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Microsecond Analysis of Transient Molecules using Bi-Directional Capillary Electrophoresis

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Abstract

We demonstrate the feasibility for minimizing electrophoretic analysis times of transient chemical species by inducing nascent, oppositely charged photochemical products to migrate in opposite directions from their point of creation. In this approach, separate probe sites are positioned within an electrophoretic channel both up-field and down-field from a photoreaction site formed by high-numerical-aperture optics, with positively charged (and in some cases neutral) components migrating toward one probe site and negatively charged species migrating in the opposite direction, toward the second probe site. As a proof-of-concept, fluorescent photoproducts of the hydroxyindoles, 5-hydroxytryptamine (serotonin), 5-hydroxytrptophan, and 5-hydroxyindole-2-carboxylic acid, are formed within a geometrically modified capillary and are transported electrophoretically and electroosmotically to probe sites several micrometers away. Although it is possible to detect all components in a single channel, or to use a two-channel imaging approach to independently detect positive and negative components, we have found the most rapid analysis approach involves a protocol in which laser light is alternately directed to opposing probe sites at high frequency (1 kHz), a strategy that allows positive and negative species to be detected with no cross-talk, even when components have overlapping detection times. Fluorescence-signal-averaging was performed on each temporal channel via summation of the two sequences of interdigitized electrophoretic traces. This approach allowed photoproducts to be detected free from interferences from oppositely charged species, allowing positive and negative species in a mixture to be analyzed electrophoretically in ca. 6 μs, a period several-fold faster than was previously feasible using uni-directional electrophoresis.

Keywords

Microsecond; electrophoresis; fluorescence; serotonin; transient; ultra-fast

INTRODUCTION

The study of short-lived chemical species requires tools capable of distinguishing structurally related molecules on time scales often not accessible using conventional analytical instrumentation. Transient spectroscopic techniques have provided capabilities for tracking metastable compounds that have known signatures, yielding important insights into mechanisms of photochemical transformations1›2, enzymatic pathways3›4, and thermal reactions5›6. Unfortunately, transient spectroscopy often is limited in its ability to speciate, particularly in cases where the relevant spectra are unknown and provide minimal molecular information (e.g., as with solution-phase fluorescence), or in mixtures containing multiple, spectrally overlapping components.

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In principle, chemical separations could offer the possibility for gaining molecular insights in such circumstances. Electrophoretic analysis, for example, provides information regarding molecular charge and drag within a viscous medium, characteristics that could aid in identification of transient intermediates when spectroscopy alone is insufficient. Such an approach has the advantage of being relatively insensitive to the presence of analytes with overlapping spectra, provided that fractionation can spatially isolate potentially interfering species. Unfortunately, separations generally are slow, typically requiring analysis times of seconds to tens of minutes – delays that generally are incompatible with the lifetimes of metastable compounds. Analytes fractionated using partition chromatography, for example, are fundamentally limited in the rate at which they can be usefully transported through a column, as efficient separation depends on the establishment of quasi-equilibrium conditions between stationary and mobile phases.

In contrast, electrophoretic analysis is performed within a single phase, and consequently, is not similarly limited by the speed at which analytes can be transported through a separation path. Indeed, efficiency in capillary electrophoresis (CE) often improves with increased velocity, being limited only by the sizes of the sample introduction plug and the detection region, and, when electric fields are sufficiently large, by Joule heating. Developments in high-speed, low-volume sample introduction strategies have enabled electrophoretic analyses to be accomplished on millisecond to low second time scales within narrow channels.[7-14]

To characterize metastable reaction products using CE, analytes must be created within brief time windows and exposed to an electric field before they decay. Although many types of reactions potentially could be characterized using high-speed CE, photochemistry represents an attractive proof-of-concept because of the high spatio-temporal control afforded by optical triggers.

It has been shown that visibly fluorescent ($\lambda_{max} \approx 500$ nm) hydroxyindole (HI) photoproducts can be generated via nonlinear excitation, and that these products decay on sub-second time scales.[8,15,16] However, the broad emission spectra and extremely small (ca. 1 fL) volumes in which these species are produced have made it impractical to characterize products using conventional means. However, because product fluorescence is spectrally distinct from reagent emission, transient species can be created and detected *in situ*, within streams of hydroxyindoles flowing under the influence of a high electric field.

