SINGLE MOLECULE SPECTROSCOPY: TOWARDS THE STUDY OF INTERFACES IN DIELECTRIC MEDIA

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Chapter 1

Introduction

1.1 About single molecule experiments

The knowledge acquired by scientist about chemical interactions comes, so far, from experiments performed on ensembles of molecules. Measurements on single atoms and molecules can be considered to be, indeed, a major technical breakthrough (take the STM and AFM techniques as a clear example of this).

The now available single molecule experiments, offer the possibility of studying distributions of properties, information that cannot be obtained with the usual (ensemble) measurements. This, by itself, represents a powerful weapon to study the underlying phenomena in systems with the same "average" (macroscopic) response: single molecule techniques may then help to either accept or discard theoretically proposed mechanisms based only on a macroscopic response of a system. A second (and very interesting) feature is that certain types of single molecule experiments (i.e. the spectroscopic ones) also offer the researchers the chance of recording dynamical information (as from the millisecond range) enabling them to study time-dependent behavior of properties with this degree of resolution (i.e. translational and rotational motions, spectral changes, etc. have already been characterized [17, 5, 2]).

As a special case, single molecule detection at room temperature has found echo in many different disciplines as biology, biochemistry and materials science [17]. An important area related to these is the one concerning the study of the orientations of dipoles of molecules. Changes in the environment can cause a dipole to be reoriented; lifetimes and quantum yields can be affected (and, therefore, dynamics followed on a truly molecular scale). Effectively relating changes in observables to dipoles’ orientations represents, therefore, an important task; it is this main task what constituted the final goal of the present work and that will be briefly reviewed and analyzed through the following sections.

1.2 Problem under investigation

In this work, we intended to perform orientation imaging and to study lifetimes of fluorescent species (up to the single molecule level) when these species are placed near interfaces between dielectric media. Lifetimes are expected to be greatly affected by boundary conditions, being the orientation of the dipoles and distances to the interface main parameters [3, 21, 17].

In the following sections, the problem of interest as well as some of the most relevant investigations carried out on the subject will be summarized. A new experimental approach applied to the problem will be introduced and its potential advantages discussed.
1.3 About interfaces and molecules therein placed

What happens when we study some material medium and, abruptly, the properties \((\varepsilon, \mu)\) of the medium change? For example, let us think of Maxwell’s equations; those were meant, in principle, for media with continuous properties. As might be expected, when these properties change abruptly \(\mathbf{E}, \mathbf{H}, \mathbf{B}\) and \(\mathbf{D}\) also do so \([24, 9, 14]\). When we consider Maxwell’s equations for a section in the interface between two media (as depicted in fig. 1.1) it is possible to find that \([14]\):

<table>
<thead>
<tr>
<th>Vector</th>
<th>Component</th>
<th>Behavior at boundary</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mathbf{E})</td>
<td>Normal</td>
<td>Discontinuous</td>
</tr>
<tr>
<td>(\mathbf{E})</td>
<td>Tangential</td>
<td>Continuous</td>
</tr>
<tr>
<td>(\mathbf{B})</td>
<td>Tangential</td>
<td>Discontinuous</td>
</tr>
<tr>
<td>(\mathbf{B})</td>
<td>Normal</td>
<td>Continuous</td>
</tr>
</tbody>
</table>

It is reasonable to think, then, that any property directly dependent on either \(\mathbf{E}\) or \(\mathbf{B}\) may also show a discontinuity at the interface.

As an example, let us consider lifetimes \(\tau\) (or decay rates, \(P = \tau^{-1}\)) of molecules. How are fluorescent molecules affected when they are placed in (or very close to) an interface between two very different media? Let us picture a fluorophore as a radiating point dipole with a transition dipole moment \(p_0\) in a position \(r_0\) at a distance \(d\) from the interface. The total decay rate can be divided into a non-radiative contribution \(P_{nr}\) and a radiative one (electromagnetic process) \(P_{em}\) \([21, 13]\):

\[
P = \tau^{-1} = P_{nr} + P_{em}
\]

(1.1)

The electromagnetic contribution can be calculated through the integration of the Poynting’s vector over a surface enclosing the dipole. The latter can be reduced to the following \([21, 13]\):

\[
\frac{P_{em}}{P_0} = 1 + \frac{6\pi\varepsilon_0\varepsilon_1}{p_0^2k_1^3} Im[p_0^*E_{br}(r_0)]
\]

(1.2)

where:

- \(E_{br}\): back-reacted electrical field at the dipole’s position (defined as the difference of the expected field in an homogeneous medium and the one in the presence of an interface); \(k_1\) represents the wave vector of the emitted radiation in medium 1.
As could be easily foreseen, discontinuities in $E$ would affect $P_{em}$ directly: in case the dipole were parallel to the surface $P_{em}$ would remain continuous while it would be discontinuous for the perpendicular case (see fig. 1.6).

Molecules (their lifetimes, in this case) would be acting as a sensitive probe for the proximity to the interface, being their response also dependent on their orientation (assuming all other affecting factors, such as the environment, could be clearly discriminated, or their contributions considered negligible).

The study of this type of systems has already been tackled and some results have come up. Some of these will be briefly summarized in the following section and, from them, further activities proposed.

### 1.3.1 Previous investigations carried out on the subject

There exist a certain number of investigations carried out on related systems (i.e., those by M. Kreiter, R. Vallée and J.J. Macklin [3, 21, 19]) which lead to results in concordance both among them and with theoretical predictions. When reviewing these studies, two of them appeared of particular interest: the early works of J.J. Macklin et al. [3] and M. Kreiter [21].

The work done by Macklin et al. [3] deals with room-temperature optical properties of single dye molecules located in a polymer-air interface. Shifts in the fluorescence spectrum (due to the varying environment) and the orientation of the transition dipole moment were correlated to variations in lifetimes. In particular, the lifetime dependence on the dipole’s orientation was found to be a consequence of the electromagnetic boundary conditions at the polymer-air interface. The samples studied consisted of randomly oriented $DiIC_{12}(3)$ molecules in a 20nm-thick PMMA film on a quartz coverslip, prepared by spin-coating. Molecules were characterized by their excited-state lifetime $\tau$ and, from their spectra, with their peak fluorescence wavelength $\lambda_p$. Comparison was made of the results for a simple polymer/air interface with the ones obtained for a polymer/index-matching oil one and a polymer/polymer one.

In this work, the authors managed to disentangle the different aspects of the problem, relating the changes of the spectra to the varying molecular environment and the pronounced changes in lifetimes to the dipoles’ orientation and the boundary conditions imposed by the proximity of an interface.

The results (depicted in 1.3) are indeed striking. Lifetimes of single molecules are plotted as a function of the peak wavelengths of their spectrum; when considering molecules placed in the polymer-air interface (B), the dispersion of the measured lifetimes is found to be approximately 1 ns, while for the other systems (where molecules are well-immersed in the polymer or oil) that value is greatly reduced. Changes in lifetimes related to variations in the spectra are neglected (see figs. 1.2 and 1.3: molecules with similar spectra - and $\lambda_p$ - show clearly different lifetimes); though no direct measurement of the orientation of dipoles was made here, such a distinctive difference among the measured lifetimes is attributed to the dipoles’ orientations near to the polymer-air interface.

The work carried out by M. Kreiter et al. [21] studied the lifetimes of single DiI molecules in a 20 nm PMMA film deposited on glass, as function of the orientation of the molecules relative to the polymer/air interface. It is found in this work that the lifetimes of the analyzed molecules follow the general expected trend (longer lifetimes for larger out-of-plane angles); significant deviations are also found, which are said to be related to the inhomogeneous environment sensed by the molecules.

This work deals with the study of dipoles’ orientations and, when doing so, introduces a distinctive methodology: the annular illumination scheme applied in the confocal microscope used to perform the measurements, [12, 21].
Figure 1.2: Measurements performed on two single molecules. Fig. B and C correspond to A; E and F to D [3]

Figure 1.3: (A) Fluorescence lifetimes versus peak emission wavelength for molecules spin-coated on PMMA, and overcoated with several micrometers of either immersion oil or PMMA (index-matching materials). (B) PMMA-air interface
This scheme, when properly optimized, leads to an electric field distribution in the focus in which the three fundamental cartesian field components are of similar strength, but present very different distribution (or "patterns") (see 1.4) [12, 21]. The distinctive pattern produced by a dipole located in the focal region of the microscope, when interacting with the electric field there present, turns out to be a combination of the three fundamental ones (see fig. 1.4). A proper fitting procedure performed with theoretically predicted and measured patterns makes the determination of dipoles’ orientation possible [12, 21]. This was the case, for example, depicted in fig. 1.5. There, the orientation of molecules (more precisely, the out-of-plane angles of dipoles) was measured according to this procedure and related to measured lifetimes [21]. As expected, it was found that molecules closer to the surface (higher $\theta$ in the picture) exhibit higher decay rates.

