Published in final edited form as:

Anal Chem. 2011 December 15; 83(24): 9485–9491. doi:10.1021/ac202212k.

Multiplexed Method to Calibrate and Quantitate Fluorescence Signal for Allergen-Specific IgE

Margo R. Monroe^{*,†}, Alexander P. Reddington[‡], Austin D. Collins[‡], Craig LaBoda[‡], Marina Cretich[§], Marcella Chiari[§], Frédéric F. Little^{||}, and M. Selim Ünlü^{†,‡,⊥}

[†]Department of Biomedical Engineering, Boston University, 44 Cummington Street, Boston, Massachusetts 02215, United States

[‡]Department of Electrical and Computer Engineering, Boston University, 8 St. Mary's Street, Boston, Massachusetts 02215, United States

[§]Consiglio Nazionale delle Ricerche, Istituto di Chimica del Riconoscimento Molecolare (ICRM), Milano, Italy

^{||}Pulmonary Center, Boston University School of Medicine, 715 Albany Street, Boston, Massachusetts 02118, United States

[⊥]Physics Department, Boston University, 590 Commonwealth Avenue, Boston, Massachusetts 02215, United States

Abstract

Using a microarray platform for allergy diagnosis allows for testing of specific IgE sensitivity to a multitude of allergens, while requiring only small volumes of serum. However, variation of probe immobilization on microarrays hinders the ability to make quantitative, assertive, and statistically relevant conclusions necessary in immunodiagnostics. To address this problem, we have developed a calibrated, inexpensive, multiplexed, and rapid protein microarray method that directly correlates surface probe density to captured labeled secondary antibody in clinical samples. We have identified three major technological advantages of our calibrated fluorescence enhancement (CaFE) technique: (i) a significant increase in fluorescence emission over a broad range of fluorophores on a layered substrate optimized specifically for fluorescence; (ii) a method to perform label-free quantification of the probes in each spot while maintaining fluorescence enhancement for a particular fluorophore; and (iii) a calibrated, quantitative technique that combines fluorescence and label-free modalities to accurately measure probe density and bound target for a variety of antibody–antigen pairs. In this paper, we establish the effectiveness of the CaFE method by presenting the strong linear dependence of the amount of bound protein to the resulting fluorescence signal of secondary antibody for IgG, β -lactoglobulin, and allergen-specific

IgEs to Ara h 1 (peanut major allergen) and Phl p 1 (timothy grass major allergen) in human serum.

Allergy, a disorder of the immune system characterized by a maladaptive immune response to otherwise harmless environmental antigens (“allergens”), affects nearly 50 million people in the U.S. with total estimated annual costs at nearly \$7B.^{1,2} The recent development of protein microarrays and the availability of recombinant allergens over the past decade have led to microarray- based allergy immunoassays testing for specific IgE in patient serum. The main advantages of these microarray *in vitro* diagnostics resides in the inherent capability to quantify allergen-specific IgE using only 10–100 μ L of serum per test and to better characterize allergen sensitization by measuring specific IgE to the component major allergens of a crude allergen extract (component-resolved diagnostics, CRD).³ However, two specific shortcomings exist with this technology. First, the typical microarray chips utilize assays with probes placed directly on a simple glass slide. In this configuration, the presence of a high index solid substrate (glass) in the immediate vicinity of the fluorophores reduces the fluorescence yield. Simple layered structures offer an inexpensive alternative to overcome the limitations imposed by glass slides and provide significant signal enhancement.⁴ Second, the more important limitation is related to the difficulty in obtaining quantitative results in conventional fluorescence based microarray tests. This difficulty arises from the variability in the amount of immobilized allergens that affects specific IgE capture and quantitation.^{3,5,6} As a result, fluorescence detection on typical glass (SiO₂) slides, the “gold-standard” technique used in microarrays, has limited sensitivity and may yield inaccurate results. These inadequacies can cause under-estimation or failure of detection for captured targets⁷ and concurrently yield unreliable clinical results.^{8–10}

