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Intraoral Fiber-Optic-Based Diagnostic for Periodontal Disease

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ABSTRACT

The purpose of this initial study was to begin development of a new, objective diagnostic instrument that will allow simultaneous quantitation of multiple proteases within a single periodontal pocket using a chemical fiber optic sensor. This approach could potentially be adapted to use specific antibodies and chemiluminescence to detect and quantitate virtually any compound and compare concentrations of different compounds within the same periodontal pocket. The device could also be used to assay secretions in salivary ducts or from a variety of wounds. The applicability is, therefore, not solely limited to dentistry and the device would be important both for clinical diagnostics and as a research tool.

Keywords: dental, biosensor, fiber optic

1. BACKGROUND AND SIGNIFICANCE

Periodontal diseases are plaque-induced disorders that cause significant loss of gingival attachment and resorption of alveolar bone, and are the number one cause of tooth loss in the United States. Detection of severe or moderate periodontal diseases is relatively easy with conventional probing techniques since at this stage significant anatomical damage is apparent [1]. The challenge is to detect the disease early enough that minimally invasive therapeutic procedures can be implemented which are less costly and often more effective than periodontal surgery [2].

The condition for a successful early detection strategy is determined by the complex etiology of periodontal diseases. Periodontal diseases are site-specific, with local anatomical, microbiological, immunological, and environmental conditions affecting disease pathogenesis [3]. Whatever diagnostic is applied should therefore result in minimal perturbation of the sampled region, since flow or volume changes can quantitatively alter the composition of the biochemical constituents present [4]. Ideally, the measurement should be a simple single stage procedure that directly and rapidly assesses disease state *in vivo*. This assessment should include as many biomarkers associated with disease activity as possible to maximize the specificity and selectivity of the measurement. In addition, since it is currently unknown whether periodontal destruction occurs in random bursts or through a slow continuous process, a larger number of measurements over a given period of time is desirable [4]. The detection method should therefore be inexpensive, robust and ultimately designed for chairside testing. Finally, the detection of potential markers should take place in the gingival crevicular fluid (GCF), where most of the useful diagnostic information is contained. This fluid bathes the tissues that lie at the interface of the host defense system and the bacterial flora, acting as a medium for transporting bacterial and host-derived products into and out of the periodontal environment.

Unfortunately, no diagnostic is currently available which includes all or even most of these requirements, although a number of test-kits have recently been commercialized [4]. These chair-side tests are designed to measure immunological or microbiological constituents of the GCF that are implicated in periodontal disease progression. Generally, all these tests use a capillary tube or filter paper to sample the GCF followed by a colorimetric reaction to determine disease activity. The measurement outcome is highly sensitive to the technique used in harvesting the sample, making reproducibility difficult [3].

Finally, the sensitivity and selectivity of these diagnostics is fundamentally limited by their inability to simultaneously measure the local concentration of multiple disease biomarkers.

The system we propose to develop, on the other hand, meets all the criteria identified above. Indicator chemistries placed at the end of imaging fiber optic bundles could be used to simultaneously measure concentrations of multiple biomarkers associated with periodontal diseases in the GCF. The parallel quantitation of relevant microbiological and immunological agents in the GCF should present a much more sensitive determination of disease activity than any previous measurement technique. In addition, the fiber optic biosensor will probe the sample GCF volume directly, allowing direct measurement of relevant biomarker concentrations in a localized region and eliminating errors associated with sampling methodologies.