As it has been shown through the previously quoted works, the response of molecules near interfaces has been studied with some detail and an overall idea of it has been established. The proximity of the dipoles to an interface between two very different media plays a major role in giving rise to very distinctive lifetimes of the molecules according to their orientation. In the work by Macklin et al. [3], the two very clear extremes were studied: molecules in the polymer/air interface (where a discontinuity is present and lifetimes of molecules are effectively affected) and molecules in a polymer/polymer or polymer/oil interface (see fig. 1.3) -where the fluorescent species could be considered to be far from a high/low index media interface- where the expected, orientation-independent bulk values are recovered [21] (see fig. 1.6).

When considering the results of the calculations (see fig. 1.6) presented by Kreiter et. al [21], it is possible to notice the very clear discontinuity in the calculated electromagnetic decay rates for dipoles oriented perpendicularly to the polymer/air interface. Calculations, as such, can predict that kind of response, but measurements could be distant from that. If interfaces could be tested on the nanometer scale, it should be possible to measure clear differences in lifetimes for different dipoles’ orientations. Although no sharp discontinuities would be expected from measurements (most probably, a rather smeared response would be detected), the possibility of studying such behavior on this small scale applying single molecule techniques is a most interesting one, and even more so when considering that the only available

Figure 1.4: Left: images obtained using the annular illumination scheme for two (perpendicular) polarization states. Right: Measured lifetimes for two molecules (A and B) with different orientations [21].
Figure 1.5: Fluorescence decay rates of DiI as a function of the relative position to the normal of the surface [21]

Figure 1.6: Calculated fluorescence decay rates (electromagnetic contribution) as a function of the distance to the polymer/air interface, for a perpendicular dipole and a parallel one [21]
information is related either to the study of molecules randomly dispersed in thin, spin-coated polymer films [21, 19] or to the comparison of the extreme cases of the polymer/air interface and the polymer/polymer one [3].

1.4 New experimental approach for the system under investigation

It is possible to build planar systems as the ones studied here using techniques other than the traditional spin-coating method.

The layer-by-layer method [4, 11, 15], is a general approach for the fabrication of multi-component films on solid supports. The method, first published by Decher [4, 11, 15], allows for the building of very thin (i.e. 10 nm [20]), smooth layers of defined molecular architecture, regardless of the substrates shape and topology: electrostatic attraction is the driving force for the molecular build-up.

A solid substrate with a charged surface is immersed in a solution containing an oppositely charged polyelectrolyte; as a result, a thin layer is adsorbed on the surface. Once the layer is built-up, some ionic groups may remain exposed to the solution, reversing the initial surface charge and allowing for the following deposition of a second layer of a different polyelectrolyte of opposite charge (the sample is, between steps, thoroughly rinsed). It is by repeating this process that it is possible to deposit a precise number of layers of well defined characteristics [4, 11, 15, 20, 1].

A schematic representation of this process is depicted in fig. 1.7; here, the polymers used are PSS (natrium polystyrenesulfonate, providing the polyanion when in a water solution) and PAH (poly (allylaminehydrochloride), providing polycations); nevertheless, other materials could be used as long as the basic charge-reversal condition is met. Films obtained through this procedure present a well defined layered structure, determined by the deposition sequence [4, 11, 15].

The result of applying such a method (with a given set of experimental parameters) is depicted in fig. 1.8 [20]. Studying X-ray reflectivity measurements it is possible to determine layers’ thicknesses: the case depicted in fig. 1.8 clearly shows that these can be within the nanometer range.
Figure 1.8: a) Small angle X-ray reflectivity spectra of samples composed of 1-2, 2-5 and 3-7 bilayers: studying these fringes ("Kiessig fringes"), it is possible to determine their thickness. b) Thickness as a function of the number of bilayers. [4]

The highly controlled layer growth and the possibility of placing fluorophores with nanometer precision in a well-known layered structure make this method very interesting for exploring the behavior of fluorescent species in the vicinities of interfaces. The previous approaches (spin-coated films containing randomly oriented molecules [21] or with molecules deposited over them [3]) did not reach the degree of accuracy achievable through the use of the layer-by-layer method.

1.4.1 Main activities - Summary

The main activities carried out (concerning experiments) consisted in the search for proper procedures for obtaining "clean" (free from fluorescent species) samples, employing the layer-by-layer method to build them. Particular interest was set on clean polyelectrolytes’ obtention. In order to make single molecule experiments feasible, proper fluorophores’ deposition on the samples was a second condition to be met. Thorough descriptions of both tasks can be found in the chapter devoted to the experimental part of the work (Chapter 2).

A separate activity (concerning the study of the theoretical aspects of the problem) was related to the calculation of electric field distributions in the focal area of a microscope (of fundamental importance for proper orientation calculation) was also tackled. Special emphasis was made to optimize the relevant variables of the system to experimentally achieve field distributions that allowed for the proper sensing of all of the dipoles’ orientations.

The orientation imaging concept and the theory related to the calculations is described in Appendix A; results of the different tests can be found in the Chapter 3, devoted to the general outcomes of the experiments carried out.

Finally, and as a consequence of a measurement-related problem, a characterization of the detectors present in device (a confocal microscope, see Chapter 2) was also carried out both to ensure the quality and the optimization of the measurements to be performed on single molecules. Details about these activities can be found in Chapter 3.
Chapter 2

Experimental

This chapter is meant to deal with the procedures, materials and devices used in the experiments.

With respect to samples, special emphasis is set in the removal of fluorescent species from the polyelectrolytes’ solutions, a fundamental requisite to be met for the type of single molecule measurement intended.

When dealing with the measurement-related issues, the device (a confocal microscope) is introduced, along with its working principle and main technical features.

2.1 Measurements

The samples were studied using a scanning confocal microscope with an epi-illumination scheme (where the excitation applied and the fluorescent signal collected both go through the sample), under two different excitation conditions: full-beam illumination (FB) and, particularly, annular illumination (AI) (such illumination scheme is obtained by interposing a disc of proper size in the excitation beam).

Two different pulsed light sources were used to perform imaging and lifetime measurements (a laser diode, $\lambda$:633 nm and an Ar-ion laser, $\lambda$:515 nm; further details about these sources can be found in Chapter 2); also, accordingly to the excitation, different dyes were employed.

The excitation power was set in most cases to 0.5 - 1 $\mu W$ and collection times to 3 - 5 ms. Typical images consisted of 200 pixels per line in a scanning range of 10 $\mu m$; all samples were exposed to a nitrogen flow while being scanned.

2.2 Sample preparation

The sample consisted of a thin-glass substrate, coated with polyelectrolyte layers and fluorophores deposited either on top or between layers (intended to study the behavior in the interface or very close to it, with nanometer precision).

The basic sample preparation procedure consisted of three steps: substrate’s treatment, layer growth and fluorophores’ attachment. An extra step would be added in case it were necessary to deposit an extra layer on top of the fluorophores; still, the basic procedures remain unaltered.

2.2.1 Substrate’s surface treatment

This procedure consists of several steps, all aimed to obtain a clean, positively charged substrate.

First, a thin glass substrate (0.13 - 0.16 mm glass coverslips, Menzel-Glaeser N1) is heated in air for at least 2 hours at 500°C to ensure that the surface is free from organic compounds. Afterwards,
the glass substrates are submerged in a $H_2O_2 : NH_3 : H_2O$ (1:1:5) solution for 30 minutes at 80 °C, carefully cleaned with purified water and dried with $N_2$. The substrates, now in a teflon holder, are placed in a clean glass container and extra-dried for about 10 min at 130 °C. The silane agent ((3-Aminopropyl)triethoxysilane, or "3-APTES") is put then in a smaller glass container near the samples; the whole device is sealed and left for 3 hours in an oven at 130 °C (this process is to be known as silanization). The now silanized substrates are extensively rinsed with high purity ethanol (to remove the remaining silane from the surface) and with purified water (in that order), dried with $N_2$ and stored for further polymer deposition.

Fig. 2.1 shows (schematically) the reactions involved and the outcome of the process.

2.2.2 Layer growth

The layer growth procedure consists of a cyclic attachment of a polyanion and a polycation from aqueous solutions. The deposition process is repeated until the desired number of layers is reached (a certain thickness achieved, [20]). The samples are, between steps, carefully rinsed with ultra-pure water and, at the end, dried with pure $N_2$ before storing.
In the present work, the polymers employed for the layer-growth were sodium-poly(styrenesulfonate) (which behaves as a polyanion and will be simply referred to as "PSS") and Poly (allylaminehydrochloride) (or "PAH", which behaves as a polycation, see fig. 2.2). The time assigned for each deposition step was set to 20 minutes; typical samples were prepared depositing two or three bilayers (a bilayer consisted of two layers: a PAH one and a PSS one).

Table 2.2.2 shows a summary of the contents of the solutions prepared to build-up the layered system; the results obtained from these experimental conditions had already been characterized and were reproduced in all of the experiments [20]. The chemicals employed to prepare such solutions can be found in the following table.