Variability in microarray technology in general has become an essential concern in producing reliable data not only due to the technical variation, such as array printing, sample processing, analytes, plate, or person, but also due to the inherent nature of proteins themselves.^{11–13} Label-based procedures have been developed to account for this variation in probe deposition and binding to the surface in order to visualize the printed slides prior to experimentation.^{14,15} Although these techniques verify the presence of uniformly bound probe, they may negatively affect the activity of the probe, fail to quantify the amount of bound probe on surface, and alter physiochemical properties. Recently, an approach that utilizes a photonic crystal biosensor surface and a high resolution label-free imaging detection instrument to formulate prehybridization images of spotted nucleic acid array was recently reported as a sensitive method of quality control.¹⁶ Aside from being demonstrated only for DNA microarrays, this quality control method merely bins the spot as being suitable or unsuitable for analysis and does not offer the quantified amount of bound probe relative to secondary antibody (i.e., fluorescence). Although a variety of techniques have attempted to advance quality assurance of microarray technologies, a need for quantitative assessment providing calibrated microarray measurements still remains.

To address these issues, we have integrated our label-free technology, the interferometric reflectance imaging sensor (IRIS), a quantitative, high-throughput, simple, robust, and versatile technology used for multiplexed detection of DNA and proteins with high sensitivity comparable to surface plasmon resonance (SPR),^{17–20} with a new enhanced fluorescence technology to develop the calibrated fluorescence enhancement (CaFE) method.²¹ By combining the sensitivity of fluorescence with the quantitative nature of IRIS, the CaFE method addresses microarray reproducibility issues by (1) quantifying the probe amount with IRIS, (2) measuring the enhanced fluorescence signal generated by labeled secondary antibodies, and (3) calibrating the fluorescence signal utilizing the quantitative assessment of the spots by IRIS. While this technique is broadly applicable to a variety of

ligand-analyte based microarray platforms, it is particularly effective for allergy chips. Detection of allergen-specific IgE molecules necessitates the use of secondary antibodies to distinguish them from the large amount of physiologic allergen-specific IgG molecules that bind to the probe but are not indicative of allergic sensitization. The additional quantification challenge imposed by the large variability of immobilized probe density makes *in vitro* allergy diagnostics a perfect candidate for the demonstration of CaFE technology proof-of-concept.

A variety of layered structure designs could be used to achieve fluorescence enhancement for DNA and protein microarray applications.^{22–26} In this paper, we choose to implement a simple oxide on Si structure due to its low-cost and well-established characteristics. Our primary design parameters include wavelength range for fluorescence enhancement and label-free detection accuracy. We have designed two types of sample platforms for enhanced fluorescence and label-free protein sensing. For design optimization we used the dipole emission model^{27,28} to simulate fluorophore emitters near a dielectric interface on a layered structure. Details of modeling for the label-free sensing by IRIS technology can be found in Daaboul et al.¹⁹ Platform-1 consists of two areas of SiO₂, one optimized specifically for label-free and the other optimized for fluorescence sensing (500 nm for IRIS and 100 nm for enhanced fluorescence^{19,21,29}), and has been designed to enhance a broad emission range of fluorophore wavelengths. Recently, this combined chip was shown to be of high practical use during the assay development process. Label-free sensing was utilized to image arrays prior to incubation with labeled antibodies to assess the robustness of the array and to quantify the amount of immobilized probes. Via this method, fluorescence measurements on the same chip yielded calibrated bioassay results in a single experiment.²¹ However, this platform relies on uniformity and repeatability within a chip since label-free and fluorescence measurements are performed on two separate parts of the same chip. Platform-2 consists of a silicon chip with a single oxide thickness optimized for single spot enhancement of a particular fluorophore(s) and label-free analysis. In this new configuration, each spot is measured with both fluorescence and label-free modalities, and hence calibrated, effectively accounting for spot-to-spot variation commonly reported in microarrays. For both platforms, regardless of having single or multiple regions, the top layer is oxide and thus chemical surface preparation is identical. This feature allows the use of specialized polymeric coatings (copoly(DMA-NAS-MAPS))^{30,31} to covalently link capture agents to the surface, while maintaining high functionality and preventing nonspecific binding. Therefore, the self-calibrated CaFE platform offers an opportunity for quantitative assessment of allergy chips which uses labeled secondary antibodies to detect captured IgE by integrating both label-free and fluorescence measurements.