2. MICROJETTING FLUORESCENT ASSAYS ON FIBER OPTIC BUNDLES

There has been intensive research in the area of optical-based chemical sensor and detection methods for biomedical applications [5, 6]. These sensors typically consist of an indicator chemistry attached to the end of a fiber optic or other optically accessible surface, where the indicator chemistry is designed to change its optical properties (i.e. fluorescence or absorption) quantitatively in response to the presence of a target ligand or analyte. Light of a suitable wavelength is used to illuminate the sensing zone. Some portion of this light is absorbed by the indicator chemistry(s). The absorption or re-emission of this radiation in the form of fluorescence is monitored via photosensitive detectors and the resulting signal used to make qualitative or quantitative determinations concerning a ligand or analyte of interest. Most conventional optical-based chemical sensors, particularly those based on fiber-optic technology, require a separate sensor for each target ligand. Recently, however, researchers at LLNL and Tufts University have developed a method for immobilizing several blood gas assays (O_2, CO_2, pH) in discrete areas on the end of a fiber optic bundle [7]. Imaging techniques allow unique identification of each assay by their geometry and location on the fiber bundle tip. This has several advantages over previous one-indicator /one-fiber sensors. The primary advantage of this technique is that different ligands in a very localized region can be simultaneously detected and/or measured *in vivo*. This is particularly important for biomedical applications, where collection or interrogation of a small sample volume (i.e. blood, gingival crevicular fluid, saliva) is desirable or necessary. A secondary advantage of this multi-analyte sensor is the ability to use the same excitation and/or emission wavelength for all the different indicator chemistries, greatly simplifying the complexity and cost of the spectroscopic components and indicator chemistry. As part of the Tufts research project, LLNL constructed a portable imaging spectrometer for measuring and analyzing fluorescent emissions from a fiber bundle.

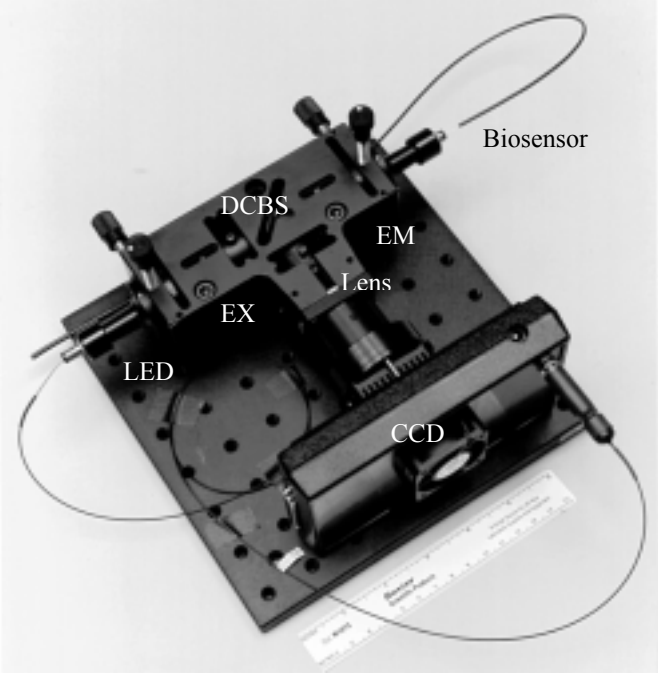


Figure 1: Portable imaging spectrometer for multianalyte sensor. Fluorescence from multiple sensing sites immobilized on the distal tip of the biosensor fiber optic bundle is filtered and directly imaged on the CCD camera. DCBS=dichroic beamsplitter, EM=emission bandpass filter, EX=excitation bandpass filter, and LED=light emitting diode.

The main reason this particular instrument never found wide-spread use was the difficulty in reproducibly and inexpensively fixing indicator chemistries on an optical substrate. These limitations make disposable sensors impractical, greatly limiting the sensors' usefulness in clinical applications where infection control is necessary. Reproducibly attaching precise amounts of a given indicator chemistry in a well-defined geometry on a fiber optic or other supportive surface is difficult, moreover, conventional methods of creating optical-based chemical sensors result in high inter- and intra-sensor variability. This variability significantly increases the complexity and cost of the manufacturing process and makes individual calibration for each sensor necessary. The large advance our probe over these earlier methods, therefore, is the reproducibility and cost-effectiveness with which microjet-fabricated sensors can be constructed.

We have, to date, performed a small pilot study to determine the feasibility of using micro-jet technology for creating multi-component optical-based chemical sensors. The goal of this project was to reproducibly print a six-around-one pattern of fluorescein/polymer microdots (< 100 μm diameter) on the end of a 500 μm diameter fiber optic bundle. The company selected for this task, Microfab, has over 13 years' experience in developing inkjet/microjet printing processes for use in a variety of manufacturing applications. They have microjetted a wide range of materials, including liquid metal solders, fluxes, adhesives, dies, pigments, and phosphors. Microfab has developed both single jet and 120-jet arrays capable of printing 40-80 μm micro-droplet sizes.