<table>
<thead>
<tr>
<th>PAH solution</th>
<th>PSS solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_2O$: 50 cm³</td>
<td>$H_2O$: 50 cm³</td>
</tr>
<tr>
<td>$PAH$: 0.0935 g</td>
<td>$PSS$: 0.2070 g</td>
</tr>
<tr>
<td>$NaBr$: 10.29 g</td>
<td>$MnCl_2$: 4.0490 g</td>
</tr>
<tr>
<td>$HCl$: 0.5 ml - 0.1 N</td>
<td>$HCl$: 0.5 ml - 0.1 N</td>
</tr>
</tbody>
</table>

Table 2.2.2 - Contents of the polyelectrolytes’ solutions

Chemicals employed:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Purity</th>
<th>Concentration</th>
<th>Obtained from</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_2O$</td>
<td>High</td>
<td>25</td>
<td>Milli-Q System</td>
</tr>
<tr>
<td>$NH_3$</td>
<td>Analytical Reagent</td>
<td>35</td>
<td>Riedel - de Hen</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>Analytical Reagent</td>
<td>99 +</td>
<td>Aldrich</td>
</tr>
<tr>
<td>$NaBr$</td>
<td>Analytical Reagent</td>
<td>99 +</td>
<td>Aldrich</td>
</tr>
<tr>
<td>$HCl$</td>
<td>Analytical Reagent</td>
<td>0.1 N</td>
<td>Fluka</td>
</tr>
<tr>
<td>Ethanol</td>
<td>HPLC grade</td>
<td>65</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>$HNO_3$</td>
<td>Analytical Reagent</td>
<td></td>
<td>Riedel - de Hen</td>
</tr>
<tr>
<td>$PAH, M_w : 70,000$</td>
<td>Analytical Reagent</td>
<td></td>
<td>Aldrich</td>
</tr>
<tr>
<td>$PSS, M_w : 70,000$</td>
<td>Analytical Reagent</td>
<td></td>
<td>Aldrich</td>
</tr>
<tr>
<td>$3 – APTES$</td>
<td>min 98</td>
<td></td>
<td>Aldrich</td>
</tr>
</tbody>
</table>

Chemicals employed in the solutions’ preparation

The polyelectrolyte solutions were used several times for the making of samples. As long as the cleanliness’ conditions were maintained, and the concentrations of species were high enough not to be affected by the repetitive use, there seemed to have been no signs of problems with using them more than once. The already acquired experience on the system [20] also showed no alteration of the expected linear behavior of the thickness’ growth upon the number of deposited layers [4, 11, 15].
Contamination

The presence of unknown fluorescent species in the polymers’ solution lead, in most cases, to non-useful samples.

The problem can appear under two basic forms: high background signals (caused by high concentrations of fluorescent contaminants present in the polyelectrolyte solutions) or isolated molecules or groups of contaminating fluorescent species. The latter, though less severe than the first one, still represented an important problem (it could affect the statistics intended to be performed on lifetimes when mistaking fluorophores by contaminant species).

Two basic approaches came up to deal with the ”contamination problem”1: effective removal of fluorescent contaminant species by purification (of polymers, salts, etc.) and the isolation of the fluorescent signal produced by the fluorophores.

Provided the first strategy is related to samples’ preparation, it will be dealt with through the following section; the discussion of the second one is to be carried out through further ones (see Section 3.X).

Clean chemicals’ obtention

Both polymers used in this work (PSS and PAH), even though obtained in the highest commercially available purity, contained unknown species that fluoresced when using either the 633 nm or the 515 nm lasers and needed, therefore, further purification before they could be used in single-molecule experiments.

In the case of PAH, the approach to a non-specific cleaning tested was a dialysis. An aqueous solution of the polymer was prepared with purified water and dialyzed during, at least, three days (renewing water between 3 and 4 times a day). To that purpose, a regenerated cellulose membrane with a molecular weight cut-off (MWCO) of 3,500 (Spectra/Por 6, Spectrum Labs) was used (see [20, 8]). The MWCO of the membrane was well below the maximum recommended limit (half the mean molecular weight of the polymer, 70000 in the case of PAH), which ensured no significant loss of polymer molecules. After the dialysis, all of the remaining components of the solution (according to 2.2.2) were added and mixed. The final product was filtered (using 0.2 µm filters), stored and tested.

This procedure showed to be very effective in removing fluorescent species; results can be found in Chapter 3.

When dealing with PSS, two different methods were tested. The first one, a dialysis, was carried out on similar conditions to the one described for PAH, but two membranes with different MWCO were tested (3500 and 12000, Spectra/Por 6, Spectrum Labs). Both attempts gave negative results; that is, they were not effective in removing the contaminants present in the PSS solution. As an alternative, a controlled precipitation method was applied: the polymer was dissolved in ultra-purified water and the solution drop-by-drop transferred into high-purity ethanol, close to its freezing temperature (-50 °C / -60 °C). The precipitated polymer was then filtered and dried in vacuum.

This procedure, though no completely effective, showed to be efficient in reducing the fluorescent background signal (see Chapter 3).

It was also found during this purificatiob process that the degree of dissolution of the polymer was a major factor affecting the cleanliness of the system: the more diluted the PSS solution was, the higher the final cleanliness obtained (typically, 300 mg in 50 ml of ”Milli-Q” water produced satisfactory results).

1The ”ideal” approach would consist of a combination of both strategies (assuming none of them would be completely effective)
Additional considerations for clean samples’ obtention

All the glassware (meant either to prepare or to store solutions) was thoroughly cleaned using aqua regia (a mixture of concentrated hydrochloric (0.1 N) and nitric acids (65 percent), in a 3 to 1 volume ratio) before use. This highly oxidizing mixture is one of the strongest known and proved to be efficient for these experiments. After cleaning the glassware with this strong acid mixture, it was extensively rinsed first with distilled and then with ultra purified water.

The cleaning procedure was applied not only to the glassware, but also to the teflon sample holders. In case it were necessary to dry any of the parts, they would be rinsed with high purity ethanol and dried with nitrogen, taking special care in not touching anything (i.e., holding the parts with clean tweezers, etc.).

2.2.3 Attachment of fluorophores

The fluorophores were the molecules of interest on which to perform orientation imaging and lifetime measurements. These molecules were meant to: give strong fluorescent signals (which, combined with a low background, would form the ideal system to study the characteristic patterns when applying an annular illumination scheme in the microscope) and be specifically located either in an air-polymer interface or very close to it (with nanometer precision), in order to study its direct influence.

The dyes, set in an aqueous solution at very low concentrations (on the order of $10^{-10}$ M), were electrostatically attached to the samples when these were dipped into the solution (see fig. 2.3). A typical "recipe" for attaching them consisted of dipping the samples for about 1 minute in the solution, rinsing them thoroughly with highly purified water and drying them with nitrogen.

Different compounds were used according to the excitation wavelength (633 nm or 515 nm); these were: DiIC$_1$(5), Alexa Fluor 633 or Rhodamine 6G.

The structure of the first tested compound (1,1,3,3,3,3- hexamethylindocarbocyanineiodide, or DiIC$_1$(5)), suitable for the 633 nm measurements, is depicted in fig. 2.4. It is positively charged when in solution, which means that it is necessary to end the layer growth process in a PSS step (negatively charged) in order to be able to attach it to the surface.

The second compound, intended also for the 633 nm measurements, was Alexa Fluor 633, in its azide form (Molecular Probes Inc.). This compound proved to be attachable both to PSS and a PAH layers (due to a secret, there is no data available on its structure).

The third compound, suitable for the 515 nm measurements, was Rhodamine 6G ($C_{28}H_{30}N_2O_3.HCl$). As it is possible to see from its structure (depicted in fig. 2.5), it is also positively charged when in solution and can be attached to a PSS layer.

![Fluorophore attachment scheme](image-url)
Results on the performance of each dye can be found in Chapter 3.

2.3 Measurements: the confocal microscope

The set-up used to perform the measurements is described through this section. It is a home-built confocal microscope: such a device allows us to perform single molecule fluorescence experiments of the kind needed.

Lifetime and spectra measurements at the single molecule level are some of the most interesting features of this device [16]; its general characteristics (and capabilities) will be briefly summarized through the following section.

2.3.1 Working principle in a confocal set-up

In this kind of set-up, light goes through an aperture pin-hole (providing a point-like source) and an objective, is focused and illuminates a sample one point at a time (see fig. 2.6). At the same time, light can be collected from the focal spot using either a second objective or the same one. A second aperture pin-hole (conjugated to the focus of the lens) acts as an effective blocker for the light coming from points other than the focus (see fig. 2.6, 2.7).

There are two basic optical arrangements for this type of device: one of the designs needs only one lens (see fig. 2.7) and the other one (the symmetric one, depicted in fig. 2.6) needs two of them. When it comes to resolution, both are equivalent, but the first one is of much simpler alignment and operation. In the case of fluorescence measurements, the excitation and fluorescence signals are split by means of a dichroic mirror, avoiding the problem of the loss of brightness related to this kind of configuration and the use of beam splitters [16], turning this the most suitable for this application.

Marvin Minsky [10] was the creator of this type of configuration for a microscope that makes it possible to drastically reduce the amount of light in the sample (very important to prevent photo bleaching in single-molecule fluorescence experiments) without reducing the brightness at the focal point.
As seen so far, light can be directed to (and collected from) one point of interest; full images are built when scanning areas of interest. There are two basic ways of scanning: moving the sample across a fixed focal spot or scanning a focused beam across a stationary sample. The first option is the one present in the device used; though slower, it produces undistorted, high quality images (the optical path remains stationary).