MATERIALS AND METHODS

Platform Design Simulations

Structure optimization is defined as the oxide thickness on Si that yields intensity enhancement across fluorescence emission wavelengths of interest and performs label-free sensing with high accuracy. These simulations address two goals regarding oxide thickness design and optimization: (1) platform-1 (called CaFE chip) should yield emission enhancement for maximum coverage of wavelengths while performing label-free sensing on a separate spot on the same chip and (2) platform-2 maintains emission enhancement for a limited wavelength range (covering 1 or 2 fluorophores) while allowing for accurate calibration of the same spot using IRIS. Platform design for Cy3 and Cy5 emission enhancement was chosen due to their extensive use in microarrays to study the ratio of expression of genes from two sources.^{32–36}

To investigate the effect of oxide thickness on fluorescence intensity, the dipole emission model^{27,28} was used to simulate fluorophore emitters near a dielectric interface on a layered SiO₂/Si structure. Simulations were carried out for oxide thicknesses ranging from 1 to 1000 nm over the entire visible wavelength range to select the optimized oxide thickness for each of the two platforms described above. In Figure 1a, we plot the wavelength dependence of fluorescence intensity for two specific oxide thicknesses in comparison to a glass substrate along with typical spectra of fluorophores that are commonly used as labels in bioassays (Alexa Fluor 359, Alexa Fluor 488, Cy3 dye, Cy5 dye, and Alexa Fluor 647). The initial optimization is performed assuming a collection angle of 0.7. For a practical fluorescence collection system, the numerical aperture (NA) is large (typical values around 0.5–0.7) (Herman, B., *Fluorescence Microscopy*, Second Edition, BIOS Scientific Publishers (1998)) and hence the theoretical enhancement calculations should consider the dependence on NA. In Figure 1b, the NA dependence of emission enhancement of Cy3 is plotted and the two optimized designs are compared at typical NA values.

The CaFE verification of fluorescence measurement is described in the Supporting Information, and details on label-free detection using IRIS have been described.¹⁹ As previously reported, 500 nm oxide on Si is optimal for IRIS sensing because the spectra of three of the LEDs (455, 525, and 632 nm) sample the linear region of the curve while the yellow LED (598 nm) helps determine the amplitude of the curve over one period. Because the linear region falls on the inflection point of the curve, any small change in height, or biomass accumulation, will be detected. To check for label-free sensing capability for the oxide thickness chosen for emission enhancement of Cy3/Cy5 fluorophores (goal-2), we generated an additional reflectivity curve at this oxide thickness as a function of illumination wavelengths and compared it to the curve produced from 500 nm oxide (Figure 1c).

Finally, to verify the results of the simulations with experimental data, platform-1 (CaFE) chips and platform-2 (320 nm) chips were fabricated as seen in Cretich et al.²¹ An experiment described in IgG Calibration was performed on each platform to validate the accuracy of label-free detection and enhancement of fluorescence (Figure 1d).

Reagents and Equipment

TRIS, BSA, Tween 20, PBS tablets, rabbit immunoglobulin G, carbonic anhydrase, and bovine serum albumin were purchased from Sigma (St. Louis, MO). Rabbit anti- β -lactoglobulin was purchased from Bethyl Laboratories (Montgomery, TX), goat anti- α -lactalbumin from GeneTex Inc. (Irvine, CA), and AbCam (Cambridge, U.K.). Secondary antibodies (Cy3-labeled goat antimouse IgG and mouse antigoat IgG) were purchased from Jackson ImmunoResearch (West Grove, PA), and anti-IgE was purchased from BD Biosciences. Allergens Bet v 1a (Bet v 1.0101), Phl p 1 (Phl p1.0101), Phl p 5 (Phl p 5.0101), and Alt a 1 (Alt a 1.0101) were recombinant allergens from Biomay, (Vienna, Austria) and allergens Bet v 2 (Bet v 2.0101), Phl p 7, nDer p 1, nDer p 2, and nFel d 1 were recombinant (or native, when prefix “n” is used) allergens from Indoor Biotechnologies Ltd. (Warminster, U.K.).