The equipment, print head device configuration, and printing process utilized in the first phase of this project (Fig. 2a) were similar to those used for the printing of micro-optical elements [8]. The key features of this system are the automated microjet print head and the motion control systems for printing accurately at pre-specified target sites and for shuttling between print head, UV-light pipe and target-viewing system. The printing axis is in the vertical plane. A horizontal microscope with stroboscopic illumination is utilized for viewing the microjetted droplets during microjetting process optimization for a particular material. A clean-air/exhaust system is necessary to prevent airborne particulate contamination of the substrate and to evacuate any fumes arising from heating of typical polymeric formulations to the temperatures required (up to 200°C) to reduce their viscosities to the 20-30 cps level needed for microjet printing. The fluids contained in the print head reservoir are typically kept under a nitrogen atmosphere prior to being emitted by the print head device orifice, in order to prevent any degradation from oxygen-driven reactions during heating. With such a drop-on-demand printing system, a droplet is emitted from the device orifice every time an appropriate driving pulse produces a displacement of the piezoelectric element in the device. The image shown in Figure 2b has an exposure time of 0.5 seconds. It is a superposition of about 1000 droplets, indicating by its clarity the time and spatial precision and reproducibility of the microjetting process.

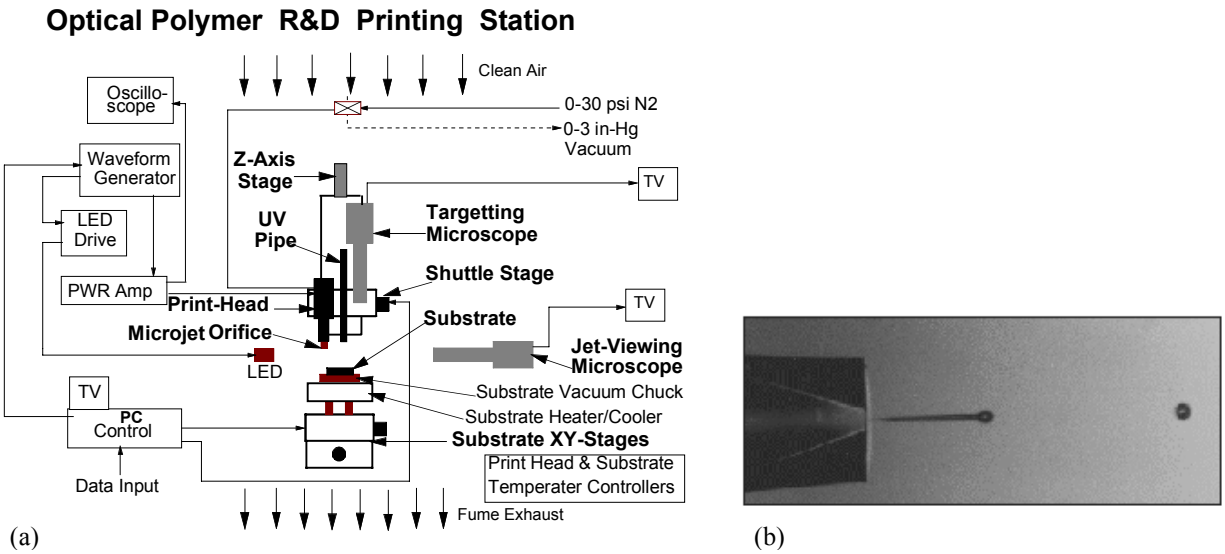
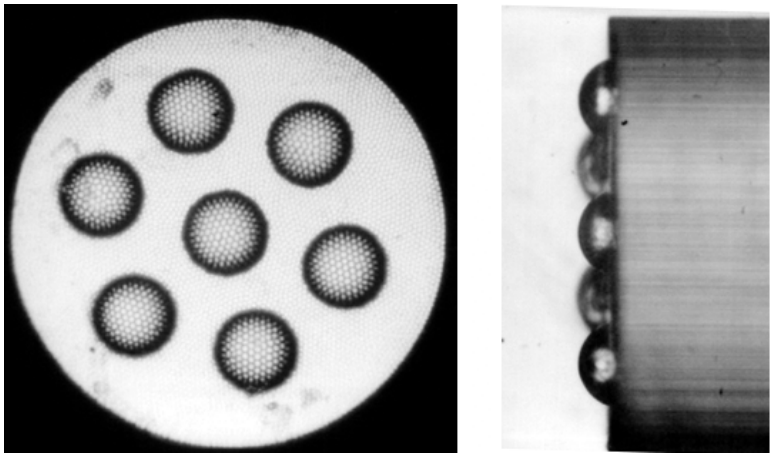


Figure 2: (a) Schematic of Polymer Printing Station used in printing patterns of dye-doped microdots onto the tips of optical fiber and (b) stroboscopically illuminated image of superposition of 50 μm droplets of fluid being emitted from a microjet print head with 50 μm orifice diameter operating at 2000 Hz.

