Fluorescence Detection of Single Molecules

Applicable to Small Volume Assays

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1. Introduction

In this chapter we give an overview of optical detection of single molecules with respect to possible application to high throughput screening of substance libraries. We restrict ourselves to single molecule detection (SMD) because the field of ultrasensitive detection is too broad to be covered by a single chapter; thus we will concentrate on the ultimate detection limit. In the future SMD will be the method of choice because of the ultimate small amount of analyte needed in connection with extremely high speed screening of vast libraries. Besides the topic of high-speed DNA sequencing (see below), the high sensitivity received attention also in the context of combinatorial chemistry (Eigen, 1984; Plückthun, 1991; Eigen, 1994; Xiang, 1995) and molecular computation (Adleman, 1994; Lipton, 1995). The basic idea of combinatorial chemistry is to synthesize large arrays of slightly different molecules, followed by screening and selection of molecules with desired properties. In the case of molecular computation, a random chemical synthesis is used to find solutions of numerically difficult problems; again, a fast and ultrasensitive screening method is needed to extract the result of the 'computation'.

Within the domain of SMD, the emphasis will be on optical detection, i.e. laser induced fluorescence (LIF) detection. LIF is one of the most sensitive detection methods in existence today. It is easy to implement, highly characteristic, and mostly non-invasive, which is important for the detection (and possible separation) of biomolecules. Moreover, based on the broad application of LIF in many fields of analytical chemistry and biochemistry, established physical technologies and a huge variety of fluorescence dyes are available for LIF.

With respect to SMD on surfaces and interfaces, we will discuss only SMD at room temperature. High resolution spectroscopy of single molecules in and on solids at low temperatures is a very broad field in itself, but probably of minor interest for applications in library screening. Although of fundamental interest for studying molecule-host interactions, the technological complexity of low-temperature, high-resolution experiments will prevent their application in biolabs and related facilities. For a recent overview of SMD and high resolution spectroscopy at low temperatures see e.g. (Orrit, 1996; Basché, 1997). Finally, we note that the emphasis of this chapter will be the physics of SMD for room temperature applications rather than its chemical aspects (e.g. design of fluorescence labels).

An overview of all current optical SMD methods at room temperature, potentially applicable to high-speed screening of biomolecular libraries, is presented in Table 1. In the following sections, each of these methods will be discussed and referenced in more detail.

Table 1: SMD methods at room-temperature with potential use in biomolecular applications

in fluids	laser induce fluorescence	fluid flow
		micro-capillary electrophoresis
		fluorescence correlation spectroscopy
		levitated micro-droplets
	laser induced Raman scattering	single molecules adsorbed on colloidal metal particles in solution
on surfaces	far-field methods	confocal microscopy
		wide-field fluorescence microscopy with low noise CCD cameras
	near-field methods	near-field scanning microscopy

There exists a large variety of other non-optical SMD methods applicable to biomolecule recognition and screening, like conventional STM, see e.g. (Cricenti, 1991; Hansma, 1991; Heckl, 1991; Li, 1991; Youngquist, 1991; Heckl, 1992; Cooper, 1994; Guckenberger, 1993; Frisbie, 1994; Thimonier, 1994; Kasaya, 1995; Venkataraman, 1995; Zuccheri, 1995; Hinterdorfer, 1996; Tanaka, 1996; Walba, 1996); STM with surface adsorbed water (Guckenberger, 1994; Heim, 1996); scanning force microscopy, see e.g. (Schaper, 1993; Radmacher, 1994a-c; Gunning, 1995; Muzzalupo, 1995); electrochemical or ion-channel SMD (Fan, 1995; Bard, 1996; Fan, 1996; Kasianowicz, 1996); and mass spectroscopy (Fenn, 1989; Jacobson, 1991). However, none of these methods combines high molecular specificity, relative technical simplicity and non-

invasiveness in such a unique way as optical SMD based on laser induced fluorescence (or possibly Raman scattering).

2. Single Molecule Detection in Fluids

Before the age of optical detection of single molecules in solution, the first SMD in liquids was done indirectly by monitoring the enzymatic activity of single protein molecules (Rotman, 1961; Rotman, 1973). This method is exploited successfully even today, see e.g. (Xue, 1995; Craig, 1996). The method relies on the amplification of sample by repetitive chemical transformations.