We have used this strategy to electrophoretically analyze mixtures of metastable photochemical products within as little as 10 μs after their creation.[17] This technique relied on the use of a pulsed laser beam, focused using high-numerical-aperture (NA) optics into the waist region of a pulled (hourglass-shaped) fused-silica capillary. Mixtures of hydroxyindoles – compounds previously demonstrated to photodegrade via moderately fluorescent reactive intermediates – migrated continuously through the hourglass region under the influence of an amplified field and high electroosmotic flow. When these reagent compounds intersected the laser focus, transient species were produced via multiphoton excitation that could be detected downstream, after electrophoretic isolation, using a second tightly focused laser beam. Because the spatial extent of “injection” plugs and the detection zone were defined by high NA optics, it was possible to reduce separation distances to as little as 6 μm while maintaining efficiencies sufficient to fractionate differentially charged photoproducts. Although separation efficiencies were modest, the theoretical plates generated per second using this analysis strategy were extremely large (>20 million theoretical plates per second), making it possible to demonstrate that hydroxyindoles degrade within microseconds into green fluorescent intermediates that have similar or identical electrophoretic mobilities as the parent species.

Here, we report the use of multiple detection foci to provide improved temporal resolution for distinguishing species of opposite mobilities, again using HI photochemical reactions as a model system for extending the capabilities of fast CE analysis. Transient hydroxyindole photoproducts are generated at a single laser focus (the "gate" position) in a capillary operated under low-electroosmotic flow conditions, and components with positive and negative electrophoretic mobilities are probed at laser spots positioned on opposing sides of the gate. Because migration velocities are determined primarily by electrophoresis, multiple analytes can be resolved over distances of only a few micrometers, even when separation efficiencies are low. In one approach, multi-site detection is achieved by directing signal from different capillary positions to separate photodetectors, with the minimum practical separation distances defined by cross-talk between detection channels. An alternate configuration in which detection foci are modulated at high frequency (> 1 kHz) is used to eliminate channel cross-talk, allowing electrophoretic information to be acquired for positive and negative components in ca. 6 μs, the fastest analysis of electrophoretic characteristics reported to date.

EXPERIMENTAL

Reagents and Solutions

All reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise noted. Stock solutions of MES buffer (pH 5.5 - 5.6) were prepared weekly and hydroxyindole solutions of 5-hydroxytryptamine [serotonin (5HT)], 5-hydroxytryptophan (5HTrp), and 5-hydroxyindole-2-carboxylic acid (5HI2CA) were prepared fresh daily. All solutions were filtered using 0.2-μm syringe filters (Corning, Corning, New York); buffer vials were cleaned by filling with filtered deionized H_2O and sonicating for 45 min before use.

Fluidics Assembly

The capillary preparation method has been described in detail previously.17 Briefly, the polyimide coating in the central 2-cm section of a 25-μm i.d., ~10-cm long capillary (Polymicro, Technologies, Phoenix, AZ) was removed using a Bunsen burner flame. The capillary was then carefully placed into a pipette puller (P-2000, Sutter Instruments, Novato, CA) where it was pulled to produce an hourglass geometry in the central region with a waist i.d. of ~ 5 μm. This modification results in a dramatic increase in the unit-length resistance within the pull region, thereby amplifying the electric field. At the applied potentials examined in this work, at least 2 kV cm^{-1} were generated per volt applied to the capillary.

Pulled capillaries were secured under a milled Plexiglas block using epoxy (Epoxy 907 Adhesive System, Miller-Stephenson, Danbury, CT) so that the hourglass region coincides with a drilled access hole (Figure 1a). A borosilicate glass coverslip (no. 0) was mounted directly beneath the capillary, and was sealed against the block to form a well surrounding the pulled region of the capillary. Refractive index matching fluid (glycerin) was placed in the well to fill narrow gap between the capillary and the underlying coverslip, an approach that minimizes optical aberrations caused by the curved surface of the capillary. Samples were electrokinetically transported between inlet and outlet vials, which were sealed with septa to confine potentials created by platinum-wire electrodes fixed permanently into the ends of the vials.

The capillary assembly, consisting of the mounted capillary and two sample reservoirs, was mounted to a 0.25-inch thick Plexiglas sheet that was bolted to a three-axis translation stage (Model 562, Newport Corp., Irvine, CA) that uses motorized actuators for stage movement and final laser alignment. Two high-voltage power supplies were used (Series EH, Glassman High Voltage, Inc., High Bridge, NJ) to supply separation voltages of opposite polarities.