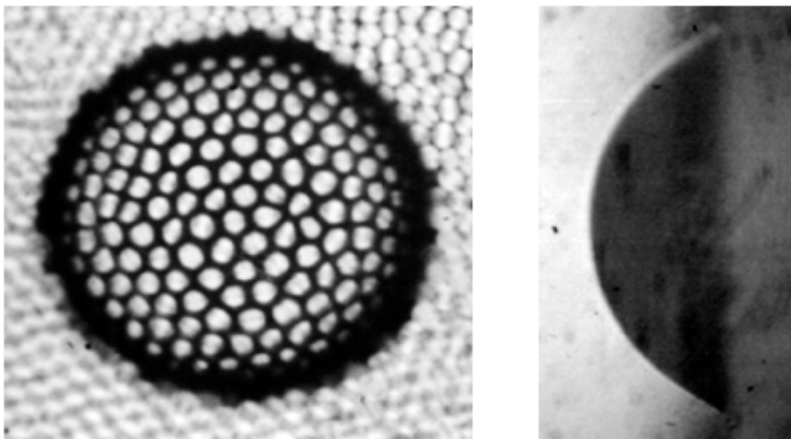
After striking a target, the material of the droplet spreads to an equilibrium diameter. This diameter depends heavily on the characteristics of the material being printed, the degree of wetability of the substrate surface by the material and the speed with which its flow may be arrested by initiation of solidification, e.g., by *in-situ* UV curing or cooling. Control of the dimensions and aspect ratio of a printed element to a given specification is obtained by adjusting the following variables:

(a) number and diameter(via device orifice size) of droplets deposited at a target site;
(b) characteristic contact angle of the low-wet coating applied to the substrate;
(c) temperature of the substrate during printing.



The results of this initial study were very promising. Microfab printed six-around-one pattern of fluorescein microdots on the end of six fiber bundles (Fig. 3). The diameter of the circle through the centers of the circumferentially printed microdots is 260 μm, indicating this pattern easily fit on the end of the 480 μm fiber bundle. The deviation between the central microdot and fiber bundle axis is approximately 2 μm.

Figure 3: (a) Top view and (b) side view of seven 90μm diameter microdots inkjet printed on a 500μm diameter fiber optic bundle. Each microdot contains a fluorophore contained in a UV curable polymer matrix.



The reproducibility of the fiber printing process was excellent for both microdot diameter and roundness (Fig 4). All fiber-average and standard deviations for these data are:

Microdot diameter = 93.3 +/- 2.2 μm

Microdot roundness [(max. diameter - min. diameter)/ avg. diameter] = .00072 +/- .00023

Figure 4: Expanded (a) top view and (b) side view of a single 90μm diameter microdot inkjet printed on a fiber optic bundle. The polymer matrix acts as a lens from the top view, providing a clear image of the individual fibers that comprise the fiber bundle.

We also made spectroscopic measurements using the imaging spectrometer (Fig.1) described above. Microdot intensity, both on a single fiber bundle and between different fiber bundles, varied by less than 2%. These first studies clearly demonstrated the capabilities of microjet technology for reproducibly printing a pattern of identical but spatially discrete sensing regions on a fiber optic bundle.

3. PERIODONTAL DISEASE ASSAYS

The GCF components that are most promising for developing diagnostic tests of periodontal disease activity include the collagenase enzymes, neutral protease's, the enzymes alkaline phosphatases and aspartate aminotransferase, cytokines, and antibodies, among others [3]. We are initially focusing on development of sensors that assess the activity of the enzymes responsible for tissue destruction and loss of tooth attachment. These enzymes, known as matrix metalloproteases (MMP), participate in the breakdown of the major protein components of the extracellular matrix. The metalloproteases account for most of the tissue degradation observed in periodontal disease and include the enzymes collagenase, gelatinase, and stromelysin. The activity of these enzymes has been directly linked to inflammation in humans with gingivitis [3].