### 2.3.2 Technical features

The main technical features of the device will be briefly named (and, in the relevant cases, more carefully described) throughout this section. The order in which the different components are introduced to the reader follows the light-path in the device, from the sources to the detectors (see fig. 2.8).

The first elements to consider are the light sources; table 2.3.2 shows a summary of the available ones.

<table>
<thead>
<tr>
<th>Source</th>
<th>(\lambda) [nm]</th>
<th>CW/PS</th>
<th>FWHM [ns]</th>
<th>f [MHz]</th>
<th>P [mW]</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon-ion laser</td>
<td>460</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Innova C90 Coherent Inc.</td>
</tr>
<tr>
<td></td>
<td>488</td>
<td></td>
<td></td>
<td>0,2</td>
<td>68</td>
<td>Mode locker: PulseDrive APE GmbH.</td>
</tr>
<tr>
<td></td>
<td>515</td>
<td>CW, PS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>He-Ne laser</td>
<td>633</td>
<td>CW</td>
<td></td>
<td>10</td>
<td></td>
<td>Uniphase inc.</td>
</tr>
<tr>
<td>Laser diode</td>
<td>633</td>
<td>PS</td>
<td>0,2 - 0,8</td>
<td>50</td>
<td>0,1 - 2,5</td>
<td>Becker Hickl GmbH BHL-150</td>
</tr>
<tr>
<td>Xenon-arc lamp</td>
<td>300 - 800</td>
<td>CW</td>
<td></td>
<td></td>
<td>15000</td>
<td>OSRAM XBO150</td>
</tr>
</tbody>
</table>

Table 2.3.2 - CW: Continuous wave, PS: pulses, FWHM: full-with half-maximum, f: frequency, P: power

The highlighted items correspond to the sources used in the experiments; the characteristics of each one will play an important role, as will be seen in Chapter 3.

The next element found in the scheme of the microscope (see fig. 2.8) are the \(\lambda\) plates. These plates (\(\lambda/2\) and \(\lambda/4\), OWIS GmbH) are placed before the coupling of the light into the optical fiber and can be adjusted to compensate the polarization effects of the fiber (the available ones were suitable for 633 nm and 515 nm). These devices are of high relevance for the intended experiments because they enable us to control the polarization state of the excitation light reaching the sample.
Figure 2.7: Illumination and collection through the same objective [10]

Figure 2.8: The confocal set-up (adapted from [16]). F: filters, L: lens, PH: pin-hole, D: diaphragm, d: disc, BS: beam splitter, M: mirror, SPG: spectrograph, AMP: amplifier, OSC: oscilloscope
Following the light-path, several other elements are found: lenses, filters and optical fibers. Lenses are used to focus or collimate light at different stages (many of these, of different focal lengths, are found throughout the device). As a common characteristic, the lenses used are meant to be achromatic and can, therefore, be used for different wavelengths.

Within the mentioned group of elements, we find different filters: line ones (to better guarantee the selection of the wavelengths of interest for excitation from the sources), notch and long-pass ones (to separate excitation from fluorescence signals), and gray ones (to adjust the intensity reaching the sample).

When considering optical fibers (which acted as point-like light sources in the confocal microscope), two types were found ready to be used in the experiments: single-mode ones (for 635 nm or 515 nm) or a photonic-crystals endless single-mode one (suitable for several wavelengths). The light transmitted by means of the them (a pure Gaussian profile) is collimated with an spherically-minimized aberration lens; the illumination beam reaching the objective could be considered to be formed by plane waves.

Before reaching the sample (and after going through a series of diaphragms meant for proper alignment of the beam), the excitation beam may encounter a disc. The disc, allows the researchers to apply annular illumination to the samples by blocking the inner part of the beam (the disk can be changed for different size ones and different illumination conditions, achieved).

The beam is directed into the objective by means of a dichroic mirror (the same mirror is used to separate the excitation from the fluorescence signal). The objective, mounted in a XYZ-stage for proper alignment, is a wide numerical aperture (NA = 1.4), oil-immersion one and is used to focus the beam on the sample and to collect the fluorescence signal [16] coming from it. Its maximum focusing angle can be calculated from the NA and the refractive index of the medium (n_{glass} = 1.5), that is, \( \theta_{\text{max}} = 68.6^\circ \).

Once the elements found along the path of the excitation beam have been described, the scanning process and the devices involved are to be named and their functions explained.

The scanning-process can be described as follows: the sample is placed in an initial position, stepwise forth and backward moved (a definite number of steps) along a "X" direction and up to a given extent (set by the scanning range); afterwards, the sample is moved in the "Y" direction and the line-scanning process is repeated. As each line is stepwise scanned, photons are counted for a given time period (set beforehand and fixed for each new measurement) at each step and are to form one pixel of the image.

Two fundamental devices are found here: the piezoelectric stage (Jena Piezosystems GmbH TRITOR 101 CAP) and the 15-bit AD/DA (Analog to Digital/Digital to Analog) converter (Jaeger GmbH ADW in-light-16). The AD/DA generates the signals controlling the piezoelectric stage (the controlling signals already being properly adapted by means of low-noise amplifiers, Jena Piezosystems GmbH ENV40CAP) and performs data acquisition (the position, counts, starting and finishing times of each pixel in a line are stored in the local memory and then transferred to the computer).

The minimum step is limited by the AD/DA converter and set to 2.4 nm, the pixel time resolution to 25 ns [16]. The maximum scanning range is 80 \( \mu \text{m} \).

While the sample is being scanned (with the excitation beam focused onto it), the fluorescence signal is collected through the sample by the objective, separated from the reflected beam by means of a dichroic mirror and the use of notch and long-pass filters (Kaiser Optical Systems Inc. Notch Plus, and Omega Optics Inc.), directed and focused towards the pinhole (150 \( \mu \text{m} \), New Focus inc.), and then, directed towards the detectors.

The available detectors are: an APD (Avalanche Photodiode), a PMT (Photomultiplier Tube) and
Figure 2.9: A: scheme of the method, B: example of photon-arrival times measurement

A transmission grating spectrograph. Both APD and PMT can be used for imaging and time-correlated measurements. A common characteristic to this type of devices is their quantum efficiency. Although they are, indeed, very sensitive, this characteristic can play a major role in the measurements to be carried out and must be considered before running an experiment. The following table summarizes some known values [16]: note the particularly low response of the PMT for the longest wavelengths.

<table>
<thead>
<tr>
<th>λ[nm]</th>
<th>Efficiency APD</th>
<th>Efficiency PMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>500</td>
<td>48</td>
<td>40</td>
</tr>
<tr>
<td>600</td>
<td>66</td>
<td>40</td>
</tr>
<tr>
<td>700</td>
<td>73</td>
<td>13</td>
</tr>
<tr>
<td>800</td>
<td>61</td>
<td>0</td>
</tr>
</tbody>
</table>

Detectors efficiency. Highlighted values correspond to intervals of interest for the 633 nm excitation measurements.

The available spectrograph enables the user to measure single molecule spectra. It consists of a volume phase holographic transmission grating (Kaiser Optical Systems inc., HVG-590, HVG-690), a photo objective (Nikon inc. 35 mm, f1.4) and a CCD (Charge-coupled device) camera (PCO Imaging GmbH SensiCam QE). The collimated beam is directed towards the grating, where light is diffracted. The objective focuses the outgoing light onto a line of the CCD camera. With the help of a calibration procedure, it is possible to measure spectra very accurately.

Once imaging and spectra measuring devices and methods have been described, time-related measurements are to be explained. The Time Correlated Single Photon Counting (TCSPC), based on the detection of single photons and the computation of their individual detection times, allows the researchers to measure excited state lifetimes. Molecules are excited with light pulses and the photons consequently emitted, detected. In this type of experiment, for each detected photon the time from the beginning of the experiment (macro-time, "Mac-T") and from the last excitation pulse (micro-time, "mic-T") are determined (see fig. 2.9). In order to do that, the device needs only two inputs: the pulse-train signal from the laser and the detector signal. If the laser pulses are narrow enough (compared to the decay times), it is possible to obtain the fluorescence decay curve by making a histogram with the detected "mic-T" times. As expected, it is important to keep in mind that the excitation light used should be pulsed at such frequency that the excited state is allowed to decay in between pulses.

The available device (Becker und Hickl GmbH, SPCM 630) can measure mic-T times with a resolution of 12 ps up to a rate of 8 MHz and mac-T times with a resolution of 50 ns [16, 18].
Chapter 3

Results

The most relevant results of the experiments carried out as well as the difficulties encountered will be described throughout this chapter.