The first successful optical detection of a single molecule (with multiple fluorescent labels) in a liquid was reported by Hirschfeld (Hirschfeld, 1976). By contrast, this method relies on repetitive emission of photons by a single molecule. In subsequent years, large progress was made in the refinement of the methodology (Dovichi, 1983; Dovichi, 1984; Mathies, 1986; Nguyen, 1987a-b; Peck, 1989; Mathies, 1990; Rigler, 1990; Shera, 1990), making single molecule detection (SMD) of single fluorescence labels in fluids a nearly routine procedure today (Hahn, 1991; Soper, 1991a; Rigler, 1992; Soper, 1992; Castro, 1993; Goodwin, 1993a; Soper, 1993; Wilkerson, 1993; Lee, 1994; Mets, 1994; Nie, 1994; Tellinghuisen, 1994; Li, 1995; Funatsu, 1995; Mertz, 1995; Soper 1995a; Berland, 1996; Chiu, 1996; Edman, 1996; Sauer, 1996; Wu, 1996; Zander, 1996). Comprehensive overviews of SMD in fluids can be found in (Barnes, 1995; Goodwin, 1996a,c; Keller, 1996).

The standard fluid-flow SMD system is similar to common flow cytometry systems. A sample stream containing the analyte molecules is injected into a surrounding sheath flow, providing hydrodynamic focusing (Kachel, 1990) of the sample stream. The sample stream is transported to the detection region, where a tightly focused laser beam excites the molecules (picoliter detection volume). Fluorescence is monitored by highly efficient collection optics and a single photon sensitive detector. Single fluorophores can emit ca. 10^8 photons/sec; the main problem in SMD is not so much the detection of the molecule's fluorescence but the efficient rejection of the background signal. The two main sources of background are fluorescence from contaminants, and Rayleigh/Raman scattering of the exciting laser beam by the solvent. The use of ultrasmall vol-

umes and efficient optical filters is effective in reducing the background. In addition to the small volume and optical filters, other methods of background rejection have been applied. One of the most common methods is the application of pulsed laser excitation together with a time gate in the detection channel that is used to reject prompt scatter, see e.g. (Harris, 1983; Shera, 1990). In (Guenard, 1996), the use of a highly efficient narrow band metal vapor filter for blocking the laser light and its applicability in SMD was investigated. For reducing impurity fluorescence of the sheath flow, in-line photobleaching before the detection region was found to be effective (Affleck, 1996). Another approach is the exploitation of two-photon excitation (Mertz, 1995; Berland, 1996; Overway, 1996), which was found to be useful for the reduction of background. Soper et al. are promoting the application of near-infrared dyes (Soper, 1995a-b), since there is a strong decrease of light scattering intensity and impurity fluorescence at longer wavelengths.

Besides the already mentioned suppression of scattered laser light by time-gating, the detection of the fluorescence decay characteristics of single molecules provides a convenient tool for distinguishing between different molecules. Recently, the application of the time-resolved single-photon counting (TCSPC), see (O'Connor, 1984), for lifetime measurements in SMD has received considerable interest. The first successful life-time measurements at the single molecule level were reported in (Soper, 1992; Wilkerson, 1993; Tellinghuisen, 1994). In (Enderlein, 1995b-c; Erdmann, 1995), new TCSPC-electronics, allowing for the continuous detection of TCSPC curves in millisecond intervals, was described and its application for SMD discussed. In (Müller, 1996), a continuous TCSPC technique was successfully applied to distinguish between molecules with different fluorescence decay times at the single molecule level. In (Seidel, 1996), nucleobase specific quenching of fluorescent dyes was studied, which could be important for the application of TCSPC-SMD to DNA sequencing (see below). Finally, (Sauer, 1996) reported the use of a diode laser as a light source in SMD, which will be of great importance for future broad biological and chemical applications of SMD, requiring simple, low cost, and compact operation.

A number of recent papers are dedicated to the theoretical study of SMD, mainly its statistics, maximum possible efficiency, and the usefulness of TCSPC in SMD (Stevenson, 1992a-b; Whitten, 1992; Köllner, 1992; Köllner, 1993; Tellinghuisen, 1993; Enderlein, 1995a), see also (Köllner, 1996) for a comparison between theory and preliminary experiments.

An already realized application of SMD in fluid flow is DNA fragment sizing (Ambrose, 1993; Castro, 1993; Goodwin, 1993b; Johnson, 1993; Petty, 1995; Huang, 1996). DNA sizing at the single molecule level became especially feasible after the introduction of an new class of intercalating dyes (Rye, 1992; Rye, 1993), which show extremely low fluorescence in their unbound state. In (Huang, 1996), fluid flow SMD was applied for the first time to the sizing of human DNA (bacterial artificial chromosome clones).