The activity of proteinases can be determined by the rate at which the enzyme cleaves a specific amide linkage that binds two amino acids of a particular sequence in the protein substrate. An enzyme usually catalyzes a single chemical reaction such as cleaving a peptide chain. However, rather than determine the rate at which an intact protein is cleaved, sensitive assays have been developed which use a short amino acid sequence that represents the substrate portion of protein recognized by the MMP [9]. These sequences are usually only six to ten amino acids in long. The polypeptide is prepared with two different fluorescent dyes, one at each end of the substrate molecule. These dyes are specially chosen because they form an energy transfer pair, such that when the dye molecules are within a minimal distance from one another, energy absorbed by one dye (the donor) is transferred directly to the nearby second dye (the acceptor).

The use of fluorescence energy transfer systems in analytical biochemistry is a common technique employed in academic laboratories as well as commercially successful products. The TaqMan DNA probe used to detect specific DNA sequences, for example, employs the energy transfer pair of fluorescein and rhodamine[10]. The growing awareness that matrix metalloproteinases play a significance role in various cancers has spurred the development of new laboratory assays based on fluorescence energy transfer pairs for specific matrix metalloproteinases.[11] These assays basically extend the approach used in earlier MMP assays which exploited the natural fluorescence of tryptophan as the indicator of enzyme activity[12]. In these initial assays, tryptophan was used as an energy donor and a nitroaromatic compound was used as the energy acceptor. The non-fluorescent energy acceptor acted to quench the fluorescence of an appropriately placed tryptophan residue on an MMP substrate. As long as the peptide chain carrying both the tryptophan donor and the nitroaromatic acceptor remained intact no tryptophan fluorescence could be detected. However, when the substrate is cleaved by the MMP, the donor and acceptor become separated on different peptide segments and the tryptophan is no longer able to transfer energy to the acceptor, and therefore returns to the electronic ground state by loosing energy as fluorescence.

In our periodontal probe, we intend to use the fluorescein/rhodamine energy transfer pair because the optical absorption and fluorescence emission occur in the visible portion of the electromagnetic spectrum which can be accessed with lost cost light emitting diodes and silicon photodiodes detector. When the substrate molecule is subjected to protease activity, the molecule will be cleaved at a specific amino acid sequence between the two dyes (Fig. 5). The fragments that result from this activity separate in solution substantially beyond the minimal distance allowed for energy transfer to occur. Consequently, the energy absorbed by fluorescein will not be transferred to rhodamine but rather will be emitted as fluorescence from fluorescein's emission manifold with a maximum at 512 nm.

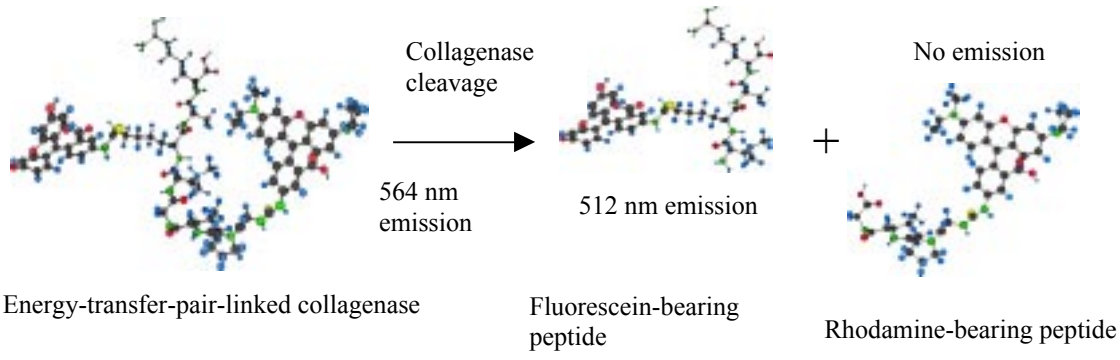


Figure 5: Collagenase cleavage reaction resulting in spectral shift from green to blue.