Si/SiO₂ Coating by Copoly(DMA-NAS-MAPS)

Si/SiO₂ slides were immersed for 30 min in a solution of copoly(DMA-NAS-MAPS) at 1% w/v concentration in a solution of deionized water and 20% saturated ammonium sulfate. Slides were washed with water and dried at 80 °C for 15 min. This polymeric coating was chosen for our CaFE chips due the feasibility and reproducibility of its synthesis and coating process. This particular polymeric coating does not change the optical properties of the setup.

Proof of Concept of CaFE Using IgG and β -lactoglobulin

To demonstrate the universal application of the CaFE method, we have performed the quantification and correlation of captured IgG and β -lactoglobulin probe to fluorescence signal of Cy3-labeled-secondary antibody. For these experiments, we utilize the CaFE chip platform to optimize for all fluorophores and achieve maximum sensitivity on the IRIS device. IgG and β -lactoglobulin proteins were chosen due to their well-established spotting protocols and reliable spotting morphologies.

IgG Calibration

As proof of concept, 20 replicates IgG of varying concentrations (0.015, 0.03, 0.063, 0.125, 0.25, and 1 mg/mL) were spotted onto 2 CaFE chips. After overnight humid chamber incubation, the chips were washed with 50 mM ethanolamine in TRIS/HCl 1M pH 9 for 1 h, rinsed with water, dried with a stream of argon gas, and then measured using IRIS. They were then incubated with 100 μ L of specific labeled antibody in incubation buffer (Tris/HCl 0.05 M pH 7.6, NaCl 0.15 M, Tween 20 0.02%) with 1% w/v BSA for 1 h at 1 μ g/mL. Another IRIS measurement was taken after washing with PBS for 10 min, rinsing with water, and drying with argon.

Fluorescence evaluation was performed by a fluorescence scanner using 40% PMT and 33% laser power for maximal fluorescence value without saturation. Mean fluorescence intensity and standard error from all 20 spots is depicted.

β -Lactoglobulin Calibration

To model a sandwich assay similar to the allergen immunoassay and to demonstrate the versatility of the CaFE method, 20 replicates of β -lactoglobulin of varying concentrations (0.015, 0.03, 0.063, 0.125, 0.25, and 1 mg/mL) were spotted onto three CaFE chips, and after overnight humid chamber incubation, the CaFE chips were washed with 50 mM ethanolamine in TRIS/HCl 1 M pH 9 for 1 h, washed with water, dried with a stream of argon gas, and then measured using IRIS. The chips were then incubated with 100 μ L of specific antibody in incubation buffer with 1% w/v BSA, for 2 h at 10 ng/mL. Slides were then washed with washing buffer (Tris/HCl 0.05 M pH 9, NaCl 0.25 M, Tween20 0.05%) for 10 min, rinsed with water, and dried with argon gas. The chips were then incubated with 100 μ L of the solution of the specific labeled secondary antibody 1 μ g/mL in incubation buffer for 1 h. Slides were then washed with PBS for 10 min, rinsed with water, dried with argon, and measured with IRIS.

Fluorescence evaluation was performed using 90% PMT and 90% laser power for maximal fluorescence value just below saturation. Mean fluorescence intensity and standard error were plotted for the 20 replicate spots.

CaFE Implemented as an Allergy Testing Platform

To evaluate CaFE as a clinical diagnostic platform, two major allergens (peanut (Ara h1) and timothy grass (Phl p1) were spotted in replicates of three at four concentrations (0.25, 0.5, 0.75, and 1.0 mg/mL) on two CaFE chips. In addition, PBS and IgG were spotted as negative and positive control parameters. After overnight humid chamber incubation, the chips were washed with 50 mM ethanolamine in TRIS/HCl 1 M pH 9 for 1 h, washed with water, dried with a stream of argon gas, and then measured using IRIS. The chips were then incubated with 100 μ L of patient serum with documented allergy to peanut (specific IgE 19.40 kU/L, Phadia ImmunoCAP) and timothy grass (positive allergen skin prick test) in incubation buffer with 1% w/v BSA for 2 h at 10 μ g/mL. (Subject recruitment was approved by the Boston University Medical Campus Institutional Review Board, Protocol H-29428.) Slides were then washed with washing buffer for 10 min, rinsed with water, and dried with

argon gas. After incubating with 1 ng/mL anti-IgE labeled with Cy3, the chips were washed with PBS (10 min), rinsed with water, dried with argon, and measured with IRIS.

