension was put on a microscope glass coverslip (Fisher Scientific, thickness range 130 - 170 µm) and dried for 30 minutes at 80°Celsius. The concentration is estimated under the assumptions that the P31 grains are spherical and have an average diameter of 5 µm and that 2.5 µl of solution are needed to cover 5 mm² on the coverslip. The coverslip then is taped to a metal plate with a hole in the center which fits the magnetic sample stage (the diameter of the hole roughly coincides with the dynamic range of the sample stage). Since the glass is opaque for x-rays, the sample is mounted in the sample holder in such a way that the coverslip surface with the P31 on it faces the incoming x-rays.

After establishing helium atmosphere, all transparent parts of the chamber (mainly the windows and the Plexiglass cover) were wrapped with at least two layers of aluminum to reduce stray light in the chamber. This procedure reduced the dark counts (i.e., no x-rays, no sample) for the detector to $< 2$ kHz.

4.2.3 Determining the Detective Quantum Efficiency

Quantum Yield of P31 Phosphor Grains The fluorescence properties of P31 have been examined earlier (see Yang et al. [36]). According to the published results, P31 has an almost linear energy response

$$R(E) = \frac{J(E)}{F(E)} = a \cdot E^m,$$

where $m = 0.995 \pm 0.015$. $J(E)$ is the measured fluorescence signal and $F(E)$ is the incident flux of photons with energy $E$. Also, it has been shown in an earlier publication (Yang et al. [37]), that the x-ray flux response of P31 is nearly linear in the range of $10^7 - 10^{10}$ photons/(sec·mm)². Taking these results into account Jacobsen et al. [28], predicted $40 \pm 10$ visible light photons for each 351 eV photon absorbed. Since not every photon might be absorbed, the expected ratio of incident photons/emitted photons has been calculated using the parameters given in [28]; which now will be elaborated upon.

The incident flux was $1.6 \cdot 10^5$ photons/pixel and the PMT (photomultiplier tube) current was corresponding to $3.5 \cdot 10^4$ photons/pixel. With a detective quantum efficiency (DQE) of the PMT of 0.15 at 520 nm, which is the main emission wavelength of P31 (see Yang et al. [38]), and taking into account that only a fraction of 4.57% of the isotropically emitted photons is detected
by the optical system (due to the limited solid angle), the quantum yield (QY) of the P31 is then calculated to be

\[
QY_{P31} = \frac{\text{energy emitted as photons}}{\text{incident energy}} = 0.22. \tag{4.24}
\]

A comparison to the predicted fraction of absorbed photons/emitted photons given in Jacobsen et al. [28] leads to the result that 81% of the incident photons are actually absorbed by the P31, which is a reasonable number because of the thickness of the individual P31 grains.

Calculating the DQE

Under the assumption that the measured linearity in x-ray flux response (Yang et al. [37]) extends to a flux of the order of \(10^{13}\) photons/(sec-mm²), which is a standard flux in the current configuration of the microscope, the detective quantum efficiency of the current SLXM detector can be determined from an \(I_0\) measurement and a fluorescence scan of some P31 grains on a glass coverslip. Both scans are shown in Fig. 4.6. The \(I_0\) measurement has been recorded with the segmented silicon detector. The maximum count rate from the left scan from Fig. 4.6 was measured to be \(R_{0,\text{measured}} = 7.11\) MHz. \(\tag{4.25}\)

The real number of photons incident on the sample is, however, higher due to the limited DQE of the SIDET and absorption of photons by helium. The DQE of the silicon detector has been estimated to be \(\approx 93\%\) for incident photons with an energy of 520 eV (see Feser [31]). The transmission of helium for x-rays can be estimated from the atomic scattering factors (see Eq. (1.5)), which have been tabulated by Henke et al. [9]. For x-rays with an energy of 520 eV the transmitted fraction is approximately 94% (assumes a distance between sample and detector of 1 cm, a chamber pressure of 650 torr, and room temperature). If this is taken into account, then the corrected value for the count rate is

\(R_{0,\text{corr}} = 8.13\) MHz. \(\tag{4.26}\)

The emission profile of P31, according to Yang et al. [36] has a width of \(\approx 60\) nm around the main emission wavelength of 520 nm, which corresponds to a photon energy of 2.39 eV. Considering the quantum yield for P31 that was calculated earlier (see Eq. (4.24)), one then would expect the rate of

\[
R_{P31} = \frac{QY_{P31} \cdot \text{incident energy}}{\text{energy of emitted photons}} = 3.89 \cdot 10^8 \frac{\text{photons}}{\text{s}} \tag{4.27}
\]
Figure 4.6 *Scans to estimate the DQE.* Both images were recorded at the same energy of 520 eV, identical slit settings and within 30 min. Left: $I_0$ measurement recorded with the silicon detector; max. counts = 7.11 MHz, pixel size: $0.5 \times 0.5$ microns. Right: Fluorescence scan of P31 phosphor grains; max. counts = 156 kHz, pixel size: $0.05 \times 0.05$ microns.

to be emitted isotropically from the P31 grains. The fraction of the solid angle that is covered by the detection unit is determined by the numerical aperture of the objective (N.A. = 0.4). By definition of the solid angle, this is

$$\Omega_{\text{det}} = \int_0^{2\pi} \int_0^{\sin^{-1}(\text{N.A.})} \sin \phi \, d\phi \, d\theta = 0.525.$$  \hspace{1cm} (4.28)

Thus, a fraction of $0.525/4\pi = 4.2\%$ of all emitted photons is projected onto the sensitive area of the detector. For an ideal detector covering the same solid angle, the count rate of detected photons would be

$$R_{P31,\Omega_{\text{det}}} = 16.3 \cdot 10^6 \frac{\text{photons}}{s}.$$  \hspace{1cm} (4.29)