3.1 Preliminary results: dyes on glass

The samples were studied using a scanning confocal microscope with an epi-illumination scheme (where excitation is applied and the fluorescent signal collected through the sample), for two different conditions: full-beam illumination (FB) and, particularly, annular illumination (AI) (see fig. 3.2). Fig. 3.3 depicts samples of images where these illumination schemes have been applied; the one on the left corresponds to an image obtained under FB and the one on the right, to AI one (the reader may observe the characteristic patterns produced when using this illumination scheme). It is also possible to note in this last picture that some of the patterns may appear as if truncated; some others, as if parts of them where missing. In the first case, the effect is due to effective bleaching of the dye; in the second case, it can be explained by dynamic-blinking of the molecule. Both phenomena are characteristic of this kind of single molecule experiments [17].

These last images, though obtained for a simple system (dyes on silanized glass) are useful to depict the kind of images we are to obtain when studying the real samples.

When looking at these simple samples, spots (under FB) and patterns (AI) were easily identified and single features detected without doubt. Several inconveniences arose, though, when studying the layered system, most of them due to contamination (providing either a decrease in the signal to noise ratio, the presence of single contaminant features or both), irregular fluorophores’ attachment, low efficiency of the detectors for the long-wavelength range and low power sources. An extra device-related issue was added to these problems, when considering time-dependent measurements.

All the problems found and their proposed solutions will be dealt with in the following sections.

3.2 Contamination in the samples

The presence of contamination in the samples can give rise not only to low signal-to-background ratios, but also to the chance of mistaking contaminant species by fluorophores.

One basic strategy to deal with the contamination problem consists of the proper cleaning and purification of all the elements involved in the samples’ preparation. The results of these efforts are depicted through the following sections.
Figure 3.1: Scheme: microscope and sample

Figure 3.2: Illumination schemes (A): Full-beam ("FB") (B): Annular ("AI")

Figure 3.3: Images obtained for Alexa 633 on silanized glass.(A): FB and (B): AI - Intensity scales were altered for clarity purposes.
3.2.1 Cleaning of chemicals

The only (and ultimate) test for checking the cleanliness of the process consisted of taking a sample to the microscope and observing it carefully.

In order to test the cleanliness the polymers after their purification, the only mechanism to trust was the electrostatic deposition of one or layer according to the standard layer-growth procedure. The same material, tested using a drop of solution on a cover glass could show significantly lower amounts of contamination than an electrostatically deposited layer (which also denoted the charged nature of the contaminating species).

3.2.2 Excitation: 633 nm

At no point it was possible to obtain completely clean samples, but it was possible to reduce by orders of magnitude the amount of contamination, just by following the purification procedures proposed in Chapter 2.

Fig. 3.4 shows results for 2 sets of samples; the first picture (A) was made on a sample where only one PSS layer deposited, the second one (B) consisted also of one PSS layer, but where the dissolution of PSS in the purification method had been increased. Picture (C) corresponds to a sample where 1 bilayer had been deposited; picture (D) correspond to a sample consisting of 2 bilayers and 1 extra PSS layer, plus the addition of DiI molecules. (B) and (C) and (D) were made under the same experimental conditions. It can be seen from (B) and (C) that the PAH solution did not seem to add a significative amount of contaminant species to the sample (its cleaning procedure could be considered to have been effective). Picture (D) is meant to compare the the signals produced by contamination (as in the case of (B) and (C)) and fluorophores. It is possible to note that single fluorophores and contaminant species can reach similar signal-to-background ratios, making of their discrimination a complex process.

The test samples here considered were also simple ones, only 1 or 2 bilayers were considered. Problems increase significantly with each added layer, and this call for effective cleaning (or signal filtering) procedures.

Fig. 3.5 shows results for a typical 2-bilayers’ sample, with fluorophores (DiI). (A) was obtained under FB illumination; it is possible to see in the profile a common signal-to-background ratio for a fluorophore. (B) depicts an image made over the same scanning range, with the same measuring parameters, but applying AI. Although fluorophores’ density is too high to give rise to clear images, it is possible to see in the profile how signal-to-noise ratios fall; making of the low-background issue, a very important one.

Signal filtering

In order to avoid the collection of data of fluorescent species other than fluorophores, a second option to chemicals’ purification appeared: the isolation of the signal produced by the fluorophores. The basic underlying idea for this approach was the following: if contaminants showed significantly different spectra from the fluorophores, their signals could probably be removed by the use of proper filter sets and; therefore, the signal produced by the fluorophores, isolated.

The single-object measurements performed using the spectrograph showed no signals at all in the measuring range of the device (350 nm - 750 nm). This procedure was repeated several times obtaining the same results. Having checked the proper alignment and calibration of the system, it was assumed that (at least) the molecules investigated had emission peaks well above 750 nm.

This was also evident when the system was tested with the available long-pass filters (695 and 665 nm, RG-695, RG-665, Schott Glass) (see fig. 3.6): most of the single features present when the system was tested without the use of filters were still clearly detectable when using them, meaning their emission was above 695 nm.
Figure 3.4: (A): 1 PSS layer, (B): 1 PSS layer (more extensive purification), (C): 1 bilayer, (D): 2 bilayers + 1 PSS layer + DiI molecules
Figure 3.5: Sample: 2 bilayers + 1 extra PSS layer + 1 min in a $5\exp{11}$ M solution (A): FB (B): AI

Figure 3.6: A: two bilayers and one PSS layer, without filters. B: Same sample as (A), but using a 695nm long-pass filter. No fluorophores were added to the sample.
If we considered that \( \text{DiI}_1(5) \) and Alexa Fluor 633 have both emission spectra between 640 nm and 670 nm \([7]\), the problem could be solved using a short-pass or a band-pass set of filters for this particular range.

The signal-isolation issue could also be treated in an alternative manner. If fluorophores exhibited clearly different lifetimes (compared to the contaminating species), it should be possible to separate the data corresponding to these species. The tests carried out following this direction, though giving no positive results, lead to the determination of some problems present in the device.

3.2.3 Excitation: 515 nm

The same purification procedures as in the 633 nm case were applied for these tests. Images made on samples without fluorophores presented seemingly homogeneous background samples, with no traces of single molecules or groups of them. Images on a simple 2 bilayer system can be found in figure 3.10 (A). Tests were repeated several times, showing similar results in all cases \(^1\).

3.3 Attachment of fluorophores

The attachment of the fluorophores to the polyelectrolyte layers proved to be a quite difficult task. The main problem found was the adjustment of the solutions’ concentrations to get a deposition on the samples that were proper for single molecule experiments (10 to 20 molecules/100(\(\mu\)m)\(^2\)).

The attachment process was found to be highly irregular—particularly when solutions had been stored—which forced to either prepare fresh solutions for every experiment or to make tests on the higher nominal concentrations old ones (as expected, some fluorophores’ degradation mechanism present in the solutions made of storing time a relevant factor).

3.3.1 Excitation: 633 nm

Two different chemicals were tested for the 633nm measurements: Alexa Fluor 633 and \( \text{DiIC}_1(5) \).

Fig. 3.7 shows a test performed using DiI: the samples consisted of 2 bilayers and one extra PSS layer. (A) shows the result of dipping the sample in a \(5 \times 10^{-11}\) M solution for 1 minute; (B) shows the result for the same procedure applied to a sample in a \(3 \times 10^{-10}\) M solution. The change of concentrations roughly by factor of 10 produces drastic changes in the amount of fluorophores attached to the samples. Tests were repeated several times, observing the same irregular behavior in every case.

Alexa Fluor showed an important advantage when compared to \( \text{DiIC}_1(5) \): changes in the solutions’ concentrations were found to cause less drastic changes in the amounts of molecules attached to the bilayers after the dipping of the samples. This property, which makes the task of finding the right fluorophores’ concentration easier, was not present for the other species tested, and seemed to last even when solutions were stored. Fig. 3.8 shows the response on silanized glass, for two solutions: (B) corresponds to the solution used in (A) when diluted 10 times. Image (A) was made using a full-illumination scheme, while (B) was made using an annular one.

Detectors’ efficiency

It was found within the frame of the 633nm measurements a detectors’ efficiency problem. Fig. 3.9 (A) and (B) shows the images obtained on a one-layer sample with fluorophores: (A) is an image obtained

\(^1\)The obtention of a homogeneous background is not significative by itself; real comparisons are to be made with systems containing fluorophores
Figure 3.7: (A): 5.10^{-11} M, 1 min (B): 3.10^{-10} M, 1 min. Same measuring conditions.

Figure 3.8: Response to Alexa 633. (A) corresponds to a silanized substrate dipped into a 10-times higher concentration solution than (B).
Figure 3.9: Sample: one PSS layer, submerged for 2 minutes in a 1exp-10 M DiI solution. Light Source: diode laser. A,B - same area, measuring parameters and detector (PMT), but different illumination schemes (A: full-beam B: annular). C,D - same measuring parameters, different detectors (C: APD, D: PMT)

with FB illumination, where the expected single feature spots are visible; (B) is an image obtained under AI: no clear signs of the expected patterns were detected. Images (A) and (B) had been made using the PMT.

When comparing the results of the same system and measuring parameters but using an APD, (fig. 3.9 (C) and (D)) the differences appear quite clearly: the lower efficiency of the PMT for the long-wavelength range make this kind of measurement problematic. As a consequence, when a good quality image in this wavelength range is to be obtained (using reasonable photon-collection times), the PMT can not be used. It is to be added to this comment that the light source employed was a low power one, fact that made proper imaging under this conditions more difficult.