Scanning for fluorescence evaluation was performed. CaFE slides were analyzed using 90% PMT and laser power to maximize the signal-to-noise ratio without pixel saturation. Mean fluorescence intensity is depicted.

RESULTS AND DISCUSSION

Platform Design Simulations

After running the radiation model of emitters near a dielectric interface, a 100 nm oxide thickness yielded emission enhancement for maximum coverage of wavelengths and a 320 nm oxide thickness yielded enhancement for emission wavelengths of Cy3 and Cy5 fluorophores (Figure 1a). This enhancement effect is a result from the interference of the emission wavelength reflected at the oxide-silicon interface and the air-oxide interface. The oxide thickness serves as a spacer in which emitted light is reflected at the air-oxide interface and refracted through the oxide layer. The refracted light travels through the spacer layer and is then reflected by the silicon layer. Depending on the wavelength of the emitted light, the thickness of the spacer will determine which wavelengths undergo constructive or destructive interference. Using this method, we designed two layered structures: (1) a 100 nm SiO₂ layer on Si that yields enhancement for maximum coverage of wavelengths and (2) a 320 nm SiO₂ layer on Si that maintains fluorescence enhancement for Cy3 and Cy5 fluorophores.

In Figure 1b, the NA dependence of emission enhancement of Cy3 is graphed and the two optimized designs are compared. At low NA, a 100 nm oxide thickness yields a greater enhancement effect compared to a 320 nm oxide layer. As the collection angle increases, the enhancement effect decreases because higher angles do not undergo complete constructive interference due to the increase in optical path length through the spacer region. The enhancement values converge as a greater percentage of the reflected emission is collected. When comparing the platforms at the typical NA used in fluorescence scanners (NA = 0.7), both platforms yield a 2-fold emission enhancement of Cy3. Furthermore, the enhancement produced by 100 nm SiO₂ is only 1.04 times greater than the enhancement yielded by 320 nm SiO₂. On the basis of these results, designing a chip with 100 nm oxide on Si would enhance all wavelengths of commonly used fluorophores and constructing a chip with 320 nm oxide on Si would maintain enhancement for Cy3 and Cy5 fluorophores.

In Figure 1c, normalized reflectivity curves as a function of illumination wavelength are generated for both platforms and compared. Because the rate of change with wavelength is slightly slower in the 320 nm oxide compared to the 500 nm oxide, the illumination wavelengths intersect the curves at slightly different locations. As a result, the spectra of the LEDs now sample the 320 nm reflectivity curve close to the minima and maxima regions. Although none of the LEDs fall directly on the inflection point of the graph, the green and yellow LED sample above and below the linear region, helping to define the slope of the curve and determine the reflectivity profile of a 320 nm oxide on Si. These reflectivity comparisons suggest that IRIS measurements on a 320 nm oxide are feasible; however, a reduction in level of detection and sensitivity may occur.

Fluorescence simulations and IRIS reflectivity curves facilitated the design of two platforms: (1) a CaFE chip with one area of 100 nm oxide on Si for fluorescence enhancement coverage over all visible wavelengths and an area of 500 nm for sensitive label-free biosensing and (2) a 320 nm oxide on Si chip that maintains fluorescence enhancement of Cy3 and Cy5 fluorophores and operates with IRIS. These designs were

tested and compared by conducting the IgG calibration experiments on each platform (Figure 1d). As expected, the CaFE chip yields a higher fluorescence signal and slightly better sensitivity compared to the 320 nm oxide chip. Standard deviation is also higher on the 320 nm oxide chip, and the measurement was not able to detect the lowest spotting concentration (0.015 mg/mL). On the basis of this data, the radiation model of emitters and IRIS reflectivity curves helped design two dual modality platforms that optimize for enhanced fluorescence of one or more wavelengths and quantify biomass accumulation, label-free.