The right hand side of Fig. 4.6 shows the fluorescence image of the P31 grains. The maximum count rate was measured to be

$$R_{P31,\text{measured}} = 156.0 \text{ kHz}.$$  \hspace{1cm} (4.30)

The ratio of the measured count rate and the expected count rate, which is just the fraction of incident photons that is detected, can then be associated
Table 4.1 A summary of all the important quantities that lead to the calculated detective quantum efficiency

<table>
<thead>
<tr>
<th>Incident flux measurements</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>initial measurement</td>
<td>7.11 MHz</td>
</tr>
<tr>
<td>corrected value</td>
<td>8.13 MHz</td>
</tr>
</tbody>
</table>

Expected count rates for P31 \((QY_{P31} = 0.22)\):

- isotropically emitted \(3.89 \times 10^8\) Hz
- emitted in solid angle subtended by detector \((R_{P31,\Omega_{det}})\) \(16.3 \times 10^6\) Hz

Measured values for P31 count rate with SLXM detector \((R_{P31,\text{measured}})\) \(156.0\) kHz

with the DQE of the detection system (see Section 4.1.4).

\[
DQE = \frac{R_{P31,\text{measured}}}{R_{P31,\Omega_{det}}} = 9.548 \times 10^{-3} \quad (4.31)
\]

which is less than 1% (Table 4.1 summarizes all the important quantities that lead to this result). Compared to the DQE of \(\approx 43\%\) that was determined by Dravins et al. [29], this seems to be a very low detection efficiency. The reason for this is not quite understood. Apart from the possibility, that the photon counting module is malfunctioning, this could also be an alignment problem. The objective can be scanned against the sample in the x-y plane, see Fig. 2.8, as well as in z direction, which changes the distance between the objective and the sample. If the detection unit is not well aligned in the x-y plane one would have a shift in x-y position of the recorded image versus the sample but this should not affect the detection efficiency. Bad alignment in z-direction would certainly worsen the detection efficiency because it blows up the spot size at the sensitive area, but this is minimized by several focusing steps. It could also be that the objective has aberrations that again would blow up the spot size. Since the sensitive area of the detector is very small (roughly 100 \(\mu m \times 100\) \(\mu m\)), a non perfect focal spot could lead to lost signal. Chapter 6 looks at some experiments that could unveil the nature of the problem.

### 4.2.4 Determining Spatial Resolution

According to Section 4.1.5 the spatial resolution of the detection system can be determined from a recorded image by calculating the power-spectral-density,
estimating the noise, and the signal and determining the spatial frequency where the signal-to-noise ratio is 5. The IDL program `tweak.pro`, written by C. Jacobsen, calculates the PSD as a square of the Fourier coefficients of the measured intensities of a 2-dimensional image and offers the possibility to apply a Wiener filter, which automatically involves the determination of signal and noise. The user can determine the signal and the noise by clicking in the plot of the PSD as can be seen in Fig. 4.7, which shows the PSD that has been calculated for the high resolution scan shown on the right side of Fig. 4.6. The program also calculates the frequency $f_c$ where the estimated curves for signal and noise coincide and also the power with which the signal declines as a function of frequency, giving

$$f_c = 4.0 \, \mu m^{-1}$$  \hspace{1cm} (4.32)
and the signal having a power law dependency

\[ \text{Signal}(f) \propto f^{-3.29}. \]  
(4.33)

The noise matching frequency \( f_c \) corresponds to a half period of

\[ \delta_{\frac{1}{2}c} = 125.0 \text{ nm}. \]  
(4.34)

Of course, this is for a signal-to-noise ratio of 1. The frequency that satisfies the Rose criterion can be calculated as

\[ f_{SR} = 5^{-\frac{1}{3.29}} f_c. \]  
(4.35)

This results in a spatial resolution of

\[ \delta_{SR} = 203.9 \text{ nm}. \]  
(4.36)

Compared to the expected spatial resolution from Eq. 4.15, this is not even close to what one would expect. There is certainly a connection between spatial resolution and detection efficiency, because a bad detection efficiency, as was calculated for this detector in Eq. 4.31, may shift the point where the signal-to-noise ratio is just 5 to a lower spatial frequency and thus worsen the spatial resolution. Whether or not this is the reason for the limited spatial resolution could not be determined. Again, Chapter 6 discusses the impact of this result on the whole idea of using quantum dots as a SLXM label.
Chapter 5

Quantum Dots

This chapter provides a basic introduction into quantum dots as far as their chemical properties, synthesis, and optophysical properties are concerned. This introduction then is followed by the experimental section, addressing questions such as quantum yield, bleaching, and cell labeling with quantum dots under x-ray exposure.

5.1 Basic Theoretical Considerations

Because the size of quantum dots is smaller than that of their emission wavelength or even of the de Broglie wavelength of their valence electrons, it is not surprising that their specific size plays a strong role in determining their optical emission properties.

5.1.1 Chemical Composition and Synthesis

Many methods have been used for synthesizing quantum dots, ranging from “physical” techniques like molecular-beam-epitaxy, to chemical processes. The explanations given in this section are taken from Klimov et al. [39]; the reader is referred to this book for more detail. Amongst all the different techniques it seems that pyrolysis of metal-organic precursors in hot coordinating solvents (120 - 360°C) yields the best results in terms of monodispersity and quality. The whole process can be characterized by an initial nucleation phase and a following aging (growing) phase. Adding chemical precursors to the hot coordinating solvents starts the nucleation phase by dissolving the precursors, creating a supersaturated monomer solution. After nucleation, the concentration of monomers is not saturated, so that monomers attach to existing nuclei instead of creating new ones. This is the growing phase. As time passes on, smaller, higher surface energy nuclei dissolve and their monomers attach to
existing larger particles, thus reducing the numbers. This is generally referred to as Oswald ripening. Time and temperature play a key role in controlling the size and dispersity of the outcome. In general, the longer the reaction time, the larger the particle size. In addition, the concentration of the precursors has an influence on the size. To decrease the size dispersion the solution is usually filtered using size-dependent precipitation, so that size dispersions of < 5% can be achieved.