To ensure the predominate mode of analyte migration was due to electrophoresis, buffer conditions were adjusted to minimize electroosmotic flow (EOF). Sufficient reductions in EOF were achieved using a moderately acidic MES buffer containing a nonionic surfactant [0.1 – 0.5% (w/v) Pluronic F-127]. Pluronic F-127 is a block copolymer (consisting of propylene oxide and ethylene oxide) that previously has been used as a CE separation medium for large biomolecules18,19 and as a coating in microfluidic channels to reduce wall adsorption of large molecules20, cell adhesion13, and to modify electroosmotic flow13,20. To further reduce EOF in some cases, a trivalent cation, Al^{3+} (from dissolved $AlCl_3$), also was used. The solution modifications made in our current studies were found to produce a stable EOF velocity (typically ~10 cm s^{-1}) for the duration of an experiment.

Instrumentation and Optical Alignment

A mode-locked titanium-sapphire (Ti:S) oscillator (Coherent Mira 900F) pumped by a 532-nm 10-W frequency-double neodymium-vanadate laser (Coherent Verdi) was operated at 755 nm for all experiments. This laser source produces ~150 fs pulses with a pulse repetition rate of 76 MHz. The Ti:S output was first split using a half-wave plate and polarizing beamsplitter into *gate* and *probe* beams (Figure 1b-c). The gate beam was passed through a Pockel's cell (350-50, Conoptics, Danbury, CT) that was controlled by a high-speed delay generator (DG-535, Stanford Research, Sunnyvale, CA) where it can be modulated electrooptically on microsecond timescales to create spatially narrow packets of photochemical intermediates within reagent streams. The gate was switched to high power (an average laser power of 300 – 400 mW, measured at the back aperture of the objective) for periods ranging from 0.7 to 10 μs.

After being split from the gate beam, the probe portion of the Ti:S beam was directed through a half-wave plate and a second polarizing beamsplitter to produce two probe beams. One of the probe beams was sent through a half-wave plate/polarizer pair to provide a means to match power and polarization to the other probe beam. These beams were recombined slightly off-axis using a 50/50 non-polarizing beamsplitter. Further along the beam path, the gate beam was combined slightly off-axis with the two probe beams using a polarizing beamsplitter. All three beams were passed through a long-pass dichroic mirror (Chroma Technology Corp., 575DCXR) and reflected by a 90/10 beamsplitter into the back aperture of a 1.3 NA 100X microscope objective (Fluar, Zeiss).

The capillary was monitored by collecting transmitted light (from a fiber-optic illumination source) through the objective and to a tube lens, which focused the capillary image onto a video-rate CCD (KP-M1U, Hitachi Denshi, Ltd., Japan). Laser back-reflections at the interface between the coverslip and glycerol (which are nearly index matched) were imaged to guide the lateral alignment of gate and probe foci. Before performing an experiment, the capillary was adjusted using three-axis electrostrictive actuators (AD30, Newport) to position the laser foci along the central axis of the capillary near the hourglass waist.

Fluorescence generated at the laser foci from hydroxyindole photoproducts was epi-collected using the objective, directed back along the laser propagation path via the 90/10 beamsplitter to the dichroic mirror and reflected through a series of optical filters (5-cm saturated aqueous $CuSO_4$ and two 3-mm-thick BG-39 filters) before striking the photomultiplier (PMT; HC-125, Hamamatsu, Middlesex, NJ). To improve signal-to-noise, data from rapid, sequential electrophoretic runs were summed on-the-fly using a zero-deadtime multichannel scaler (SR430, Stanford Research Systems) allowing electrophoretic properties of analytes to be probed on microsecond time scales. Three different strategies were implemented to acquire fluorescence data, as described below.

Detection Strategies

Configuration I: Dual Probe Beam/Single-Detector Instrument—Using a single detector configuration (Figure 1b), fluorescence emission from both primary and secondary probe beams was delivered using a 20-cm focal length plano-convex lens to the photocathode of the PMT.

Configuration II: Dual Probe Beam/Dual-Detector Instrument—Here, fluorescence generated from each probe beam was spatially isolated, and delivered to two separate PMTs, allowing positive and negative analytes to be detected independently, even when they arrived at their respective detection sites simultaneously. In this approach, fluorescence collected by the objective was reflected along a 2-m optical path, after which signal from the two probe foci was separated laterally by ~1 cm (Figure 1c). The two fluorescence beams then were passed though separate plano-convex lenses (7.5-cm focal length) off-axis, aligned through separate (~3-mm diameter) pinholes, and delivered to separate PMTs. Signal acquisition was performed using two SR430 multichannel scalers.