One of the most exciting potential applications of SMD in fluid flow is DNA sequencing (Jett, 1989; Davis, 1991; Soper, 1991b; Harding, 1992; Ambrose, 1993; Goodwin, 1993c; Eigen 1994; Goodwin, 1995; Goodwin, 1996b). Although much progress has been made in recent years to achieve this goal, no group has yet reported the successful sequencing of a single DNA molecule. Nonetheless, SMD promises to be a high speed method for reading long (> 10 kbase) DNA sequences.

In addition to SMD in fluid flow, a number of groups have reported SMD in gel electrophoresis experiments (Guo, 1992; Castro, 1995a-b; Haab, 1995; Soper 1995b; Chen, 1996). Guo, Castro and Haab applied the method to DNA sizing. Chen studied the limitations of quantitative analysis at the single molecule level.

A completely different method of SMD was applied by Ramsey and coworkers. They used levitated diluted microdroplets for SMD (Whitten, 1991; Kin, 1992; Ng, 1992; Barnes, 1993; Barnes, 1996; Hell, 1996). An advantage of the method is the low background level due to the small volume of illuminated liquid. The main applications of their technique is ultrasensitive chemical analysis, and investigations of quantum confinement effects.

Related to SMD in fluid flows is SMD in fluorescence correlation spectroscopy (FCS). The main setup of FCS is similar to SMD in fluid flows, but without hydrodynamic flow. The molecules move in and out of the detection region by diffusion. For a comprehensive review of FCS see e.g. (Thompson, 1991). One advantage of FCS is the use of a much smaller laser focus (of the order of 0.5 μm) and thus detection volume of femtoliters, reducing significantly the background signal. This is in contrast to fluid flow SMD where the laser focus is set large enough to detect all molecules in the sample stream. The disadvantage of FCS is its intrinsically 'non-sequential' character - one has to wait until a specific molecule diffuses into the detection region.

Nonetheless, FCS was applied successfully to kinetic studies at a single molecule level, such as probe-target binding and triplet state kinetics (Rigler, 1992; Rigler, 1993; Mets, 1994; Widengren, 1994; Edman, 1995; Kinjo, 1995; Rigler, 1995; Widengren, 1995; Edman, 1996), see also (Nie, 1994; Nie, 1995).

At the end of this section, we mention a new technique of optical SMD in fluids: the excitation and detection of surface-enhanced Raman signals from single molecules adsorbed on colloidal metal particles diffusing through a focused laser beam. First experimental studies approaching this technique were reported in (Kneipp, 1994; Kneipp, 1995a-d). Because of the adsorption of the molecules on metal particles, this method can be considered as a hybrid between SMD on surfaces (which is discussed below) and SMD in fluids.

3. Single Molecule Detection on Surfaces

As mentioned in the Introduction, we will consider SMD at room temperature only. The two main methods for optically detecting single molecules on surfaces are far-field and near-field microscopy.

3.1. Far-Field Microscopy

In far-field microscopy, two different approaches have been used for SMD: confocal microscopy and conventional wide-field microscopy. In confocal microscopy, the sample is illuminated by a tightly focused laser beam that is scanned over the surface for recording a complete image. For recent reviews of confocal optical microscopy see (Inoue, 1995; Webb, 1996). The first successful detection of single Rhodamine-6G molecules by a confocal scanning system was reported in (Dapprich, 1995). (Ambrose, 1996) and (Macklin, 1996) used this technique to measure time-resolved fluorescence of single molecules. The advantage of the technique is the relatively low background due to the small illuminated area combined with spatial filtering, and the possibility to obtain time-resolved fluorescence data. This is not possible in conventional wide field microscopy due to the current absence of commercially available single-photon sensitive cameras with sub-

nanosecond time-resolution, but see (Ho, 1993), and (Köllner, 1994; Kalusche, 1995) for plans of a construction of such a camera for SMD applications.

Many groups are using conventional wide field microscopy together with high-sensitivity low-noise optical cameras. The first report of disodium fluorescein detection on a silicon single-crystal wafer with such a system was (Ishikawa, 1994). In (Schmidt, 1995; Schmidt, 1996a-b), this technique was applied to the detection and tracking (on a millisecond time scale) of single molecules at an air-liquid interface. Fluorescence collection by a conventional wide field microscope objective was also the basis of a 3-dimensional monitoring of single molecules in a gel layer (Dickson, 1996), where fluorescence excitation was achieved by the evanescent field of total internal reflection. Ueda reported the monitoring of single DNA molecule phase transitions (Ueda, 1996).