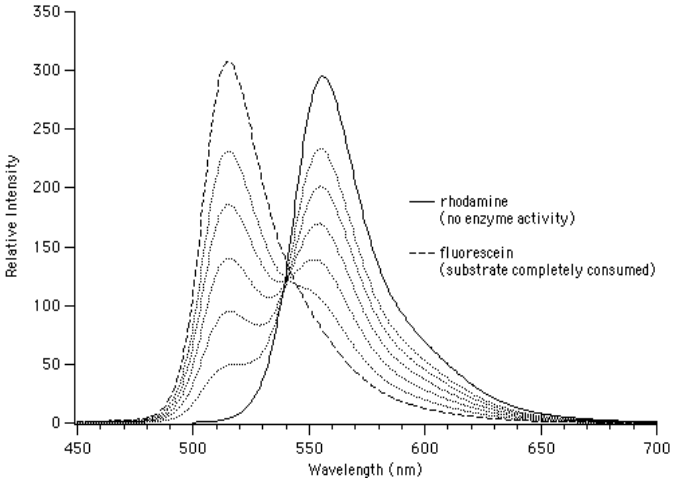


Figure 6: The intensity of the rhodamine signal decreases as the probe substrate is cleaved by the MMP enzyme. With the rhodamine no longer attached to the probe, it diffuses away leaving fluorescein to loose its absorbed energy through fluorescence. The intensity of the rhodamine emission diminishes while the fluorescence emission from fluorescein increases as the energy-transfer-pair-linked substrate is cleaved by enzyme.

As the indicator chemistry on the probe continues to be subjected to collagenase activity, the observed probe spectra will shift from complete emission from rhodamine to complete emission from fluorescein (Fig. 6). The change in the ratio of blue light emitted from fluorescein (512 nm) and green light emitted from rhodamine (564 nm) is a measure of enzyme activity.

Although, as with many enzyme substrates, the enzyme specificity is not absolute, the activity of a particular enzyme for an substrate can be optimized by manipulating the structure of the substrate. The collagenase enzyme, for example, exhibits a high specificity constant k_{cat}/K_m for the amino acid sequence Pro-Leu-Ala-Leu-Lys-Ala-Arg-OH. Because other MMP enzymes associated with periodontal disease recognize a different substrate amino acid sequence [9], we can use a similar strategy to add assays for other MMP enzymes, such as gelatinase and stromelysin. Table 1 lists fluorogenic substrate probes that have been evaluated and used to measure the activity of several matrix metalloproteinases [9,13,14,15].

Table 1. Fluorogenic substrates for various MMPs.

MMP	Family	Specific Enzyme	Mwt(kDa)	Probe Sequence
MMP-1	collagenase	interstitial collagenase	42	Dnp-Pro-Leu-Ala-Leu-Trp-Ala-Arg- NH_2
MMP-2	gelatinase	gelatinase A	72	Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2
MMP-3	stromelysin	stromelysin-1	45	Dnp-Pro-Tyr-Ala-Tyr-Trp-Met-Arg-OH
MMP-7	gelatinase	matrilysin	19	Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2
MMP-8	collagenase	PMN collagenase	65	Dnp-Pro-Leu-Ala-Tyr-Trp-Ala-Arg--NH_2
MMP-9	gelatinase	gelatinase B	92	Dnp-Pro-Leu-Gly-Met-Trp-Ser-Arg- NH_2

The activity measurements of MMP enzymes are normally acquired from assays preformed in homogeneous aqueous media with the appropriate parameters optimized (e.g., pH, ionic strength, metal ion co-factors, temperature). This situation will not be possible with the periodontal assay because the substrate molecules will be attached to a polymer matrix fixed onto the end of an optical probe. Under these conditions the enzyme activity may be reduced and its substrate selectivity altered. However, by selecting polymers that are wettable, only slightly cross-linked, and biologically compatible, it is possible to minimize the effects of substrate immobilization and maintain a solution-phase-like environment so that the enzyme kinetics are only slightly modified. There are several types of polymers to choose from which are compatible with enzymes, including polyacrylamides, polyhydroxyethylmethacrylate, and various phosphazene polymers.