3.3.2 Excitation: 515 nm

The fluorescent species used for the 515 nm experiments was a well-known dye: Rhodamine 6G.

Images made on systems without fluorophores (see fig. 3.10, (A)) showed seemly homogeneous samples, with no traces of agglomerations of fluorescent features.
Figure 3.10 also shows the results for different fluorophores’ concentrations and dipping times: the increase of concentration by a factor of 10 does not reveal signs of fluorophores’ attachment (see (B) and (C)). Concentrations of $10^{-9}$ M caused massive attachment; fig. 3.10 (D) shows the result of dipping the sample in the solution for only 5 seconds: some groups of molecules are observed, no clear sign of single features were present.

The tests here presented were repeated several times, using fresh fluorophore’s and polymers’ solutions every time and minimizing the storing time of the samples. The results were similar in all cases: massive attachment was achieved with $10^{-9}$ M solutions while no signs of single molecules were detected when using $10^{-10}$ M solutions. The latter could be explained under two basic assumptions: very irregular surface attachment behavior (as in the case of DiI for the 633nm-excitation measurements) and/or low fluorophore’s emission intensity to background ratio.

Although rhodamine is known to be a powerful dye [7], no clear signs of fluorophores were detected in the samples dipped into the low concentration solutions; then, the idea that the signals provided by Rhodamine 6G might not be much stronger than the background can not be discarded without further proof.

3.4 Measurement of lifetimes

A very important part of the work to be carried in the work consisted of the proper measurement of lifetimes of single molecules in the nearby area of interfaces. Lifetimes’ measurement proved to be not an easy task; two problems related to the device were found while attempting to do so. These problems will be briefly described through the following section and possible solutions, proposed.

The first problem found was related to the performance of the 633nm notch filter employed in the device. Fig. 3.11 shows the results of two lifetime measurements, one on a background spot (where no signal is expected) and one on a fluorescent feature: both results are very similar. The latter could be explained if the notch filter were not effective at blocking the 633 nm excitation light; a signal superposed to the photons due to fluorescence would be recorded.

This feature was found to be highly sensitive to the orientation of the notch filter with respect to the beam; unfortunately, it was not possible to remove it completely by tilting it (see figure 3.11). In order to make proper lifetime measurements when using 633nm excitation, a different notch filter or a long-pass one (provided the signal of interest is not filtered) should be used.

The second problem found in the device was related to the APD detector. When checking the width of the pulses emitted by the diode laser (by directing the beam straight to the detector, see fig. 3.13), it was possible to see that those sensed by the TCSPC module (SPCM 630, Becker und Hickl GmbH) were wider than warranted by the manufacturer (FWHM: full-width at half-maximum; expected: 0.3 ns; measured: 0.8 - 0.9 ns). This represented a major problem: it is necessary to have narrow excitation pulses to perform direct reading of lifetimes from measurements. When this is not the case (that is, when the width of the sensed pulse is greater than approximately 1/5 of the decay time) a deconvolution procedure is to be applied to extract meaningful information.

Fig. 3.12 is intended to depict (schematically) the detected problem; fig. 3.13 shows the set of devices used to measure the width of the pulses. The signal produced by the APD (2) is directed to a router (3) which transforms the TTL pulses provided by the APD into suitable pulses for the TCSPC device. In this scheme, any of the devices involved in the measurement could be the source of the distortion and had to be tested, including cables and connectors. The replacement of the APD (for a device of identical characteristics) and of cables and connectors lead to no changes in the results.
Figure 3.10: Tests performed with 515 nm excitation. Fluorophore: Rho 6G. Samples: two bilayers and a PSS layer. A: no fluorophores. B: 30 s submerged in a 1e-11 M solution. C: 30 s in a 1e-10 M solution. D: 5 s in a 1e-9 M solution. P: 0.3e-6 W, 1.8 ms

Figure 3.11: The 633 nm notch filter problem. A: lifetime measurement of a molecule. B: measurement performed on a spot of the background. Detector: APD.
The detection electronics and laser pulses were tested by installing a PMT (Photomultiplier Tube) with its correspondent amplifier. The result of this test was a positive one: the FWHM of the pulses produced by the diode laser measured by the TCSPC module was as described by the lasers manufacturer.

When considering that the only significative change made to the set-up consisted of an exchange of detectors (and their correspondent "adapters", necessary to feed the TCSPC device with proper signals) and that two APD devices were tested (showing the similar results), it could be inferred, that this behavior was an intrinsic feature of the APD and the available router.

The "broadening" of the pulse measured when using the APD and its router may be due to some time-instability of their outputs. The TCSPC module applies a timing method aimed to reduce time-jitter caused by the amplitude jitter of the detectors pulses; in case APDs jitter could not be properly minimized an effective broadening of the pulse would be sensed (for further details on the working mechanism of the SPCM, please refer to [18]) and the measurements, affected.

This feature is something worth to be kept in mind for future measurements: it could be necessary to apply deconvolution procedures to the time-dependant signals acquired with the APD when, for example, attempting lifetime measurements.

The direct consequence of the APD feature was that some changes (regarding the detection scheme) were performed to the set-up: a PMT was added to the device. Fig. 2.8 schematically describes the lay-out after such changes; before these changes, only the APD was present in the microscope.

The signals produced by the PMT do not present APD’s particular response for time-related measurements (no need of data treatment).

As a disadvantage, the PMT device shows a low efficiency in the long wavelength range (lower than the one for APD), which prevents us from making high quality images (see fig. 3.9).

Since the APD does exhibit a higher sensitivity in the 600nm-range than the PMT and would allow for better measurements in that range, it would be proper to develop such algorithm if further experiments were attempted.

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2Unfortunately, no record was kept of these tests, but the changes applied to the set-up allow for easily checking what is here stated.
Chapter 4

Calculations

This chapter deals with the theory related to the calculation of electric field distributions near focus; how this is applied to the system of interest and the results obtained when running such calculations for layered structures with different external parameters.

4.1 Field distributions near focus

Far from saturation, the excitation rate of a fluorophore with a transition dipole moment $\mu$ ($\mu = \mu \mathbf{d}$) is given by:

$$ R(\mathbf{r}) = C \left\lvert \mathbf{E}(\mathbf{r}) \cdot \mathbf{d} \right\rvert^2 $$

(4.1)

where $\mathbf{E}(\mathbf{r})$ represents the electric field at the position $\mathbf{r}$ (dipole’s position).

The system under study consists of a pile of layers (the sample on which measurements were performed consisted of a glass coverslip and polyelectrolyte layers) in which linearly polarized light (the excitation) is being focused through with a wide numerical aperture objective (see fig. 4.2).

In order to calculate electric field distributions ($\mathbf{E}(\mathbf{r})$) produced by this excitation (and to be able to make calculations as $R(\mathbf{r})$, it is possible to think of the total electric field at any given point in the nearby area of the focus as the result of the added contributions of the refracted rays towards that spot. The latter can be expressed as:

$$ \mathbf{E}(\mathbf{r}) = C \int f \mathbf{E}_0 e^{i \mathbf{k} \cdot \mathbf{R}} d\Omega $$

(4.2)

where $\mathbf{E}(\mathbf{r})$ represents the electric field at a given point $\mathbf{P}$ near the focus, with a $\mathbf{r}$ position vector; $\mathbf{E}_0$ the field reaching that point, $\mathbf{k}$ the wave vector of the incoming radiation, $\Omega$ a solid angle and $f$, the so called ”strength function” (accounting for the contribution of rays focused from different directions).

These calculations, meant for an ideal, aberration-free optical system under the assumptions of geometric optics, were done following the pioneering work of E. Wolf [24, 23] and some standard procedures for optical devices [6, 24, 23, 25, 22, 21, 12].

The excitation (linearly polarized light), before being focused by the objective of the microscope, could be simply described as:

$$ E_0 = |E_0|(E_x, 0, 0) $$

(4.3)

or, by means of its $s$ and $p$ components (please, refer to fig. 4.1), as:

$$ E_p = E_0 \cos \psi $$

(4.4)

$$ E_s = -E_0 \sin \psi $$

(4.5)
The system in which light is being focused through consists of a pile of layers (see fig. 4.2): glass, polyelectrolytes and air. Both glass and air are to be considered as semi infinite, only the thickness of the polyelectrolyte layer is to be taken into account.

The electric field at a given point in a layered system in which light is impinging can be calculated following the standard matrix theory for the analysis of multilayered systems [24, 23].

As light is being focused through such type of architecture, its influence is to be considered (E₀ in 4.1 can be considerably affected).

The response of this pile of thin films (layers) can be modelled as:

\[ E_f = ME_0 \]  \hspace{1cm} (4.6)

\[ M_T = \Pi M_i \]  \hspace{1cm} (4.7)

where \( M_i \) represents the response of the i-th film, \( E_f \) the field at an exit point of interest and \( E_0 \) the external electric field reaching the layers. The relevant variables in this problem are: the angle of incidence of the incoming rays (\( \theta \)), the wavelength (\( \lambda \)), the thickness of each film (\( d_j \)) and the refractive indices of the materials involved (\( n_j \)).