A very common semiconductor material used for quantum dots synthesis is the II-VI compound CdSe. All the quantum dots used for the experiments discussed below had a CdSe core. To produce CdSe quantum dots by pyrolysis, different cadmium precursors, like cadmium oxide for example, can be used together with a selenium precursor made of selenium powder dissolved in trioctylphosphine or a similar surfactant-solvent combination. The choice of coordinating ligands has a strong influence on the quantum yield (QY) of the resulting particle, since they help passivate the surface of the particle and thus reduce trapped states for charge carriers. Quantum yields of 70 - 80% have been reported.

Another important way to passivate the surface and to increase the QY of the particles is the use of inorganic surface modification. Overcoating the CdSe core with a ZnS or CdS hull is common practice and potentially increases the QY of the particles by up to an order of magnitude. As for the organic ligands, the overcoating deactivates unbound surface bonds, but it also leads to a better confinement of the electrons and holes inside the core. The latter is due to a higher bandgap for ZnS or CdS compared to the bandgap of the core material CdSe. Figure 5.1 (reprinted from Dabbousi et al. [40]) shows a comparison of absorption and fluorescence spectra for capped and uncapped quantum dots of different size. It is noteworthy that the capping increases the QY for the fluorescence, but does not have a significant influence on the emission wavelength of the QD, as can be seen in the left graph. It does, however, increase the linewidth of the emission peaks, which is due to an increase in size dispersion during the capping process.

Chemically, the capping process is similar to the production of the core particles itself, i.e. pyrolysis from metal organic precursors. To prevent nucleation of homogeneous shell particles instead of heterogeneous core-shell particles, the temperature for the reaction and the concentration of the precursor material is kept low (for ZnS shell, diethylzinc and bis(trimethylsilyl) sulfide are used as precursors). All quantum dots used for the experiments came with ZnS capping (see Section 5.2).
Figure 5.1 Fluorescence and absorption spectra of capped and un-capped CdSe quantum dots. Left: Fluorescence spectra for quantum dots of different sizes (diameter of core in nm; a: 2.3, b: 4.2, c: 4.8, d: 5.5). The luminescence yield is generally higher for capped QDs. There is only a slight shift in emission wavelength between capped and un-capped QDs. The linewidth of capped QDs is increased due to higher size dispersion. Right: Absorption spectra for quantum dots of different sizes. The absorptivity for capped QDs is generally higher. Reprinted from Dabbousi et al. [40]

5.1.2 Electronical and Optical Properties

The discussion thus far of the properties of quantum dots has been based on the review of Klimov et al. [39]; we now consider electronic properties in more detail based on the treatment by Eisberg and Resnick [10].

At a first glance, one of the most important properties of quantum dots is their size dependent energy gap between the valence and the conduction band, which for bulk materials is usually size independent. By using a simple quantum box model, this dependency can be expressed as

$$E_g(R) = E_{g,0} + \frac{\hbar^2 \pi^2}{2 \cdot m_{eh} R^2},$$

(5.1)

with $m_{eh} = \frac{m_e m_h}{m_e + m_h}$,

(5.2)

where $m_{eh}$ is the combined effective electron-hole mass and $E_{g,0}$ is the energy gap for the “bulk” semiconductor. Due to the limited number of atoms, the energy bands are reduced to discrete energy levels, which gives rise to their
Figure 5.2 Electronic band structure for a direct semiconductor like CdSe. This simple two band model is derived under the assumptions of the particle-in-a-sphere model (see text). If a photon of a certain energy $h\nu$ is absorbed, it promotes an electron from the valence band to the conduction band.

narrow absorption and emission spectra. This atomic-like behaviour can be understood in terms of quantum confinement.

When a photon is absorbed, an electron hole pair is created. The radius of this electron-hole pair or exciton can be calculated from the Bohr radius as

$$a_B = \epsilon \frac{m}{m^*} a_0,$$

(5.3)

where $\epsilon$ is the dielectric constant of the material, $m$ is the rest mass of the electron, and $m^*$ is the reduced mass of the electron-hole pair. Quantum confinement effects occur if the size of the particle becomes smaller than the size of the electron and hole, which for the exciton is given in Eq. (5.3) (e.g., for CdSe nanocrystals, $a_{\text{exc}} = 6$ nm). The electron/hole/exciton is then confined by the boundaries of the quantum dot, giving rise to discrete energy levels, rather than energy bands like for the bulk material.

A simplistic approach to calculating the electronic structure and the wave functions for electrons and holes of a quantum dot is the particle-in-a-sphere
model, assuming a spherical potential well of radius $a$ with $V(r) = 0$ for $r < a$ and $V(r) = \infty$ for $r > a$. According to the Bloch theorem, the wave functions in a periodic crystal can be expressed as

$$\Psi_{nk}(\vec{r}) = u_{nk}(\vec{r})e^{i\vec{k}\cdot\vec{r}},$$

(5.4)

where $u_{nk}$ is a function with the same periodicity as the crystal lattice, $n$ is the band index, and $\vec{k}$ is the wave vector. The energies of these wave solutions as a function of $\vec{k}$ can be plotted in band diagrams. In order to do so, another approximation has yet to be made: It is convenient to incorporate the periodic potential of the atoms into the so-called effective mass of the electron or hole. The effective mass expresses the force of the potential acting on the charge carrier. Thus, the energies of the conduction and the valence band for instance can be approximated to

$$E_{\text{cond}} = \frac{\hbar^2k^2}{2m_{\text{cond}}^\text{eff}} + E_g, \quad \text{and}$$

