Single Molecular Spectroscopy

Parallel Lifetime and Imaging of Single Molecules

Conventional microscopy and spectroscopy techniques can accurately analyze the properties of polymeric materials but incapable of distinguishing between properties in bulk and nanoscale. For this purpose, single molecular spectroscopy has been developed, a method that is able to circumvent these limitations. In this paper we present an automated experimental method capable of characterizing in real time a large number of individual molecules.

Introduction

Conventional microscopy and spectroscopy, as well as scanning probe techniques are capable of accurately characterizing polymeric films. But the intrinsic properties of the ensemble differ greatly from those of individual molecules, which are heavily influenced by the various degrees of disorder available to a single polymer chain. Therefore, the optical properties of thin film and that of a polymer’s will be radically different [1]. For example, figure 1 shows the optical properties in bulk and at nanoscale of a commercially applicable conjugated polymer. The thin film displays a relatively featureless spectrum, containing a broad dominant peak at 680 nm as well as several minor shoulders. At single molecule level, the same material shows four distinct fluorescence peaks, at lower wavelengths.

To overcome some of the limitations associated with conventional microscopy and scanning probe microscopy, a new experimental method emerged in the early 1990’s: Single Molecular Spectroscopy (SMS), initially as a cryogenic method [1-3]. Figure 2 illustrates the basic operating principle of SMS.

Conventional microscopes are capable of detecting single molecules but due to the small distance between single chains, below the diffraction limit, the system will be unable to resolve individual molecules and an ensemble measurement will be taken averaging the optical properties off all the molecules within the diffraction limited collection region. This limitation can be overcome by diluting the material of study to a level where the space between emitting molecules is higher than the device’s optical resolution.
Single molecule fluorescence spectroscopy measurements require a large number of chromophores to be analyzed in order to get a statistically relevant data set. Due to the manual operation of the typical experimental set-ups, single molecule spectroscopy is a lengthy procedure and data acquisition is a very time consuming process.

In order to automate and optimize the method, we are using a custom built optical microscope based on Zeiss optics. Automation was achieved by implementing a specific hardware configuration, working in conjunction with a series of software packages. In its current form, our experimental method allows the automatic acquisition of data in several modes of operation: fluorescence intensity acquisition; image capture, spectroscopy and photoluminesence (PL) lifetime measurements.

**Sample Position**
An important function of the system is accurate control the samples position. This is achieved by means of a Zaber A-series microscope stage for coarse positioning, and a nPoint piezo stage for fine positioning. In this configuration, there are two distinct modes of operation: manual and automatic. In manual mode, the user can freely move the sample either in coarse steps or in finer sub-micron steps; this allows the user to select areas of interest within a sample.

The second operating mode involves the automatic movement, important in data acquisition. For this purpose, the software accompanying the piezo stage (nP Control) is equipped with a raster scanning mode. The sample is moved on one of the horizontal axes (noted for convenience as X) in equal steps of pre-determined size. Between the steps, a controller (nP LC403 controller) will trigger a Princeton Instrument ProEM 512 EMCCD camera or a secondary capture device, for acquiring data. When motion on the X axis is complete the sample will be moved one step on the perpendicular axis and the cycle is repeated. The result will be a raster pattern on the film surface. The system is highly flexible with all parameters
of the raster pattern being determined by the user. That includes the number of steps on the X axis, the number of lines in the pattern (steps on Y axis) as well as the dwell time between each step (used in data acquisition). If necessary the raster pattern can be extended on the vertical axis, automatically repeating the horizontal raster scan. This operating mode is useful for acquiring data in “slices”, to create a 3D scan of the analyzed sample.

**Fluorescence Intensity and Extended Lifetime**

For all measurements a laser is focused to a tight spot on to the sample. When measuring fluorescence intensity, the diffraction limited laser spot is imaged using the EMCCD camera at each point of the scanners raster pattern. Optical filters are used to remove the laser light allowing only PL to be detected on the camera. The intensity of the spot is integrated over the point-spread function of the microscope to build up a map of PL intensity. A typical intensity image is given in figure 3a.

Extended lifetime measurements are performed by means of Avalanche Photo Diode (APD) detector, provided by Photonic Solutions. We use time-correlated single photon counting methods to measure PL lifetimes (TCSPC), method based on detecting individual photons of a periodic signal, measuring detection times and reconstructing the waveform from the time measurements. TCSPC is possible because the intensity of low level high repetition rate signals is usually so low that the probability of detecting more photons in a single signal period is insignificant. Upon detecting a photon, a detector pulse in the signal period is measured. When a large enough number of photons has been measured, their distribution over the signal period time builds up and the result is a distribution probability, in the shape of a waveform, of the optical pulse [4]. Again for each point on the scanners raster pattern a full PL lifetime curve is collected, this requires a longer delay time, on the order of 300 ms. A typical lifetime curve is given in figure 3b.

Figure 3 shows an example of data acquired by the described module. The controlling (Becker & Hickl) SPCM software, together with the nPoint controller and piezo stage, will automatically scan the sample surface providing a fluorescence intensity map (fig. 3A), as a preliminary analysis, as well as lifetime data (fig. 3B) for various types of samples.

**Spectroscopy**

For spectroscopy measurements, our custom set-up is equipped with a ProEM 512 electron-multiplying charged couple device camera and an Acton SP2500 spectrometer, both provided by Princeton Instruments [5]. The spectrometer is composed of a slit, for minimizing collection on both horizontal axes as well as a multi-grating turret containing a mirror for conventional imaging and a series of two gratings (150 and 300 grooves/mm respectively) for fluorescence spectroscopy
measurements. Again for each point of the raster scan the camera is triggered to collect a PL spectrum from the spot excited by the laser.

**Conclusions**

We have optimized and successfully implemented a custom experimental set-up and method for single molecular spectroscopy. By using a specific hardware configuration and software packages, the system is capable of automatically collect data from a large number of emitters, in various modes of acquisition: fluorescence intensity, fluorescence spectroscopy, image capture or lifetime measurements. The set-up offers a high degree of flexibility, allowing the characterization of many types of samples from thin polymeric films biological samples or fluorescent beads.

**References**


**Authors**

**Adrian Mantsch**

**Dr. Ashley Cadby**

The University of Sheffield

Department of Physics and Astronomy

Sheffield, United Kingdom

**Further information on microscopy of single molecules:** [http://www.imaging-git.com/](http://www.imaging-git.com/)

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