The output of this algorithm is the electric field at a given point of interest (\( \tilde{E_x}, \tilde{E_y}, \tilde{E_z} \)) (all of these components are functions of the variables previously mentioned; provided constant system parameters \( \theta \).

---

1The algorithm uses a rotated (about the z axis) coordinate system; the new y' axis lying along the s component of the field. In this context, the p component (of the already refracted beam) has contributions both along x' and z'.
the angle of incidence, becomes the main one and will be the only variable pointed out in the following expressions).

If the output of this procedure were expressed in the original system of reference, the result could be expressed as follows:

\[ E_x(\theta) = E_{x'}(\theta) \cos \psi - E_{y'}(\theta) \sin \psi \quad (4.8) \]
\[ E_y(\theta) = E_{x'}(\theta) \sin \psi + E_{y'}(\theta) \cos \psi \quad (4.9) \]
\[ E_z(\theta) = E_{z'}(\theta) \cos \psi \quad (4.10) \]

Considering the form of the \(s\) and \(p\) components, the previous equations take the form:

\[ E_x(\theta) = E_{x'}(\theta) \cos^2 \psi + E_{y'}(\theta) \sin^2 \psi \quad (4.11) \]
\[ E_y(\theta) = [E_{x'}(\theta) - E_{y'}(\theta)] \sin \psi \cos \psi \quad (4.12) \]
\[ E_z(\theta) = E_{z'}(\theta) \cos \psi \quad (4.13) \]

Once again, each of these components is a function not only of \(\theta\) and \(\psi\), but also of the properties of the layered system and the incoming light \((E_i(\theta, \lambda, d, n))\).

Up to this point, the functional form of the at a given point of the layer system has been obtained; in order to be able to calculate the total electric field in a given point 4.1, it is necessary to express the relevant phase factor present in 4.1 \((k \cdot R)\) in a more accurate way.

To that purpose, consider the basic configuration displayed in figure 4.1 and an arbitrary point \((P)\) with its associated position vector \((R)\):

\[ R = (r \cos \varphi, r \sin \varphi, z_P) \quad (4.14) \]
\[ k = |k|(\cos \psi \sin \theta, \sin \psi \sin \theta, \cos \theta) \quad (4.15) \]
\[ |k| = 2\pi/\lambda \quad (4.16) \]
\[ k \cdot R = r \cos \varphi k \cos \psi \sin \theta + r \sin \varphi k \sin \psi \sin \theta + z_P k \cos \theta = k(r \sin \theta(\cos \varphi \cos \psi + \sin \varphi \sin \psi) + z_P \cos \theta) \quad (4.17) \]
\[ = k(r \sin \theta(\cos(\psi - \varphi)) + z_P \cos \theta) \quad (4.18) \]
\[ e^{ik \cdot R} = e^{ik(r \sin \theta(\cos(\psi - \varphi)) + z_P \cos \theta)} \quad (4.20) \]

According to this, it is possible to obtain:

\[ E_i(P) = C \int E_i^f e^{ik(r \sin \theta(\cos(\psi - \varphi)) + z_P \cos \theta)} dS \quad (4.21) \]

which takes the following form when

\[ z_P = 0 \quad (4.22) \]

(a given point in the focal plane):

\[ E_i(P) = C \int E_i^f e^{ik(r \sin \theta(\cos(\psi - \varphi))} dS \quad (4.23) \]
The contributions of interest can be set as wished; if we are to consider an annular illumination scheme, the region taken into consideration results (see fig. 3.2):

\[ \theta_{\text{min}} < \theta < \theta_{\text{max}} \]
\[ 0 < \psi < 2\pi \]  
(4.24)  
(4.25)

or, in a similar way:

\[ k_{\rho_{\text{min}}} < k_{\rho} < k_{\rho_{\text{max}}} \]
\[ 0 < \psi < 2\pi \]  
(4.26)  
(4.27)

where \( k_{\rho} = k \sin \theta \)

Not yet concluded, an extra factor is to be added to this calculation: the strength function, which takes the following form [22]:

\[ f(\theta) = \sqrt{\cos \theta} \]
(4.28)

Having obtained all the functional forms of the terms in the general expression for the electric field in a given point in the focus 4.1, the latter takes the form:

\[ E_i(P) = C \int \sqrt{\cos \theta} E_i^f e^{ik(r \sin \theta (\cos \psi - \varphi))} dS \]  
(4.29)

It is by applying this very same procedure to any given point P surrounding the focus that it is possible to calculate the field distribution in the area of interest.

Even when this calculation procedure seems simple and easy, running it for every point in the nearby area of the focus may require quite a lot of effort and time. It is possible, though, to obtain the desired field distribution performing a smaller number of calculations, considering symmetry properties of the system.

4.1.1 Symmetry considerations

If we consider a source field polarized along the x axis (as it has been so far done), this source gives rise to in-plane fields along the x and y axis that have only x components (due to mirror symmetry of the problem about the x axis, y components of the electric field for points located along the x and y axis vanish).

Along x \( E_x^x \) (x component of the field, points along the x axis)
\( E_x^z \) (z component of the field, points along the x axis)

Along y \( E_y^x \) (x component of the field, points along the y axis)
\( E_y^z \) (z component of the field, points along the y axis)

The in-plane field distribution could, therefore, be described using only these non-vanishing components: \( E_x^x \) and \( E_x^z \).

Again, let us consider any given point (P), and place a new coordinate system where this point is located, so as to set z parallel to z’ and P along x’ (see fig. 4.3).

Let \( \alpha \) be the angle between x and x’. If it were possible to describe the field at any point on this new x’ axis for any given \( \alpha \), it would be possible, then, to describe the field for any point of the plane and the problem would be solved.
According to this new reference system (given by $x'$, $y'$ and $z'$), the source field ($E_s$) could be decomposed into two main components $E_{s-x'}$ and $E_{s-y'}$; and so could be the problem. Considering once again that no perpendicular components to the source field are generated, the picture would go on as follows:

\[
E^x_x = E^x_x \cos \alpha \quad \text{(4.30)}
\]
\[
E^y_x = E^y_x \sin \alpha \quad \text{(4.31)}
\]

It is of interest to obtain $E_x$ and $E_y$ for every point along this arbitrary $x'$ direction. Considering that:

\[
E_x(r, \alpha, z_P) = E^x_x \cos \alpha + E^y_x \sin \alpha \quad \text{(4.32)}
\]

and introducing 4.30 into the equation, it is possible to deduce that:

\[
E_x(r, \alpha, z_P) = E^x_x (\cos \alpha)^2 + E^y_x (\sin \alpha)^2 \quad \text{(4.33)}
\]

In a similar way:

\[
E_y(r, \alpha, z_P) = E^y_x \sin \alpha E^y_x \cos \alpha \quad \text{(4.34)}
\]
\[
E_y(r, \alpha, z_P) = (E^x_x - E^y_x) \sin \alpha \cos \alpha \quad \text{(4.35)}
\]

When considering the z component, it is easy to see (due to symmetry reasons) that both $E^y_z$ and $E^z_z$ vanish and that $E^x_z$ is the only remaining component;

\[
E_z(r, \alpha, z_P) = E^x_z \cos \alpha \quad \text{(4.36)}
\]

Finally,

\[
E_x(r, \alpha, z_P) = E^x_x (\cos \alpha)^2 + E^y_x (\sin \alpha)^2 \quad \text{(4.37)}
\]
\[
E_y(r, \alpha, z_P) = (E^x_x - E^y_x) \sin \alpha \cos \alpha \quad \text{(4.38)}
\]
\[
E_z(r, \alpha, z_P) = E^x_z \cos \alpha \quad \text{(4.39)}
\]

It has been shown that all components of the electrical field have been described based only on two in-plane components (for points along an arbitrary direction given by $\alpha$, and, therefore, for all the plane) reducing and simplifying calculations considerably. Once being able to calculate these distributions, and obtaining images applying the annular illumination scheme, dipoles’ orientations could be calculated.

Figure 4.4 shows the results of this type of calculation for a homogeneous system; fig. 4.5 is meant to compare the results of calculations and measurements.
Figure 4.4: Calculated patterns, homogeneous medium. A: patterns plotted using their own intensity scale (meant for clarity purposes) B: patterns plotted a common scale (the strongest one, correspondent to Z patterns)

Figure 4.5: Comparison between measured and calculated patterns (molecules on silanized glass)
4.2 Results

The following part of this chapter will deal with the results of the focal electric-field distribution calculations, focusing on the effect of the different illumination conditions for imaging.

4.2.1 Testing the system: illumination

The main aim of this set of calculations was finding the right combination of external parameters (in particular, illumination conditions) to obtain field distributions where the cartesian components of the electric field presented comparable intensities.

If this were not the case, that is, when the external settings of the measurement were such that the electric field distribution had at least one remarkably weaker component (compared to the other ones), it would only be possible to effectively sense orientations of the dipoles close to the stronger component/s. This could represent not only a great loss of valuable information but also lead to wrong assumptions; if the sensing were preferential, it could be readily assumed that dipoles could be oriented only according to those directions.