Proof of Concept of Quantification and Calibration of CaFE using IgG and β -Lactoglobulin

Images of both label-free measurements (Figure 2a) and fluorescence measurements (Figure 2b) show a gradient that correlates with varying concentration of immobilized rabbit IgG and captured Cy3-anti-rabbit IgG. The fluorescence enhancement of the 100 nm oxide islands is noticeable when compared to the 500 nm oxide island. Similar effects are seen in the β -lactoglobulin array but are not shown.

Theoretically, each chip should bind the same probe density on the surface. However, IRIS measurements show that both IgG and β -lactoglobulin showed a large immobilization variation between chips resulting in varied fluorescence measurement of secondary antibody. Quadrant-to-quadrant variation on chip was also present in the IgG data. The CaFE method was then applied to fluorescence and IRIS data in order to quantify and calibrate specific secondary antibody for both IgG and β -lactoglobulin systems (Figure 2c,d). In these plots, all error bars correspond to spot-to-spot variability, while each subset shows chip-to-chip variability. In both cases, each of the proteins demonstrates a calibrated, strong linear response ($R^2 \geq 90\%$) between fluorescence and probe density despite chip-to-chip variability. Because each protein has different finite binding capacities, the slopes of the CaFE curves differ. This capacity also may result in possible saturation point at higher concentrations. Overall, these results suggest that the CaFE method will be an effective and versatile platform to quantify and correlate bound probe to secondary labeled antibody and has potential application in immunodiagnostics. In addition, on-chip variation could be addressed by implementing platform-2, which would assess each spot individually.

CaFE Implemented as an Allergy Testing Platform

The performance of the two chips, defined by fluorescence signal of secondary IgE, varies between the allergens because different levels of IgE in serum for each particular allergen are present. This is confirmed with the ImmunoCAP results (Figure 2-S in the Supporting Information). When analyzing the fluorescence signal of anti-IgE in the fashion of typical ELISA and microarray assays, measurement variation of allergen-specific IgE of the same allergen between chips is present, particularly in Phl p1 allergen (Figure 3a). The degree of chip-to-chip variation between allergens most likely occurs due to the physiochemical properties of the allergens themselves (i.e., affinity to immobilize to the surface) and technical variation (i.e., spotting). While only slight chip-to-chip fluorescence variation is seen for Ara h1 allergen (R^2 of 0.88), a significant chip-to-chip fluorescence variation is seen for Phl p1 allergen (R^2 of 0.24), despite the same conditions, reagents, and serum samples used in this single experiment. On the basis of the fluorescence data alone, it is unknown as to why Phl p1 allergen chips yield different fluorescence responses.

To account for this deviation, the CaFE method is applied to collected fluorescence and IRIS data. As a result, a calibrated, linear response between allergen-specific IgE and amount of allergen immobilized on surface emerges (Figure 3b). In both Ara h1 and Phl p1 examples, the R^2 value increases to at least 90%, demonstrating the higher degree of correlation between fluorescence signal and immobilization density compared to spotting concentration.

Although the CaFE method only slightly improves upon Ara h1 allergen data, the effects of including label-free IRIS measurement are dramatically seen in the Phl p1 data. Most importantly, use of the CaFE method clarifies that higher immobilization density of Phl p1 allergen on chip 2 results in higher fluorescence signal, indicating that any variation in immobilization density will affect the amount of IgE captured. In accordance with the literature, this data supports that high variation in allergen immobilization microarrays is a concerning issue. The strong linear correlation between fluorescence and immobilization density demonstrates the value of the CaFE method as an opportunity for calibrated quantitative assessment of serum allergen-specific IgE and should improve accuracy in predicting clinical reactivity in susceptible individuals.

