

Electrophoretically Actuated Nanoscale Optotropic Transduction Mechanisms

Benjamin Sullivan,* Dietrich Dehlinger,* Sanja Zlatanovic,** Sadik Esener** and Michael J. Heller.*

*Department of Bioengineering

**Department of Electrical Engineering

University of California, San Diego

9500 Gilman Drive, La Jolla, CA, 92093. bdsulliv@ucsd.edu

ABSTRACT

We have shown that DNA assembled fluorescent resonant energy transfer systems oscillate in response to electrophoretic actuation in an acrylamide model of *in situ* hybridization. Integration of fluorescence over time produced clear peaks in Fourier space with signal to noise ratios far above static levels. Frequency responses were demonstrated between 5 Hz to 10 Hz. Actuation speed was likely limited by the ionic flux under low field strength of the relatively macroscale system. The ability to pull signals out of a high background of autofluorescence has important implications for improving the resolution of FISH, and in particular using oligonucleotide probes for the analysis of early stage genetic transitions from hyperplasia to carcinoma.

Keywords: FISH, FRET, electrophoresis, biosensors, nanoparticles

1. INTRODUCTION

In situ hybridization and immunohistochemical analyses represent some of the most sensitive techniques for the detection of protein and nucleic acid targets within intact cells. fluorescent in situ hybridization (FISH) tests have traditionally focused on the assessment of insertions/deletions, loss of heterozygosity, translocations, or the semi-quantitative classification of copy number [1-7]. Clinically, FISH has found utility as a confirmatory, secondary test for guiding therapeutic strategies, as well as within panels of diagnostic tests for acute leukemias [8-9].

Absent from traditional FISH assays are high resolution genetic mappings of the transitions from hyperplasia to carcinoma and metastasis, and specifically, the ability to perform single nucleotide polymorphism profiles of biopsied tissue. As a result of the autofluorescent background and propensity for nonspecific binding within cellular matrices, the shortest individually detectable FISH probes are on the order of a few hundred base pairs [10]. This also makes multiplexing within FISH difficult, as traditional kilobase probes offer little spatial discrimination on submicron length scales.

The lack of resolution of passive molecular FISH probes has, perhaps, limited its clinical utility; for instance, in situ hybridization also plays a role in the quantitative estimation

of minimal residual disease following leukemia remission, where the test carries significant specificity advantages over both reverse transcriptase PCR (RT-PCR) and traditional metaphase cytogenetics [11]. However, while RT-PCR is able to distinguish the subtype of the t(9;22) derived protein product (i.e., p190 vs. the less active p210 tyrosine kinase) FISH lacks the resolution to do much more than verify the existence of a translocation [11]. As such, FISH is not recommended as a cost-effective first line diagnostic for myeloid lymphomas, despite evidence of its superior receiver operating characteristic.

In-situ hybridization is also very time consuming and cumbersome. Tissue and cell preparations must be carried out with great care. Adding to the complexity, reagent binding conditions must be controlled with regard to reaction time, probe concentration, temperature, pH, ionic strength of electrolytes, and the presence of additional denaturing or chaotropic agents. In conjunction with these parameters, the subsequent washing steps determine the ultimate sensitivity and specificity of the FISH assay. Small changes in procedure or the nature of the cell preparation introduce intraobserver variation over time and between clinical laboratories [12,13].

Multiplexed oligonucleotide analysis of gene expression has been recently demonstrated in situ [14]. However, mRNA analyses lack the ability to monitor upstream promoter mutations that may contribute to dysregulation, and reliance upon the intrinsic amplification from multiple message molecules restricts multiplexed analysis to dominant transcribed products. Additionally, the high cost of labor restricts the number of nuclei that can be examined, which may skew statistical inferences gathered from the assay.

Despite the clear need for faster, higher resolution FISH methods, technical advancements for whole cell molecular assays have largely focused on improving the detectability of the post-hybridization label. Quantum dots, padlock probes, biotin, digoxigenin, and enzyme amplification have all been used for this purpose [10, 15-17]. These technologies have not, however, translated into effective single nucleotide polymorphism (SNP) profiling procedures, and intrinsic single molecule resolution has yet to produce viable clinical diagnostic assays at this level of sensitivity.

The inherent disadvantage of fluorescent over radiolabeled in situ hybridization is that one cannot expose a fluorescent tissue section for an extended period of time

to recover specific photons due largely to the presence of autofluorescent background noise from the cell matrix. Whereas individual radioactive grains can be exposed for weeks at a time to produce very strong responses from single probes, photobleaching of modern fluorophores, even nanoparticles, eliminate the possibility of direct integration over noise.