(5.5)

$$E_{\text{val}} = -\frac{\hbar^2k^2}{2m_{\text{val}}^\text{eff}},$$

(5.6)

where $E_g$ is the bandgap between the conduction and the valence band. Note, that one can think of $m^n_{\text{eff}}$ as being the slope of the $E_n(k)$ dependency. A schematic of such a two-band system is shown in Fig. 5.2. This approximation only holds if we can treat the quantum dot like a bulk sample of the same material (which actually holds for the conduction band of CdSe). If so, then the wavefunction for a single electron or hole can be calculated as a linear superposition of the electronic wave functions from Eq. (5.4). Assuming a weak $k$ dependence for the periodic Bloch functions $u_{nk}$, the result is

$$\Psi_{e/h}(\vec{r}) = u_{n0}(\vec{r}) \sum_k C_{nk}e^{i\vec{k}\cdot\vec{r}} = u_{n0}(\vec{r})f_{e/h}(\vec{r}),$$

(5.7)

where $C_{nk}$ are the expansion coefficients which are determined by boundary conditions. Accordingly, the functions $f_{e/h}(\vec{r})$ are just the solutions to the initially stated particle-in-a-sphere problem, given by

$$\Phi_{n,l,m}(r, \theta, \phi) = \text{const.} \cdot j_l(k_{n,l}, r)Y^m_l(\theta, \phi),$$

(5.8)

where $Y^m_l(\theta, \phi)$ are spherical harmonics and $j_l(k_{n,l}, r)$ is the $l$th-order spherical Bessel function. Finally, the energies of these atomic-like orbitals is are given
by

\[ E_{n,l} = \frac{\hbar^2 \alpha_{n,l}^2}{2m_{\text{eff}} a^2}, \]  

(5.9)

where \( \alpha_{n,l} \) is the \( n \)th zero of \( j_l \). A comparison of Eq. (5.1) and Eq. (5.9) shows the same size dependence of the energy levels, namely a scaling of \( \propto R^{-2} \), where \( R = a \) is the radius of the quantum dot.

As a first approximation, this gives only an idea of the electronic structure. It does not, for instance, take into account the Coulombic attraction between the electron and the hole (leading to excitons) or atomic effects like spin-orbit coupling or mixing of the quantum-mechanical states. Those effects can be incorporated by applying more complicated theoretical models.

At the same time the particle-in-a-sphere model leads to some estimation of the transition probabilities according to Eq. (1.11), since it is possible to calculate the eigenfunctions of such a system. For the specific case of an electron-hole recombination, where the electron and the hole are treated as single particles, the transition probability can be written as

\[ P = |\langle \Psi_e | \vec{e} \cdot p | \Psi_h \rangle|^2, \]  

(5.10)

where \( \vec{e} \) is the polarization vector of the light and \( p \) is the momentum operator. For the particle-in-a-sphere model, where the wavefunctions \( \Psi_{e/h} \) are given by Eq. (5.7) as a product of the zero order Bloch function \( u_{n0} \) and an envelope function \( f_{e/h} \), which in turn is given by Eq. (5.8) (and note that the \( f_{e/h} \) are orthogonal), the probability of the electron-hole recombination is

\[ P = |\langle u_e | \vec{e} \cdot p | u_h \rangle|^2 \delta_{n_e,n_h} \delta_{L_e,L_h}, \]  

(5.11)

where \( n_{e/h} \) and \( L_{e/h} \) are the quantum numbers of the electron and the hole state.

### 5.2 Experiments with Quantum Dots

The quantum dots used for the experiments were bought from Quantum Dot Corporation, Hayward CA. They consist of a CdSe core, a ZnS shell, a polymer coating, and a coating of streptavidins. The diameter of the whole particle is between 15 and 20 nm, which is the size of a large macromolecule or protein.
5.2.1 Quantum Yield Estimation

After having determined the DQE of the optical system in Section 4.2.3, it is now possible to estimate the quantum yield (QY) of quantum dots by the same technique: Recording the $R_0$ flux without sample, followed by recording a fluorescence image of the sample. The samples consisted of quantum dots of wavelength 655 nm and 705 nm as they were provided by the Quantum Dot corporation. Samples were prepared by putting a drop of quantum dot solution (different concentrations were achieved by diluting the stock solution with methanol) on a glass coverslip or a SiN-window and letting it air-dry. Fig. 5.3 shows a fluorescence picture of non-diluted quantum dots with a specified emission wavelength of 655 nm, that were excited with x-rays of 509.02 eV. To achieve maximum count rates for focusing purposes, the slits were fully opened to get the highest x-ray flux incident on the quantum dots (this and other shortcomings are discussed further below). The maximum count rate was then measured to be

$$R_{\text{max}} = 13.95 \cdot 10^3 \frac{\text{photons}}{s}. \quad (5.12)$$

The corresponding $R_0$ measurement with identical slit settings could not be recorded at the same time but instead was recorded 3 months later. Restrict-
tions in experiment interpretation arising from that fact are explained below.
Taking into account limited detection quantum efficiency of the silicon detector and x-ray absorption in helium, as explained in Section 4.2.3, the $R_0$ flux is

$$R_0 = 44.851 \cdot 10^6 \frac{\text{photons}}{s}. \quad (5.13)$$

According to Eq. (4.24) the quantum yield (QY) is the ratio of the energy emitted as photons against the incident energy. Since the optical system has a limited detective quantum efficiency, which is given by Eq. (4.31), the QY of the quantum dots can be calculated as

$$QY_{QD} = \frac{\text{counted photons} \cdot \hbar \nu_{\text{em}}}{0.042 \cdot \text{incident energy} \cdot \text{DQE}^{-1}} = 2.88 \cdot 10^{-3}. \quad (5.14)$$

Assuming an emission wavelength of 655 nm (1.89 eV), this corresponds to 1 emitted photon for 1 - 2 incident x-ray photons.