Configuration III: Modulated Dual Beam/Single-Detector Instrument—In this approach, the two probe beams were modulated using an optical rotator from a 350-50 Pockel's cell so that the beams alternate between high and low intensity (i.e., 180° out of phase with each other), enabling each probe to detect photoproduct in half of the electrophoretic repetitions as signal averaging is performed. Here, the optical rotator was placed in the probe-beam path immediately before the second half-wave plate/polarizing beamsplitter pair (Figure 1b). This configuration caused the beam to be either transmitted or reflected at the second beamsplitter, depending on whether vertically or horizontally polarized light emerges from the optical rotator. The optical rotator was controlled using a function generator (DG-345, Stanford Research, Sunnyvale, CA) synchronized to the delay generator controlling the gate-beam Pockel's cell (see above). Switching frequencies of 2 kHz for the gate Pockel's cell and 1 kHz for the probe optical rotator were used, allowing each probe spot to excite photoproduct fluorescence after every other gate pulse. Fluorescence was passed through the same optical system used in Configuration I, described above.

RESULTS AND DISCUSSION

We previously have shown that mixtures of positive and neutral transient photoproducts can be analyzed in ~10 μs using extremely large electric-field and ultrashort separation paths.17 Here, we examine the feasibility of bi-directional electrophoresis for characterizing transient positive and negative species on even faster time scales using three different detection strategies under conditions in which the predominant mode of analyte migration is due to electrophoresis.

Detection Strategies

Configuration I: Dual Probe Beam/Single-Detector Instrument—In the simplest approach for performing bi-directional high-speed electrophoretic separations (Configuration I, Figure 1b, no optical rotator), fluorescence generated at the two distinct probe spots is collected with no attempt to distinguish signal from the two sites. The feasibility of this initial strategy is shown in Figure 2. Here, a sample containing the positively charged 5HT and negatively charged 5HI2CA is photochemically converted at the gate site and the resultant transient products are electrophoretically analyzed using an applied potential of 15 kV between buffer reservoirs.

Under these separation conditions, this two-component mixture was separated in less than 60 μs at a resolution of 1.5. Separation efficiencies – ~20 and 130 theoretical plates for 5HI2CA and 5HT, respectively – are low compared to traditional capillary electrophoresis in part because of the short migration times and large extent of the injection and detection functions relative to the separation distance.17 However, extremely fast analyte velocities result in large numbers of theoretical plates generated per unit time (~1 and 2.5×10^6 plates s^{-1} for 5HI2CA and 5HT photoproducts, respectively).

Although bi-directional analyses of oppositely charged transient products can be performed using this approach, the requirement that no more than one probe site generate signal at any given time limits both the speed and the complexity of analyses that can be carried out. Even for simple samples, ones for example that contain two hydroxyindoles of opposite charge, relatively large differences in separation distances must exist to achieve baseline resolution. Moreover, for samples containing additional components, it may be prohibitively difficult to identify geometries that avoid significant signal overlap from the two channels.

Configuration II: Dual Probe Beam/Dual-Detector Instrument—To address limitations associated with temporal overlap of bands, we explored the feasibility of spatially isolating signal from the two probe sites to allow positive and negative components to be detected using separate PMTs (Configuration II, Figure 1c). Here, 5HT and 5HI2CA again were photochemically altered near the waist of a pulled capillary and separated using a continuously applied potential of 25 kV, and signal from photoproducts was collected along two separate confocal paths. The resultant electropherogram shown in Figure 3 (top plot) reveals the ability of this approach to decrease analysis times by allowing species of opposite charge to be detected simultaneously.

Bi-directional separation of 5HT and 5HI2CA photoproducts was performed using migration distances of 11 μm and 3 μm, respectively, yielding analysis times of 40 – 45 μs for both components. Because of residual EOF in the positive direction, 5HI2CA photoproduct migrates more slowly and with greater temporal bandwidth compared to the 5HT photoproduct.

In principal, spatial isolation of signal from positive and negative electrophoretic channels would eliminate signal overlap for analytes that simultaneously occupy the two probe sites. Unfortunately, for high concentration analytes, crosstalk between channels became detectable when the distance between probe sites was ca. ≤12 μm due to scattering in the optical path. This phenomenon is shown in Figure 3, middle and lower plots. Using migration distances of 6 μm in both directions and an applied potential of 10 kV, migration times for 5HI2CA and 5HT were 95 μs and 125 μs, respectively. In both detection channels a minor but detectable peak is observed at the migration time for the component from the alternate channel, a signal representing crosstalk of several percent.