In accordance with these views, our research on active microelectronic arrays and modulated nanoscale transduction systems should significantly enhance the sensitivity and resolution of whole cell assays. The primary objective of this study was to examine the feasibility of actuating DNA assembled FRET systems using electrophoretic methods.

2. METHODS

An acrylamide model of *in situ* hybridization was developed in order to study the effects of vertically oriented electrophoretic fields on surface bound nanoscale transduction mechanisms. 14 μM biotin dextran [Sigma-Aldrich] was prepared in a 19:1 acrylamide:bis, TBE buffer solution. All buffers were prepared with 18.2 $\text{M}\Omega$ deionized water [Millipore]. 100 μL of the acrylamide solution was combined with 0.35 μL of TEMED [Sigma-Aldrich] and 0.35 μL of ammonium persulfate. The activators were stirred into the acrylamide rather than using aspiration to avoid excess oxygenation during mixing. 15 μL aliquots of biotin dextran acrylamide were immediately placed within a circular, 5 mm diameter wells fashioned from a hole punched, residual stress-free insulating tape adhered to gold coated slides [BioGold, Erie Microarray]. A partially wrapped borosilicate glass slide [Parafilm-M] was rapidly tilted on top of the acrylamide and a 200 g weight placed on top of the assembly to provide a uniform hydrophobic surface that was reasonably sealed from the oxygen atmosphere. The assembly was cured for 1.25 hours at room temperature, upon which time a hybridization solution (0.5M NaCl, 0.05 M sodium phosphate, pH 7.0) was poured over the slides. Capillary action lifted the top glass apart enough to allow the slides to be sheared apart with minimal effort. Normal forces were avoided during separation. Excess polymerized gel around the circumference of the well was carefully removed using a lint-free cloth. The polymerized dextran acrylamide was washed twice with hybridization solution. Cy3 labeled streptavidin was functionalized with a 1:1 concentration of biotin modified DNA taken from the p53 gene, with the wild type sequence p53B51WT: 5'-Biotin-GAA-CAG-CTT-TGA-GGT-GCG-TGT-TTG-TGC-CTG-TCC-TGG-GAG-AGA-CCG-GCG-CAC-3'. Complementary and 1 base pair mismatch sequences are shown below, with site-specific mismatches printed in lowercase:

p53cQSY7:
3'-QSY7-CTT-GTC-GAA-ACT-CCA-CGC-AC-5'

p53m1bpQSY7:
3'-QSY7-CTc-GTC-GAA-ACT-CCA-CGC-AC-5'

All DNA was purchased lyophilized from Trilink Biotechnologies, San Diego, CA. Stock solutions of DNA were reconstituted in pH 7.0, 0.1 M sodium phosphate and concentrations verified by measuring absorbance at 260 nm on a Perkin Elmer Lambda 800 spectrophotometer. Samples used for concentration verification were twice diluted to approximately 100 nM to ensure that UV adsorption was within a linear range.

Independently, equimolar amounts of p53B51WT functionalized Cy3 streptavidin were mixed with 1:1 ratios of complementary or quencher modified DNA, vortexed, briefly spun, and then allowed to hybridize for 3 hours. Samples were stored at 4°C in o-ring sealed plastic tubes prior to use. After washing the acrylamide constructs with hybridization solution, excess fluid on top of the gel layer was removed via suction at the top right corner provided by a lint-free cloth. 5 μL of the preassembled Cy3 streptavidin/capture probe/quencher system was added to the center of the gel and incubated for five minutes before washing and immersing in hybridization solution at room temperature until analysis. We found that higher concentrations and short incubations reduced diffusion into the subsurface of the acrylamide.

Shortly following the gel surface modification, the DNA constructs were analyzed under a custom built epifluorescent microscope mounted on an air-stabilized optical table. Gold slides are attached to an electrically insulated, current controlled Peltier stage [LDT 5910B, ILX Lightwave, Bozeman MT] located under a rotary Leica head fitted with long-range air objectives. Light from a 100W arc lamp is passed through a dichroic mirror and focused onto the sample, with the return light passing vertically through the dichroic and a green (Cy3) emission filter before being recorded by a Orca-ER CCD camera [Hamamatsu C4742, Hamamatsu City, Japan] driven by custom written software. A 64x64 pixel subarray of the 12-bit camera field is set to 8x8 binning mode to provide maximal contrast at ≈ 100 Hz A/D conversion through a National Instruments PCI-1422 board [National Instruments, Austin TX].

