Long-term observation of protein dynamics via thermal-snapshot single-molecule spectroscopy

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SUMMARY

Optics-based single-molecule detection and imaging methods currently form a fundamental pillar in experimental research in biophysics and biomedical sciences, by removing the molecular averaging effects in the systems of interest. This dissertation revolves around the design and implementation of novel measurement techniques and scientific instrumentation capable of single-molecule detection.

Chapter 1 begins with an exposition of the historical development in the field of single-molecule detection and analysis, and continues with a presentation of current microscopy techniques in confocal and super-resolution configurations. A presentation of the mechanism of Förster resonance energy transfer (FRET) is included, together with a general overview of the electronics working principles of time correlation single photon counting (TCSPC) and polarization-based measurements. Additionally, the temperature dependence of the viscosity of glycerol, used in the thermal-cycle calibration included in Chapter 3, is presented.

A fluorescence-based room temperature (RT) confocal microscope was designed and built during the course of this doctoral work, and its implementation and testing are presented in detail in Chapter 2. This instrument, with single-molecule detection capability, is able to simultaneously carry out fluorescence-based area scans combined with time-correlated single-photon counting (TCSPC), polarization-based measurements, and fluorescence correlation spectroscopy (FCS), thanks to its optical equipment and associated advanced electronics with picosecond temporal resolution. At its present state, a 485 nm blue laser is used as the excitation source, and its polarization state is suitably controlled. In future, additional excitation wavelengths could be installed, thus expanding this instrument's capabilities towards also including Förster resonance energy transfer (FRET)-based experiments.

The optical quality of this RT microscope was tested by using fluorescence beads with a nominal diameter of 100 nm, and carrying out both in-plane and vertical area scans, for obtaining the associated point spread function (PSF), which gave an experimental agreement with the expected theoretical PSF. Further testing of the single-molecule detection capabilities of this instrument was done by using both ATTO 488 and Rhodamine 6G dyes. Single-molecule area scan detection, time traces that include clear signatures of the blinking effect, and single-molecule lifetime decays, are also included in this Chapter.

In line with the spirit of this dissertation regarding developing novel instrumentation and related techniques for single-(bio)molecule exploration, Chapter 3 presents the thermal-cycle confocal microscope designed and built during the course of this doctoral research, capable of exerting controlled extreme thermal gradients for forcing molecules out of equilibrium, by using purely optical means. By the time these lines are being written, and to the author of this dissertation´s best of knowledge, this aforementioned novel research instrument and related thermal-cycles-based technique developed at Leiden University, are both truly original and unique in their type in the world.

This technique aims to detect out-of-equilibrium molecular configurations, by detecting changes in the measured fluorescence emissions of a pair of dye molecules attached to the molecule of interest, and as such related to a conformational change which takes place during a thermally favorable evolution period forced by the activation of a near-infrared (NIR) laser.

A fraction of the incident radiation is absorbed by a thin metal layer deposited on the opposite side of a glass substrate where the sample molecules are located. When the NIR laser is switched off, the sample, located inside a dedicated cryostat with an inner liquid nitrogen temperature, is frozen again and a second laser, operating in the visible range, excites the attached dyes and their emitted fluorescence signals are related to a change in their relative distance,

within the framework of the Förster resonance energy transfer (FRET) phenomenon.

After this detection is made the NIR radiation is sent again followed by a new cooling period, and so on, allowing the capture of non-equilibrium configurations in a way akin to “thermal snapshots”. In particular, the application of this technique to proteins would allow to experimentally reconstruct their free-energy landscape within the funnel model.

A 488 nm laser beam is used to excite fluorescence from the sample of interest, and is sent towards the cryostat bottom entrance by an external steering mirror also used for fluorescence-based area scans. Three independent nanopositioners attached to the cryostat insert provide degrees of freedom in the cartesian reference system, for sample lateral displacements and focusing by a custom-made thermal-fatigue-resistant objective, which is also responsible for collecting the fluorescence emission that is subsequently passed through a suitable combination of optical elements and fine optomechanics, and then sent towards single-photon detectors.

On the other hand, a suitable dichroic mirror installed in the detection path provides spectral separation for donor and acceptor channels. A fourth nanopositioner is also installed in the insert, and is capable of providing independent relative vertical displacement between the specimen of interest and an internal aspheric mirror used for focusing a 671 nm heating beam on the metal-coated side of the substrate. The modulation and alternation of excitation and heating beam pulses are achieved by two acousto-optics modulators (AOMs), and an in-house custom-developed synchronization software, respectively.

The optimization of the detection path was done by using fluorescent beads with diameters of 1 μm and 100 nm, respectively. In particular, the latter allowed us to obtain a point-spread function (PSF) with a slight elongation, which is attributed to an aberration caused by an internal displacement of the lenses in the custom-made

objective. Considering that the diffraction-limited spot for the case of blue radiation is on the order of 300 nm, a full width at half-maximum (FWHM) of 340 nm was obtained along one lateral displacement, whereas the corresponding FWHM obtained in the orthogonal lateral axis was 620 nm. On the other hand, the temperature cycle calibration was done by using spin-coated Rhodamine 6G embedded in glycerol with 6% - 8% water, starting at the liquid nitrogen temperature of 170 K in the cryostat sample chamber. The heating beam incident power was regulated by a variable attenuator installed in the heating optical path, and the first order of diffraction in the heating AOM was aligned and sent towards the lateral entrance of the cryostat.

This doctoral work continues in Chapter 4 with the experimental test of a prediction made by Theodor Förster in 1949, regarding the non-exponential fluorescence decay of donor molecules surrounded by a spatially fixed random distribution of an ensemble configuration of acceptor molecules, and its possible deviation from the stretched exponential behavior at the single-molecule level. For the test, Rhodamine 6G was used as the donor, whereas ATTO 575Q quencher was the acceptor molecule. The lifetime fluorescence decays were obtained at room temperature with the microscope presented in Chapter 2.

Both donor and acceptor molecules were embedded in a solid matrix of poly(methyl methacrylate) - PMMA spin-coated on a circular glass microscopy slide, and subsequently excited with 485 nm radiation. The fitting of the lifetime decays was improved by considering the presence of both quenched and unquenched donor molecules, in comparison to the Förster expression.

The latter could be an indication of the incompleteness of the Förster model, although this might be inconclusive due to the detected parasitic emission of the quencher when embedded in a solid matrix. To circumvent this issue, an original configuration of Rhd 6G - black hole quencher (BHQ-1) embedded in a solid matrix of polyvinyl acetate (PVAc) was used, for the first time to the author

of this dissertation´s best of knowledge. Additional suggestions for the improvement of the surface morphology of this combination are also given, and presented as a future approach in this research direction. This concludes the dissertation.