**Discussing the Result**  As already mentioned before, the calculations and results presented above can only be seen as a rough estimate for the QY of the quantum dots for several reasons: For instance, the measurement of the incident flux was recorded 3 months later than the experiment. During that time, there might have been changes in the electron orbit, that could have lead to intensity variations. Also, changes to the beamline itself required more than once a re-alignment of the microscope. Finally, when the incident flux was recorded the ring current was higher than that one with which the image of the quantum dots was recorded. Ideally, the output intensity of the undulator is linear with the ring current, but in combination with the factors mentioned above, this leads to an unquantifiable uncertainty in the incident flux.

Even more important in terms of restricting the possibilities of interpretation of the results is the fact that only one sample has been used for the experiment described here. Most of the SiN samples broke during the drying or transportation process. For the rest of them as well as for most of the glass coverslip samples, it was impossible to find a fluorescence signal, *i.e.* to focus on the sample. Small quantum yield for the quantum dots and small DQE of the detector led to low count rates (for the scan from Fig. 5.3 the signal to noise ratio was just above 5). Thus, a fluorescence signal was only observed for the two undiluted samples on glass coverslip (655 and 705 nm) and of those, only the 655 sample was used for this experiment. Apart from the fact, that these experiments have never been double checked with another sample, the results are questionable, because the sample was prepared in a suboptimal
way. By air drying 1 µl of undiluted (1 µM) quantum dot solution on a glass coverslip, one produces a layer of dried quantum dot solution of ≈ 0.32 µm which is 32 × Ø_{QD}, where the diameter of the quantum dots (Ø_{QD}) was assumed to be 10 nm (this includes the hull of bioconjugating proteins). Effects like multiple absorption and emission or fluorescence contributions from the proteins can neither be quantified nor neglected. Finally, there are hints that the quantum dots actually emit light of different wavelength upon x-ray excitation than the one specified for visible light excitation, as no fluorescence signal could be recorded, when the sample was scanned using a spectral filter that only transmitted light from a 20 nm interval around the main transition wavelength of 655 nm.

All in all, the QY that has been calculated in Eq. (5.14) can therefore only be seen as a rough estimate. The interpretation would be that, taking into account the experimental shortcomings, the quantum yield of x-ray excited quantum dots seems to be fairly low compared to the relatively high values that can be achieved for visible light excitation (70 - 80% have been reported). It also seems low compared to other dyes that have been characterized for soft x-ray excitation (see Section 1.2). However their quantum yield seems to be of the same order of magnitude. For possible applications of SLXM of quantum dot labeled specimens, this would result in inconvenient focusing and alignment problems (as has already been experienced) and it would make it more difficult to record significant data at high spatial resolution, especially when quantum dots of different wavelengths are used. Chapter 6 provides a general discussion based on the experiments conducted, whether or not quantum dots are qualified to be SLXM labels. It also describes possible future experiments to answer some remaining questions and names some ways of how to overcome the experimental shortcomings that hampered the analysis of the data in the present case.

### 5.2.2 Bleaching Properties

The question of bleaching properties (i.e. how long can the label be excited before it is destroyed by the incident light energy) is an important one for biological labels. It affects the life span and possible applications of the stained sample. Quantum dots are known for their long life time in visible light fluorescence microscopy (it is possible to record movies of several hours length; see Michalet et al. [27]). To determine their bleaching properties for x-ray excitation a small area from undiluted QDot 705 on glass coverslip was scanned over and over again (see Fig. 5.4). The extract was 5 µm × 5 µm with a pixel size of 100 nm × 100 nm. Every pixel was exposed for 10 ms. All in all the
extract has been scanned 92 times. The results can be seen in Fig. 5.5. The dose estimate has been calculated from

\[
\text{dose} = \frac{\text{incident energy}}{\mu^{-1} \cdot A \cdot \rho},
\]

where \(\mu^{-1}\) is the absorption length \((\mu^{-1} = 0.101 \mu\text{m for CdSe})\), \(A\) is the pixel area and \(\rho\) is the mass density of the sample \((\rho = 5.82 \text{ g/cm}^3\) for CdSe).

**Discussing the Result**  As expected, the measured count rate decreases for longer overall exposure times. A fit with an exponential to the declining part of the curve indicates that the count rates decreases with a bleaching coefficient of \(1.46 \cdot 10^{-9}/\text{Gy}\). However, during the first 180 ms (or 111.6 \(\times\) \(10^6\) Gy) the count rate rises from \(\approx 7\) kHz to more than 13 kHz. A possible explanation might be that this is an effect of having multiple layers of quantum dots and proteins on top of each other. Like for the sample described in the previous section, air drying the undiluted quantum dot solution led to a thick deposit consisting of several layers of quantum dots and their biocoating. The intense beam of x-rays destroys surface layers of non-fluorescent proteins and of quantum dots whose emitted photons get re-absorbed or scattered before they make their way through all layers to the glass. As these layers are removed, the beam can ‘reach’ more quantum dots that were buried underneath and whose emitted
Figure 5.5 Bleaching of quantum dot luminescence over time. The increase in signal during the first 180 ms (or $111.6 \cdot 10^6$ Gy) is probably an effect of having multiple layers of quantum dots and proteins on top of each other. An exponential has been fitted to the declining part of the curve. It shows, that the count rate decreases with a bleaching coefficient of $-1.46 \cdot 10^{-9}$/Gy.

photons have a higher possibility of reaching the glass coverslip (because they are closer to it).