Finally, in order to verify that the CaFE method differentiates between allergic and nonallergic samples, five allergens were arrayed in replicates of three onto a CaFE chip. This chip was incubated with characterized serum in which there was selective allergen-specific IgE. Results were compared to ImmunoCAP or skin test characterization (Figure 2-S in the Supporting Information).

CONCLUSIONS

We have designed two platforms: (1) a chip with multiple sections to optimize label-free measurement and fluorescence over a broad range of wavelengths and (2) a silicon chip that has been engineered specifically for the enhancement of a particular fluorophore and is operational in the label-free modality and allows for calibration of the same spot, to create a calibrated fluorescence enhancement, or CaFE, method that improves upon quality and quantity control in microarrays. In our experiments, we utilize one of the two platforms, the CaFE chip, to demonstrate that the correlation between the fluorescence signal and immobilized probe density is linear despite chip-to-chip variance. We also show that these experiments are repeatable on a single oxide thickness platform. Without the ability to quantify the amount of capture probes (major allergens), the typical assay quality control would be limited to controlling the spotting conditions, i.e., the *intended* concentration of the probes. Thus, the CaFE method is an effective, self-calibrated, multiplexed, and sensitive platform and offers technological advances that are not currently or readily available.

Additional applications of this technology are by no means limited to the described model of allergy diagnosis; this platform could be effectively applied where readout from a labeled secondary antibody must be calibrated against quantity of immobilized capture probe for accurate diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Carlos A. Lopez and George Daaboul for intellectual discussion. We would also like to thank the reviewers and editors for their helpful suggestions. Financial support was from the Wallace H. Coulter Foundation 2010 Coulter Translational Award and by the Smart Lighting ERC funded via the NSF under Cooperative Agreement EEC-0812056.

References

1. U.S. Department of Health and Human Services. Airborne Allergens: Something in the Air. National Institutes of Health and Infectious Diseases; 2003.
2. National Center for Health Statistics. National Health Interview Survey. CDC; 2005.