3.2. Near-field Microscopy

Another form of optical microscopy that has been used widely for SMD is near-field microscopy. The idea is to illuminate the sample with a light source of sub-wavelength spatial extent, thus circumventing the Abbe limit of spatial resolution in conventional microscopy. This approach was first described in papers by Synge (Synge, 1928; Synge, 1932). Today, there are several different techniques for near-field microscopy. The most frequently used in SMD is the transmission near-field scanning optical microscope (NSOM or SNOM). In NSOM, the sample is illuminated using a tapered metal coated optical fiber. A small aperture in the metal coating at the apex provides a light source of approximately 100 nanometers across. In transmission NSOM, the excited fluorescence of the sample is monitored by a conventional microscope through the optically transparent support of the sample. By scanning the exciting fiber over the sample surface, a spatially resolved image of the sample is generated. For recent reviews of the NSOM technique see (Harris, 1994; Kopelman, 1994; Paesler, 1996; Trautman, 1997). With this method, the detection of single molecules has been widely investigated (Betzig, 1993; Ambrose, 1994a-b; Betzig, 1994; Dunn, 1994; Trautman, 1994; Xie, 1994; Ambrose, 1995; Bian, 1995; Dunn, 1995; Meixner, 1995; Bopp, 1996; Lu, 1997). A recent review can be found in (Xie, 1996). Again, as in far-field confocal microscopy, the point probe character of the NSOM allows

for time-resolved detection of the molecules fluorescence (Ambrose, 1994b; Dunn, 1994; Trautman, 1996). Ha et al. used this technique for monitoring the fluorescence polarization of single molecules, and energy transfer between two different single molecules (Ha, 1996a-b).

At present, SMD with NSOM has been reported only for apertures ≥ 100 nm in diameter. Quenching of fluorescence by the metal coating at smaller diameters and a larger relative background (Trautman, 1997) may prevent further size reduction for SMD. A possible improvement in future generation NSOMs could be the introduction of new optical probes, like the tetrahedral tip of (Koglin, 1996a-b), using surface plasmons for generating a sub-wavelength light source, or the exploitation of micro-photodiodes (Davis, 1995; Akamine, 1996).

Besides the NSOM technique, the so called apertured photon scanning tunneling microscope (apertured PSTM) was used for detecting Rhodamine-6G molecule aggregates (Tsai, 1995). In this technique, the sample surface is illuminated by the evanescent field of a totally reflected light wave, which is incident from beneath the transparent sample support. The fluorescence is then collected by a metal coated tapered fiber, which is equivalent to the excitation probe in an NSOM. The low collection efficiency of this setup may prevent its application to real single fluorophore detection.

There are two promising alternatives to the NSOM and PSTM technique, which are worthy of mention in the context of SMD. The first class of new techniques can be called near-field disturbance methods. The idea is to use a small (nanometer-range) metallic probe disturbing a near-field configuration (and thus generating extremely confined electromagnetic fields), and to measure the interaction of disturbance with the sample (Pedarnig, 1992; Specht, 1992; Pedarnig, 1993; Bachelot, 1994; Inoyue, 1994; Zenhausern, 1994; Bachelot, 1995a-b; Zenhausern, 1995; Wickramasinghe, 1996). The advantage of this methods is the potential very high spatial resolution, which can be better than a nanometer. It remains to be seen whether it has the sensitivity to detect single molecules.

The second class of techniques uses the emission of photons in an scanning tunneling microscope (STM) (Gimzewski, 1989; Berndt, 1993; Berndt, 1994; Berndt, 1995). One expects that this emission will depend critically on the close environment of the STM metal probe, including

single molecules. Again, the achievable spatial resolution could be in the sub-nanometer range comparable to standard STM.

4. Conclusion

In the present chapter, we presented an overview of techniques for SMD at room temperature, potentially applicable to high-speed and high-throughput screening of large molecular libraries. The detection speed and throughput of fluid flow SMD, FCS, and wide-field microscopy have the greatest potential for such applications. Already, fluid flow SMD is successfully applied to DNA fragment sizing. A promising application of SMD is DNA sequencing, which could lead to a method of rapid sequencing of long DNA fragments. In addition, imaging techniques with single molecule sensitivity have the possibility for interrogating large libraries of molecules.

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