All in all it takes $\approx 740$ ms (or $458.8 \cdot 10^6$ Gy) to cut in half the count rate of emitted photons. The significance of this number however must not be overemphasized. Similar to the discussion of the previous section, the result suffers from ill-defined experimental conditions. The multilayer nature of the sample prohibits any quantization of the results. As far as possible applications are concerned (and taking into account the aforementioned experimental shortcomings), the bleaching properties seem to qualify quantum dots as a SLXM label, provided that pictures of good significance and resolution can be recorded at count rates on the order of 10 ms dwell time per pixel. Again, the reader is referred to Chapter 6 for a discussion of how to deal with some of the problems mentioned above.
5.2.3 Cell Labeling with Quantum Dots

A large amount of time was spent on cell staining and fixation. This work was done in collaboration with Prof. Helmut Strey at the Department of Biomedical Engineering (BME) at Stony Brook. All the cells that were stained were either 3T3 mouse fibroblasts or RCJ rat chondrocytes, two cell lines that eventually differentiate to form bone structure. Cell culturing and maintaining was done by BME grad students; see the Acknowledgements on page xi.

A diluted solution of undifferentiated cells was allowed to settle on either glass cover slips or electron microscopy grids. Although cells successfully attached to formvar coated grids that had a thin layer of collagen on them, further experiments focused on cells on glass coverslips, because they can be readily imaged with the visible light microscope (a big advantage for the search for the right staining protocol and drying technique). The following explanations are thus restricted to coverslip samples. Nonetheless, EM-grids will be part of future experiments as is explained in Chapter 6.

After a certain incubation time, the cells are attached to the surface and start to form a monolayer. Before differentiation occurs, they are fixed and stained, and some of them are then air-dried. Several factors have to be taken into consideration in order to obtain quality samples which allow for reliable information about processes in living cells. A quality sample is characterized, above all, by good preservation of its ultrastructure and by the fact that it is as close to “reality” as possible, which means that changes in the actual cell structure due to chemical and physical processing have to be minimal. Also, one has to make sure that the label specifically stains the desired target and that artifacts from unbound quantum dots or autofluorescence are minimized.

Quantum dots themselves are not transfected over the cell membrane. However, there are several ways to introduce them into the cell: Besides electroporation of the cell membrane, or attaching the quantum dots to a carrier protein that is naturally transfected, one can also simply destroy the cell membrane; this was done for the samples described here. To break the membrane, the cell is incubated in a non-ionic surfactant, Triton X-100. Since this step destabilizes the whole cell, it has to be simultaneously fixed by a cross-linking agent such as glutaraldehyde or formaldehyde. The choice of cross-linking agent depends on several factors such as what kind of antibody is used and how the cell is imaged afterwards. For instance, glutaraldehyde forms a complex 3-dimensional network which results in better stabilization than cross-linking with formaldehyde, which in turn only forms linear bonds. However, it has been reported that glutaraldehyde changes the actual form of...
some target antigens or sterically hinders the accessibility of others, making it an unusable cross-linking agent for certain target antigens. A more detailed discussion of that topic can be found in Griffiths [41]. All the cell-samples described here have been cross-linked with formaldehyde for membrane permeabilization and staining purposes (this is not true for post-staining fixation, which had to be done for samples to be dried, as described below). Two blocking steps, one before the incubation with the primary antibody and one after, minimize unspecific staining effects. It is also important to thoroughly wash the cells with PBS (phosphate buffered saline) in between different steps to make sure that all unbound antibodies are removed. The best results achieved are shown in Fig. 5.6. Those cells have been stained according to the protocol described in Appendix A.1. The filamentous structures are labeled actin fibers. The cells thus show a well preserved actin skeleton. Improvements in terms of reducing the background fluorescence could be made by increasing the duration of the two blocking steps and the wash steps. As already mentioned above, these samples are the best in a series of wet samples that have been prepared in order to find out the best staining protocol.

The situation gets a little more difficult if the cells are to be dried. Fig. 5.7 shows cells that have been stained and dried according to protocol A.3. There is no greater structure in evidence; only some short unconnected fibers are visible. Both images seem to be from the same sample (since they show the
same lack of structure), yet they were prepared differently. The cells from the left image, after being stained, were simply washed in 50/50 acetone/methanol and then air dried. The sample shown in the right image had an additional post-staining fixation process with 4% glutaraldehyde for 8 minutes, afterwards it was also washed in 50/50 acetone/methanol and air dried. Obviously, the additional fixation was not able to preserve the structure of the cell. What happens is that the cytoskeleton of the cell gets destroyed due to surface tension effects, when it dries. By washing the cells with acetone/methanol one can reduce the surface tension of the surrounding medium by a factor of 2/3, but since the surface is so small and the surface tension is $\propto \text{m}^{-1}$ (or the surface energy $\propto \text{area}^{-1}$) it is still not sufficient.

Again there are different techniques to overcome this problem. Electron microscopists usually rely on critical point drying, where the sample in 100% acetone is washed with liquid CO$_2$ and then brought to the critical point where the CO$_2$ evaporates without surface tension. An easier approach that was followed here (see protocol A.2) is to wash the sample in ethanols of increasing concentration starting with 20% up to 100% in 20% jumps to make sure that all the water in the medium is replaced by ethanol. Then the ethanol itself is washed out with HMDS (hexa-methyldisilazane), a liquid with lower surface tension than acetone or methanol. The sample then can be air dried.
Figure 5.8  VLM Fluorescence picture of quantum dot stained cells that have been prepared according to the protocol in Appendix A.2. Although the visibility of the stained actin filaments is hampered due to background fluorescence, there is good structural information present. The image is 24 µm × 18 µm.