The speed and sensitivity of an assay are dependent on the values of K_M and k_{cat} of the enzyme catalyzed reaction. The K_M is the substrate concentration at which the reaction progresses at one half of its maximum rate. Therefore, a substrate-enzyme reaction that exhibits a very low K_M will generally be more sensitive than one with a higher value. However, the K_M says nothing directly about the reaction rate, k_{cat}. Obviously, under the right conditions a reaction with a large rate constant will proceed more rapidly than one with a smaller rate constant, but the reaction may not necessarily be capable of proceeding in a reasonable timeframe if the value of K_M is to large and the actual substrate concentration is low. The ratio of k_{cat}/K_M is a measure of the enzyme's selectivity for a substrate and a large value suggests a reaction that is both relatively fast and sensitive. Table 2 shows values of these parameters for specific enzyme-substrate reactions.

Table 2. Kinetic values for reaction of various MMPs with fluorogenic substrates [9].

Enzyme		Substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})/K_M (μM)
MMP-1	EC 3.4.24.7	Dnp-Pro-Leu-Ala-Leu-Trp-Ala-Arg- NH_2	1.2	130	9400
MMP-2	EC 3.4.24.24	Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(DNP)-NH_2	2.70	50	58000
MMP-3	EC 3.4.24.17	Dnp-Pro-Tyr-Ala-Tyr-Trp-Met-Arg- NH_2	0.24	100	2400
MMP-7	EC 3.4.24.23	Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2	ND	ND	169000
MMP-8	EC 3.4.24.34	Dnp-Pro-Leu-Ala-Tyr-Trp-Ala-Arg--NH_2	3.1	7.7	400000
MMP-9	EC 3.4.24.35	Dnp-Pro-Leu-Gly-Met-Trp-Ser-Arg- NH_2	0.07	5.2	13000

4. PORTABLE SPECTROMETER

Currently at LLNL we have a single excitation, single emission imaging spectrometer for imaging fluorescent intensities from the end of a fiber optic bundle. This design will be modified in this portion of the project to incorporate a second imaging path for simultaneously detecting both fluorescein and rhodamine emissions (Fig 7). In our biosensor a collagenase assay will be printed on the distal end of an imaging fiber optic bundle. Excitation light from a light emitting diode (LED) is propagated down the fiber bundle, illuminating the distal end. The target proteases will then react with the indicator chemistries immobilized on the fiber tip and produce a spectroscopic shift in the induced fluorescence. By coupling these imaging fibers to a set of charge coupling device (CCD) cameras, the ability to spatially discriminate the multiple sensing sites on the fiber tip is possible, allowing simultaneous monitoring of multiple biomarkers. The LED and CCD cameras will both be controlled and monitored using Labview software interfaced to a computer. Acquisition rates of 5 Hz are possible with this system, making "real-time" measurements of collagenase concentrations feasible.

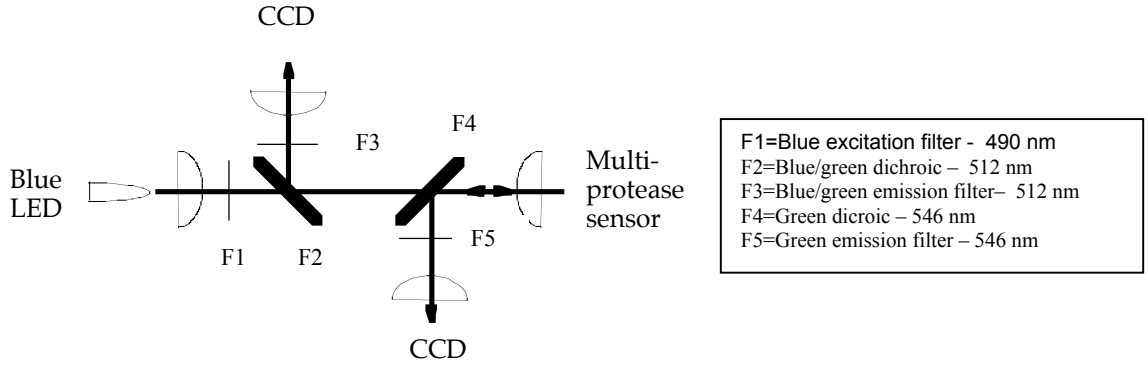


Figure 7: Schematic of single excitation, dual emission imaging spectrometer.

5. ACKNOWLEDGEMENTS

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