3. Shreffler WG. *J Allergy Clin Immunol.* 2011; 127(4):843–849. [PubMed: 21458654]
4. Chiari M, et al. *Anal Chem.* 2009; 81(13):5197–5203. [PubMed: 19485342]
5. Balboni I, et al. *Proteomics.* 2008; 8(17):3443–3449. [PubMed: 18752214]
6. Lin J, Bardina L, Shreffler WG. *Methods Mol Biol.* 2009; 524:259–272. [PubMed: 19377951]
7. Wang XJ, et al. Comprehensive quality control utilizing the prehybridization third-dye image leads to accurate gene expression measurements by cDNA microarrays. *BMC Bioinf.* 2006; 7:378–388.
8. Shi LM, et al. The balance of reproducibility, sensitivity, and specificity of lists of differentially expressed genes in microarray studies. *BMC Bioinf.* 2008; 9(Suppl 9):S10.
9. Shi LM, et al. *Curr Opin Biotechnol.* 2008; 19(1):10–18. [PubMed: 18155896]
10. Foy CA, Wilkes T, Laux H. *OMICS: J Integr Biol.* 2007; 11(1):1–13.
11. Kricka LJ, Master SR. *Clin Chem.* 2009; 55(6):1053–1055. [PubMed: 19395435]
12. Emmert-Buck MR, et al. *Nat Genet.* 2002; 32:509–514. [PubMed: 12454646]
13. Klee GG, et al. *Clin Chem.* 2009; 55(6):1092–1099. [PubMed: 19372187]
14. Schabacker DS, et al. *Anal Biochem.* 2006; 359(1):84–93. [PubMed: 17034751]
15. Hessner MJ, et al. Utilization of a labeled tracking oligonucleotide for visualization and quality control of spotted 70-mer arrays. *BMC Genomics.* 2004; 5:12. [PubMed: 15018646]
16. Cunningham BT, et al. *Anal Chem.* 2010; 82(20):8551–8557. [PubMed: 20857905]
17. Ozkumur, IE., et al. Label-free and High-throughput Screening of Biomolecular Interactions. *Lasers and Electro-Optics & Quantum Electronics and Laser Science Conference*; 2008. p. 58-59.
18. Ozkumur E, et al. *IEEE J Sel Top Quantum Electron.* 2010; 16(3):635–646.
19. Daaboul GG, et al. *Biosens Bioelectron.* 2011; 26(5):2221–2227. [PubMed: 20980139]
20. Lopez CA, et al. *Biosens Bioelectron.* 2011; 26(8):3432–3437. [PubMed: 21342761]
21. Cretich M, et al. *Biosens Bioelectron.* 2011; 26(9):3938–3943.
22. Moal E, et al. Mirror slides for high-sensitivity cell and tissue fluorescence imaging. *J Biomed Opt.* 2007; 12(2):024030. [PubMed: 17477745]
23. Akimoto T, Yasuda M. *Appl Opt.* 2010; 49(1):80–85. [PubMed: 20062493]
24. Sullivan K, Hall DG. *J Opt Soc Am B.* 1997; 14(5):1149–1159.
25. Rajendra R, et al. *J Biomol Tech.* 2006; 17:122–130. [PubMed: 16741239]
26. Choumane H, et al. Double interference fluorescence enhancement from reflective slides: Application to bicolor microarrays. *Appl Phys Lett.* 2005; 87(3):031102.
27. Hellen EH, Axelrod D. *J Opt Soc Am B.* 1987; 4(3):337–350.
28. Moiseev L, et al. *J Appl Phys.* 2004; 96(9):5311–5315.
29. Yalcin, A., et al. Fluorescence Enhancement on Reflecting Substrates for Microarray Applications. *IEEE Leos Annual Meeting Conference Proceedings*; 2009. p. 465-466.
30. Ozkumur E, et al. *Biosens Bioelectron.* 2009; 25(1):167–172. [PubMed: 19628383]
31. Yalcin A, et al. *Anal Chem.* 2009; 81(2):625–630. [PubMed: 19061409]
32. Brownstein MJ, et al. *Nat Biotechnol.* 2002; 20(7):738–742. [PubMed: 12089562]
33. Muckenthaler M, et al. *Biotechniques.* 2002; 33(3):620–630. [PubMed: 12238772]
34. Swaroop A, et al. *Mol Vision.* 2002; 8(17):130–137.
35. Tang T, et al. *Bioinformatics.* 2007; 23(20):2686. [PubMed: 17698492]
36. 't Hoen PAC, et al. Fluorescent labelling of cRNA for microarray applications. *Nucleic Acids Res.* 2003; 31(5):e20. [PubMed: 12595569]

Figure 1.

(a) Simulations model the theoretical fluorescence of 100 nm oxide, 320 nm oxide, and glass at normal incidence as a function of emission wavelengths. From left to right, the emission spectra of commonly used fluorophores (Alexa Fluor 359, Alexa Fluor 488, Cy3 dye, Cy5 dye, and Alexa Fluor 647). (b) Fluorescence emission enhancement of Cy3 for 100 and 320 nm oxide is 2-fold compared to glass at NA=0.7. (c) Normalized reflectivity curves for 500 nm oxide and 320 nm oxide are fitted to illumination wavelengths used in IRIS. (d) The CaFE method is performed and compared for both chip platforms.

Figure 2.

(a) Label-free IRIS image of varying concentrations of rabbit IgG on a 500 nm island of Platform-1(CaFE) chip. (b) Fluorescence image of spotting array on CaFE chip for varying concentrations of captured Cy3-antirabbit IgG. CaFE measurement for rabbit IgG (c) and β -lactoglobulin (d). Error bars represent the SD of 20 spots. For IgG calibration of Chip 3, two quadrants (Q1 and Q2) analyze on chip variability. Results show that the CaFE platform yields calibrated, linear responses from chip-to-chip for a variety of proteins.

Figure 3.

(a) The standard “calibration” of secondary antibody for diagnosis of allergy is measured as the degree of fluorescence as a function of spotting concentration is shown for Ara h 1 peanut allergen (left) and Phl p 1 timothy grass allergen (right) for two chips. Using the self-calibration method provided by CaFE (b), results show calibrated, linear responses for allergy testing analysis compared to traditional “semi-quantifiable” analysis in part a.