The results of this technique can be seen in Fig. 5.8. The cells clearly have preserved their structure throughout the drying process, as can be seen from the long actin fibers stretching out across the cell. However, they are not as numerous and clearly visible as in the wet samples from Fig. 5.6. This maybe partly due to a high level of background fluorescence, but it also seems that the quantum dots did not attach to the actin as numerously as they did in the wet sample. It might just be this particular sample, but it could also be a result from the repeated washing and the accompanying mechanical stress during the drying procedures. If the binding energy of the quantum dots to the antigen is not high enough, some of them might just have been washed away. If the quantum dots prove to be a feasible label for scanning luminescence x-ray microscopy (see Chapter 6) then there will be also further attempts to try and modify the staining and drying procedures, and also to prepare samples on electron microscopy grids for high quality SLXM samples.

So far, only one attempt has been made to image stained cells with the SLXM system. It was also the first experiment after the detector has been built in and the cells are actually prepared on an EM grid. The outcome, which is shown in Fig. 5.9, indicated that the staining had to be improved on wet cells first and that the right drying technique had to be determined by using the visible light microscope. The cells were prepared according to protocol A.4, which apparently did not satisfy the requirements for structural preservation.
Figure 5.9 *STXM and SLXM image of quantum dot stained cells that have been prepared according to the protocol in Appendix A.4.* Left: STXM image @ 520 eV. Cell structure not obvious; instead, clusters of quantum dots appear. Right: Fluorescence scan of the same section @ 509 eV. The clusters are fluorescing, with a maximum countrate of 6.15 kHz.

and highly specific staining. The left image shows an x-ray absorption contrast image of the sample. There is no cell structure like nucleus or cell boundaries visible. However, there are several absorbing clusters of various sizes. These clusters, as can be seen on the right image (which is a fluorescence image of the same region on the sample) are fluorescent. Since autofluorescence is not very likely for soft x-ray excitation, the interpretation is that these clusters actually are quantum dots. Since the size of the clusters is ≈ 1 µm in diameter, one cluster consists of several quantum dots that are bound together. This image, however, does not give an indication whether or not quantum dots are an appropriate SLXM label. The reader is referred to Chapter 6 for further discussion.
Chapter 6

Anticipated Experiments and Modifications

This chapter sums up the experiments, discusses the results and proposes new experiments or modifications of existing experiments.

6.1 So, Are Quantum Dots Feasible SLXM Labels?

First, what is a feasible SLXM label? There are certain conditions which should be satisfied. In general, the labeling process and the label itself should not take away the advantages of soft x-rays for biological applications such as high spatial resolution compared to visible light microscopy and easy sample preparation compared to electron microscopy, as well as chemical state mapping capabilities. Ideally, it would improve upon all those features and add new features, which of course for a fluorescent label of high specificity would be the capability to map the spatial distribution of certain proteins inside a cell (this could also improve the significance of XANES spectra).

The experiments described in this thesis addressed those questions, answered some of them and provided hints on how to answer the others. Properties like specificity and easy labeling process have already been shown in many applications. Section 5.2.3 shows that, once the correct protocol is figured out, the preparation of quantum dot stained cells for x-ray microscopy is easy and can be done within two days. Issues arising from the necessary drying step can be minimized by a combination of chemical fixation and of drying with chemicals of very low surface tension. This of course has already been known from visible light and electron microscopy.

As it turns out, the performance of quantum dots as a SLXM label, apart from the labeling process itself, cannot yet be determined in a quantitative way from the experiments conducted. The bad performance of the detection
system in terms of detective quantum efficiency and spatial resolution limits the ability to answer these questions. Unless the detection system is improved, the remaining questions regarding quantum dots cannot be answered. The next section will propose some experiments and modifications that deal with a possible improvement of the detection system.

Besides that, there are other things that have to be figured out first. Although it seems that, on the one hand, quantum dots have a low quantum yield for x-ray excitation, which is a disadvantage for SLXM, and that, on the other hand, their bleaching properties are good enough to qualify them as a SLXM label, the respective parameters cannot be quantified from the experiments, as is explained in the respective sections.

Another important question arises from the fact that it was not possible to record a fluorescence image, if the matching filters (i.e. those for the specified emission wavelength of the quantum dots) were used in the experiments. This could mean two things: First, the fluorescence is actually not emitted from the quantum dots, but rather from their bioconjugating hull or from cell proteins (autofluorescence). And secondly, the quantum dots emit light of a different wavelength when they are excited with x-rays compared to when they are excited with visible light. Experiments to address these problems are also described in the next section.

6.2 What Comes Next?

Having specified all the experimental shortcomings it is now time to think about ways of how to overcome them. This is not only of hypothetical value; some of the equipment needed was already purchased.

Re-determining Detective Quantum Efficiency An independent experiment is planned to re-determine the detective quantum efficiency of the detection unit. As light source one might use a light bulb with frequency filter that illuminates a pinhole. Then in a black box the intensity is first measured with a calibrated photodiode and afterwards with the detection unit. Since the actual flux is known from the first measurement, the detective quantum efficiency can be calculated from the second measurement. To account for different sensitivities of the photodiode and the photon counter, different pinhole sizes or neutral density filters could be used. Depending on the result further experiments might be necessary. One might, for instance, use the detector objective together with a photodiode to see if the objective causes the problem, or play with different distances between objective and the sensitive area of the
single photon counting module.

**Improving Sample Preparation** Preparation of labeled and dried cells has been mastered to some extend as is shown in Section 5.2.3. One might try critical point drying as a different drying method, but the results with HMDS are satisfying enough so far. It would definitely be an advantage to prepare the cells on electron microscopy grids, since those can also be used for regular x-ray absorption contrast imaging, which has two major advantages. First, one could bring the sample in x-ray focus with the segmented silicon detector, which is much easier than with the fluorescence detector alone, and secondly, one could directly compare fluorescence and x-ray absorption images from the same sample, as was already done in Fig. 5.9.