Electrical connections are facilitated by two 32 gauge platinum wires [13-766-10B Fisher Scientific] guided by micromanipulator probes [P110/360MT, Probing Solutions, Dayton NV] were connected in series with a programmable 1 kV, 100 mA power supply [237 SMU, Keithley Instruments, Cleveland OH] as well as in series with an external 100 Ω resistor. Parallel connections across the Keithley and resistor were fed into the analog inputs of a USB DAQ [6009 National Instruments] in order to simultaneously record applied power and estimate aliasing over time. The platinum electrode from the high Keithley terminal was connected to an exposed half of the gold slide with insulating electrical tape. Immediately before analysis, a second, circular, 3 mm diameter hole made within

residual stress-free insulating tape was placed over the center of acrylamide construct to mitigate low-resistance current paths originating from the acrylamide-well interface. 400 μ L of 0.01X TBE buffer was pipetted on top of the 3mm well, taking care to create a prolate spheroid of buffer with an off-center minor axis such that any bubbles that may release during electrophoresis are guided away from the objective's focal point. Finally, the upper electrode was positioned flat against the surface of the second layer of insulating tape, roughly 3 mm removed from the outer edge of the gel construct with the long axis of the wire spanning the diameter of the acrylamide.

Constructs were first exposed to an electronic stringency protocol in order to set a specific free energy threshold. The gold slide surface was biased negative compared to the upper electrode from -1 V to -6 V in 0.1 V/s increments. Following stringency, any bubbles that were observed to accumulate along the edge of the tape were removed by gentle agitation from a pipette tip.

Acrylamide constructs were then subjected to a sequence of three hundred monophasic square wave oscillations; (-3 V, 5 Hz), or (-6 V, 10 Hz), with the lower gold electrode biased as the cathode. Earlier qualitative findings had demonstrated that certain fully hybridized constructs did not oscillate when -3 V, 10 Hz was applied across the electrodes, but began to respond when given a higher power. These results indicated a complex spatial interaction between the ionic flux and the nanoscale transduction mechanisms, wherein the geometry of the device was the rate-limiting factor in coupling electrophoretic fields to the constructs.

3. RESULTS

DNA FRET constructs were shown to oscillate (Figure 1 & Figure 2) at 5 Hz and 10 Hz, respectively.

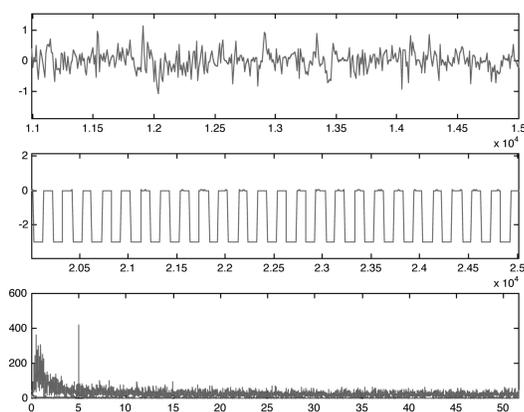


Figure 1. 5 Hz fluorescent response. (Top) Inset of detrended, low pass filtered fluorescent data. Horizontal axis represents sample number at ≈ 100 Hz. (Middle) Inset of electrical actuation. (Bottom) Absolute value of the discrete Fourier transform.

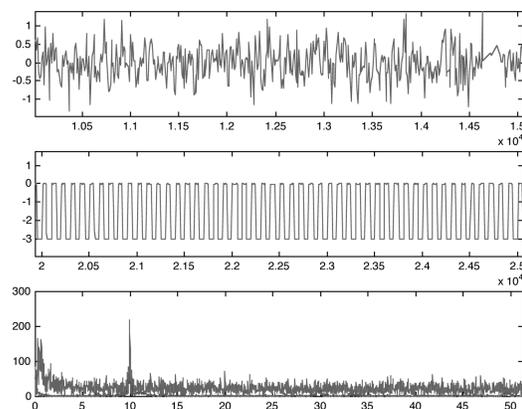


Figure 2. 10 Hz fluorescent response. (Top) Inset of detrended, low pass filtered fluorescent data. Horizontal axis represents sample number at ≈ 100 Hz. (Middle) Inset of electrical actuation. (Bottom) Absolute value of the discrete Fourier transform.