Figure 4.6 depicts results of the field distributions calculations for two illumination schemes. (A) represents the full-beam case (FB), for a polymer/air interface: both x and z components of the field are remarkably stronger than the y one (almost two orders of magnitude between maxima, which makes the sensing of closely y-oriented dipoles very difficult); (B) is the result of an almost completely blocked excitation beam (AI); patterns show different intensities, though differences appear less severe than in the full-beam illumination scheme (within a factor of 10). In both cases the proper sensing of y-oriented dipoles would be difficult, but the full-illumination scheme (A) would be clearly disadvantageous when compared to (A).

The parameters readily alterable when performing measurements, provided a given system of layers is fixed (materials; their correspondent thicknesses and dielectric constants) are: $\theta_{\text{max}}$ (maximum angle for annular illumination), $\theta_{\text{min}}$ (blocked angle for the annular illumination scheme, see fig. 3.11) and $\lambda$ (wavelength of the excitation source).

Changes in wavelength ($\lambda$) only give rise to changes in the size of patterns according to the wavelengths’ ratio (see fig. 3.12), therefore, it was of main interest to study different illumination schemes (apertures and blocked angles).

Table 4.2.1. summarizes the calculations carried out for different systems and illumination conditions.

<table>
<thead>
<tr>
<th>633 nm</th>
<th>$\theta_{\text{max}}$</th>
<th>$\theta_{\text{min}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>homogeneous medium (air)</td>
<td>68.6$^\circ$</td>
<td>50$^\circ$</td>
</tr>
<tr>
<td>Spacer 1:10 nm, air side</td>
<td>68.6$^\circ$</td>
<td>60$^\circ$, 55$^\circ$, 50$^\circ$, 45$^\circ$, 40$^\circ$, 35$^\circ$, 30$^\circ$, 0$^\circ$</td>
</tr>
<tr>
<td>Spacer 1:10 nm, Spacer 2:5nm, interface</td>
<td>68.6$^\circ$</td>
<td>60$^\circ$, 55$^\circ$, 50$^\circ$, 45$^\circ$, 40$^\circ$, 35$^\circ$, 0$^\circ$, 30$^\circ$, 25$^\circ$</td>
</tr>
<tr>
<td>Spacer 1:24 nm, air side</td>
<td>68.6$^\circ$</td>
<td>65$^\circ$, 60$^\circ$, 55$^\circ$, 50$^\circ$, 45$^\circ$, 40$^\circ$, 35$^\circ$, 30$^\circ$, 25$^\circ$</td>
</tr>
<tr>
<td>Spacer 1:44 nm, Spacer 2:24nm, interface</td>
<td>68.6$^\circ$</td>
<td>65$^\circ$, 60$^\circ$, 55$^\circ$, 50$^\circ$, 45$^\circ$, 40$^\circ$, 35$^\circ$, 30$^\circ$, 25$^\circ$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>515 nm</th>
<th>$\theta_{\text{max}}$</th>
<th>$\theta_{\text{min}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spacer 1:10 nm, air side</td>
<td>68.6$^\circ$</td>
<td>60$^\circ$, 55$^\circ$, 50$^\circ$, 45$^\circ$, 40$^\circ$, 35$^\circ$, 55$^\circ$</td>
</tr>
<tr>
<td>Spacer 1:10 nm, Spacer 2:10nm, interface</td>
<td>68.6$^\circ$</td>
<td>60$^\circ$, 55$^\circ$, 50$^\circ$, 45$^\circ$, 40$^\circ$, 35$^\circ$, 30$^\circ$, 25$^\circ$</td>
</tr>
</tbody>
</table>
Figure 4.6: Electric field cartesian components (x, y and z) in the air/polymer interface: spacer-10 nm, 633 nm; (A) Max: 68.6 deg., Min: 0 deg. (full-beam illumination) (B) Max: 68.6 deg., Min: 60 deg. (annular illumination scheme). Axis - vertical: intensity, horizontal: pixels (10 = 50 nm). Y patterns were rotated 45 degrees for simplicity.
Figure 4.7: Calculated z component for the 633 nm (A) and 515 nm (B) excitations.

Figure 4.8: Normalized maxima, for maximum aperture and different blocked angles. (A) polymer/air interface (layers thickness: 10 nm) (B) layers 1 and 2: 10 nm

Table 4.2.1

The data here presented through fig. 4.8 represents the general trend of the calculations performed. For clarity purposes, only two of these were shown.

It is plotted in fig. 4.8 the normalized maxima (to the strongest component, in this case, x) for each calculation performed; this graphic was set to compare calculated data for the $\lambda$: 515 nm case. (A) is intended to depict the relative maxima obtained for different blocked angles, when focusing on top of a 10 nm polymer layer, in the polymer/air interface ($\theta_{\text{max}}$: 68.6°, both for (A) and (B)). (B) considers a point immersed in the polymer (or, between two layers 10 nm thick each). Note in this last picture the remarkable difference observed for the maxima of the z component: it becomes comparable to the y one (both much lower than the x one).

From this data, it could be considered that imaging of molecules placed on top of the polymer layer should present no major inconveniences (the y maxima are weaker than the x and z ones, but the difference is still within a factor of 10). When considering points close to the interface (as the one placed in between 10 nm layers, see fig. 4.8, (B)), x and y components become comparable and much lower than the x one, which would make the sensing of such directions harder.
As shown in Table 4.2.1, the system has not been thoroughly explored. While studying the air/polymer interface, it was possible to see that for the explored experimental conditions, x and z maxima show comparable strength; y maxima appear weaker (around a factor of 10). This, though limiting when considering the background-signal problem for the measurements, could still make it possible to observe y patterns.

When considering points immersed between the thin spacers (a few nanometers distant to the interface with air), x maxima appear as the strongest ones (see Fig. 4.8 (B)). Though the observed difference remains within a factor of 10 for most cases, the use of such experimental parameters might lead to a rather preferential sensing of dipoles oriented parallel to this direction.

It is to be added to these facts an extra one: the fraction of light collected by the objective for the different dipoles’ orientations. These calculations, though not performed here, show that the fraction of light going to the glass side (directly related to the fraction collected by the objective) for the perpendicular and parallel dipoles are roughly comparable, not affecting, therefore, the considerations previously stated [16].
Chapter 5

Conclusions

General outcomes

The basic goal pursued in this work (the study of single molecules placed in or near to a dielectric interface, by applying orientation imaging and lifetimes’ measurement) could not be achieved; still, some contributions were made to the study of the problem, particularly some related to the use of the layer-by-layer method as an alternative approach to spin-casted films.

- Polymer’s purification
  The controlled precipitation method (applied to PSS) and the dialysis (applied to PAH) showed positive results for the red-light measurements. The samples prepared using polymers purified in this manner usually presented between two and three contaminant fluorescent features in a 10 x 10 μm² area for a two/three bilayers sample.

- Fluorophores’ attachment behavior
  Dyes were found to attach to samples in a highly irregular way; making of this a not very reproducible way for obtaining the desired surface density for single molecule measurements.

- 515 nm measurements
  The green-laser measurements showed no positive results. The same materials and methods as in the red-light case were applied, obtaining samples with seemingly homogeneous backgrounds, but no signs of single molecules were detected after the deposition of fluorophores.

- Technical features
  Related to detectors’ efficiencies, lasers’ intensities and the removal of detector’s signal time-jitter issue, the 515 nm measurements represent a more suitable choice than the 633 ones.

- Calculations
  These procedures, initially meant to fit calculations to measurements and obtain the correspondent dipoles’ orientation, showed to be suitable for studying the influence of the experimental factors, such as illumination conditions and proximity to interfaces.
Chapter 6

Proposed activities

As seen from the general outcomes of the tests performed during this work, some general issues could be pointed out to be worth to continue exploring:

• Regarding polymer cleaning: testing of higher MWCO membranes (recommended by the manufacturers: up to half the MW of the polymer ensures minimum loss of polymer) and higher dissolution degrees for the controlled precipitation method (for PSS). These procedures could be more extensively tested on both PSS and PAH, and the 515 nm measurements repeated (this might help to elucidate whether the problems are related strictly to poor fluorophores attachment, to a high degree of contamination or to both of them).

• Regarding the use of filters for measurements: for the 633 nm measurements: testing the use of short-pass or band pass filters that could help to isolate the fluorophores signals. For the 515 nm measurements, the same criterion could be applied if high levels of contamination were effectively detected.

• APD issue: given its intrinsic higher efficiency (at least, higher than for PMT, see Chapter 2), when intended to perform lifetime measurements using this device, the elaboration of a proper deconvolution algorithm would be necessary.

• 515 nm measurements: even though no positive results were obtained for this system, it would be worth continuing with these attempts testing, for example, other fluorescent species.

• Calculations: further calculations of the type performed would be in place to establish suitable measuring conditions; by doing this, it could be possible to discard the most unfavorable configurations, which could not only save time and effort, but also provide a stronger basis to analyze measurements.
Bibliography


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  - Showing me how useful the combination of some Loctite and a hammer can be...
  - Not attempting to kill me every time I made a stupid question (and there were many of those!)...
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