For a re-determination of quantum yield and bleaching, but also for the experiment described in the next paragraph, it would be an advantage to have blank quantum dots without any biological coating. Thus any fluorescence would clearly come from the quantum dots themselves and not from their protein hull. Furthermore, the samples have to be prepared in a way that no more than a monolayer of quantum dots is present on the support. For the support it would be nice to have some samples on SiN windows - again, because these could be used with the segmented silicon detector for x-ray absorption contrast imaging. It is now also possible to prepare samples with a wider range of sizes, since quantum dots with visible light emission wavelengths of 525, 565, 585, 605, 655 and 705 nm have been purchased.

**Determining Quantum Dot Emission Wavelength** The experiments described in Section 5.2 give rise to the assumption that the quantum dots do not emit on their specified wavelength when they are excited with x-rays. This experiment attempts to measure the spectrum of quantum dot emission with a spectrometer. The fluorescence detector will be replaced by an objective, that couples any incident light into a fiber. The fiber connects to an optical feedthrough to which, from the outside, the spectrometer is attached. The spectrometer is the HR2000 from OceanOptics, which runs from 200 - 1100 nm with a resolution of \( \approx 0.2 \) nm. Depending on the results, different filters can be purchased and further experiments will be conducted.
Bibliography


Appendix A

Staining protocols

A.1 Staining protocol for wet cells - good

This protocol is recommended by the Quantum Dot Corporation. It has been slightly modified to optimize the results.

1. Wash cells with PBS - 3 × 5 min.

2. Fix cells with 3.7% formaldehyde/0.1% Triton X-100/PBS for 10 minutes at RT.

3. Wash cells with PBS - 3 × 5 min.

4. Block cells with 2% BSA/0.1% Triton X-100/PBS for 30 minutes at RT.

5. Incubate cells ON at 4°C with 1:20 Biotin-Phalloidin (10 units/ml) diluted in PBS.

6. Wash cells with PBS - 3 × 5 min.

7. Block cells with 1X Sigma Blocking Buffer for 30 minutes.

8. Wash cells with PBS - 3 × 5 min.

9. Incubate cells with 20 nM QDot Streptavidin Conjugate/1% BSA diluted in PBS for 30 minutes at RT.

10. Wash cells with PBS - 3 × 5 min.

11. Mount with coverslip using 90% glycerol in PBS.
A.2 Staining protocol for dried cells - good

This protocol works after the same principle as the one before, but is has been modified to prepare the cells for drying.

1. Wash cells with PBS - 3 × 5 min.
2. Fix cells with 3.7% formaldehyde/0.1% Triton X-100/PBS for 10 minutes at RT.
3. Wash cells with PBS - 3 × 5 min.
4. Block cells with 2% BSA/0.1% Triton X-100/PBS for 30 minutes at RT.
5. Incubate cells ON at 4°C with 1:20 Biotin-Phalloidin (10 units/ml) diluted in PBS.
6. Wash cells with PBS - 3 × 5 min.
7. Block cells with 1X Sigma Blocking Buffer for 30 minutes.
8. Wash cells with PBS - 3 × 5 min.
9. Incubate cells with 20 nM QDot Streptavidin Conjugate/1% BSA diluted in PBS for 30 minutes at RT.
10. Post-staining fixation with 2.5% Glutaraldehyde/2.0% Paraformaldehyde in PBS for 20 minutes.
11. Wash cells with PBS - 3 × 5 min.
12. Rinse cells with graded ethanols 20%, 40%, 60%, 80% for 5 minutes each. Then rinse with 100% ethanol for 20 minutes.
13. Repeately wash cells with HMDS.
A.3 Staining protocol for dried cells - bad

Same as before but with different drying approach.

1. Wash cells with PBS - 3 × 5 min.

2. Fix cells with 3.7% formaldehyde/0.1% Triton X-100/PBS for 10 minutes at RT.

3. Wash cells with PBS - 3 × 5 min.

4. Block cells with 2% BSA/0.1% Triton X-100/PBS for 30 minutes at RT.

5. Incubate cells ON at 4°C with 1:20 Biotin-Phalloidin (10 units/ml) diluted in PBS.

6. Wash cells with PBS - 3 × 5 min.

7. Block cells with 1X Sigma Blocking Buffer for 30 minutes.

8. Wash cells with PBS - 3 × 5 min.

9. Incubate cells with 20 nM QDot Streptavidin Conjugate/1% BSA diluted in PBS for 30 minutes at RT.

10. (Post-fixation with 4% glutaraldehyde for 8 minutes.)

11. Wash cells with PBS - 3 × 5 min.

12. Rinse cells in 50/50 acetone/methanol for 30 seconds.

13. Let dry.
A.4  Staining protocol for SLXM attempt

1. Wash cells with PBS - 2 × 5 min.
2. Fix cells with 4% formaldehyde for 10 minutes at RT.
3. Wash cells with PBS - 2 × 5 min.
4. Incubate cells with 0.1% Triton X-100 for 5 minutes at RT.
5. Incubate cells with 10 μl biotin -XX phalloidin, diluted in 200 μl of 100 mM Tris-HCL (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 1% FBS.
6. Wash cells with PBS - 2 × 5 min.
7. Incubate with 20 nM QDot Streptavidin Conjugate diluted in PBS for 30 minutes at RT.
8. Wash cells with PBS - 3 × 5 min.
9. Rinse cells in 50/50 acetone/methanol for 30 seconds.
10. Let dry.