While not immediately discernable within the time series, frequency spectra reveal very clear responses as a result of electrophoretic actuation. These data indicate that low signal-to-noise ratio signals can be modulated to provide higher sensitivity within a model of *in situ* hybridization. As other groups have shown solution phase lock-in amplification using tethered molecules [18], it is interesting to note such an effect using freely hybridized DNA constructs. We are currently exploring additional experiments to support the proposed mechanism of action, as well as to examine the amplitude of oscillation due to environmental modulation of radiation rate as compared to intramolecular FRET transfer.

REFERENCES

- [1] Hirsch FR, Witta S. Biomarkers for prediction of sensitivity to EGFR inhibitors in non-small cell lung cancer. *Curr. Op. Oncology*. 17 (2): 118-122 MAR 2005
- [2] Dei Tos AP, Ellis I. Assessing epidermal growth factor receptor expression in tumours: What is the value of current test methods? *Eur. J. Cancer*. 41 (10): 1383-1392 JUL 2005
- [3] Munne S. Analysis of chromosome segregation during preimplantation genetic diagnosis in both male and female translocation heterozygotes. *Cytogenet Genome Res*. 2005;111(3-4):305-9.
- [4] Yeon CH, Pegram MD. Anti-erbB-2 antibody trastuzumab in the treatment of HER2-amplified breast cancer. *Investigational New Drugs* 23 (5): 391-409 OCT 2005
- [5] Ronski K, Sanders M, Burleson JA, Moyo V, Benn P, Fang M. Early growth response gene 1 (EGR1) is deleted in estrogen receptor-negative human breast carcinoma. *Cancer*. 2005 Sep 1;104(5):925-30.

- [6] Hartmann A, Stoehr R, Wild PJ, Dietmaier W, Knuechel R. Microdissection for detecting genetic aberrations in early and advanced human urinary bladder cancer. *Methods Mol Biol.* 2005;293:79-92.
- [7] Huang S, Li Y, Chen Y, Podsypanina K, Chamorro M, Olshen AB, Desai KV, Tann A, Petersen D, Green JE, Varmus HE. Changes in gene expression during the development of mammary tumors in MMTV-Wnt-1 transgenic mice. *Genome Biol.* 2005;6(10):R84
- [8] Navolanic PM, McCubrey JA. Pharmacological breast cancer therapy. *Int J Oncol.* 2005 Nov;27(5):1341-4.
- [9] Nachman J. Clinical characteristics, biologic features and outcome for young adult patients with acute lymphoblastic leukaemia. *Br J Haematol.* 2005 Jul;130(2):166-73.
- [10] Tanke HJ, Dirks RW, Raap T. FISH and immunocytochemistry: towards visualising single target molecules in living cells. *Curr Opin Biotechnol.* 2005 Feb;16(1):49-54.
- [11] Wang YL, Bagg A, Pear W, Nowell PC, Hess JL. Chronic Myelogenous Leukemia: Laboratory Diagnosis and Monitoring. *Genes, Chromosomes & Cancer* 32: 97–111. 2001
- [12] Thomson TA, Hayes MM, Spinelli JJ, Hilland E, Sawrenko C, Phillips D, Dupuis B, Parker RL. HER-2/neu in breast cancer: interobserver variability and performance of immunohistochemistry with 4 antibodies compared with fluorescent in situ hybridization. *Mod Pathol.* 2001 Nov;14(11):1079-86.
- [13] College Of American Pathologists: Cell Markers And Cytogenetics Committees. Clinical laboratory assays for HER-2/neu amplification and overexpression: quality assurance, standardization, and proficiency testing. *Arch Pathol Lab Med.* 2002 Jul;126(7):803-8.
- [14] Levisky JM, Shenoy SM, Pezo RC, Singer RH. Single-cell gene expression profiling. *Science.* 2002 Aug 2;297(5582):836-40.
- [15] Alivisatos AP, Gu W, Larabell C. Quantum dots as cellular probes. *Annu Rev Biomed Eng.* 2005;7:55-76.
- [16] Lherminier J, Bonfiglioli RG, Daire X, Symons RH, Boudon-Padieu E. Oligodeoxynucleotides as probes for in situ hybridization with transmission electron microscopy to specifically localize phytoplasma in plant cells. *Mol Cell Probes.* 1999 Feb;13(1):41-7.
- [17] Antson DO, Isaksson A, Landegren U, Nilsson M. PCR-generated padlock probes detect single nucleotide variation in genomic DNA. *Nucleic Acids Res.* Jun 15;28(12):E58, 2000
- [18] Braun, D and Libchaber, A. Lock-in by molecular multiplication. *Applied Physics Letters*, 83 (26): 5554-5556, 2003