Configuration III: Modulated Dual Beam/Single-Detector Instrument—In contrast to spatially isolating signal, it is possible to temporally isolate detection of positive and negative components by high-speed interdigitization of electrophoretic traces. In this approach, signal is generated alternately in the two channels by pulsing the probe beams to high intensity out of phase with each other using an optical rotator (Configuration III, Figure 1b, with optical rotator). By detecting signal for each migration direction on alternate, high-speed electrophoretic traces, the fluorescence-collection pathway is simplified, analyte sensitivity is improved (i.e., by eliminating the confocal pinholes), and interference between the two channels is completely avoided.

These results are shown in Figure 4, where a three-component mixture of 5HT, 5HTrp, and 5HI2CA was photochemically altered near the hourglass waist. Because a small residual EOF component exists in this study, the neutral 5HTrp photoproduct migrates in the same direction as the positive 5HT product. Separation distances were 2 μm for negative species and 3 μm for positive and neutral species, a geometry that enabled the positive and negative components to be analyzed within 10 μs and all three components to be resolved within 30 μs.

No detectable signal was measured from the low-intensity residual laser light transmitted to a probe site during its "off" periods. The magnitudes of the electrophoretic mobilities for 5HT and 5HI2CA transient photoproducts were experimentally indistinguishable, a result consistent with similar molecular structures and magnitude of charge states (+1 and -1, respectively) of the parent hydroxyindoles. However, because of the small EOF in the direction of the cathode, 5HT migrates at a greater velocity (0.4 m s^{-1}) than 5HI2CA (0.2 m s^{-1}) and produces a narrower temporal bandwidth.

Finally, by reducing the separation distances for positive and negative components to 2.2 and 2.0 μm, respectively, transient photoproducts created from a two-component mixture (5HT and 5HI2CA) could be analyzed with both peaks arriving at their respective detection sites in ca. 6 μs (Figure 5).

CONCLUSIONS

We demonstrate an approach for rapidly characterizing transient compounds based on their electrophoretic velocities toward an anode or cathode from a centrally positioned photoreaction site. By designing a system capable of detecting analytes as they migrate in opposite directions within an electric field, analysis times for mixtures containing species of opposite charge states are minimized.

In the simplest strategy, product fluorescence is probed using a single detector, but this approach is limited by its absolute requirement that positive and negative species do not simultaneously occupy their respective detection sites. In principle, spatial isolation of signal from multiple detection sites (i.e., by imaging fluorescence in different detection channels) would both simplify analysis and minimize analysis times. However, small but measurable levels of channel cross-talk limit the minimum distances between detection sites, preventing analysis times from approaching their theoretical limits.

In contrast, temporal isolation of analyte signal – achieved by rapidly alternating the detection site between the anodic to cathodic directions – provides a means to efficiently fractionate multi-component mixtures with no detectable channel cross-talk. Moreover, by eliminating the need for spatial filtering, the fluorescence throughput of the system is improved, resulting in greater sensitivity. Using high-speed CE with a large electroosmotic flow component and a single-detection site, we previously demonstrated feasibility for fractionating positive and neutral species within 10 μs.17 Although a negative component was not present in those earlier studies, we estimate the analysis time for 5HI2CA would have been nearly 20 μs. The time scale on which electrophoretic information is obtained in the current studies – ca. 6 μs for both positive and negative species – represents a several-fold decrease in the time needed to characterize oppositely charged components in a mixture.

This high-speed approach for characterizing the mobility of transient intermediates should be applicable to a range of reactions. In addition to photochemical processes such as those examined in this work, it should prove feasible to study intermediates in non-photonic reactions using capillary or on-chip electrophoresis. In such cases, thermal and enzyme-

catalyzed reactions could be initiated rapidly either by high-speed mixing or by photolytically exposing a reactive moiety on one or more dissolved solutes. In addition, by appending reagent molecules with caged fluorophores, the fluorescence properties of solutes in flowing streams could be dynamically altered in brief time windows – a requirement for detecting fluorescent products on top of a baseline of low fluorescence.

Finally, the value of multiple detection sites in microsecond separations should extend beyond multi-component analyses, as it provides new capabilities for tracking the temporal evolution of a single analyte as it migrates through an electric field (data not shown). By using multiple probe beams positioned along a capillary axis, it may be possible, for example, to examine the conformational state of a macromolecule as it folds or unfolds over microsecond-to-millisecond time scales.

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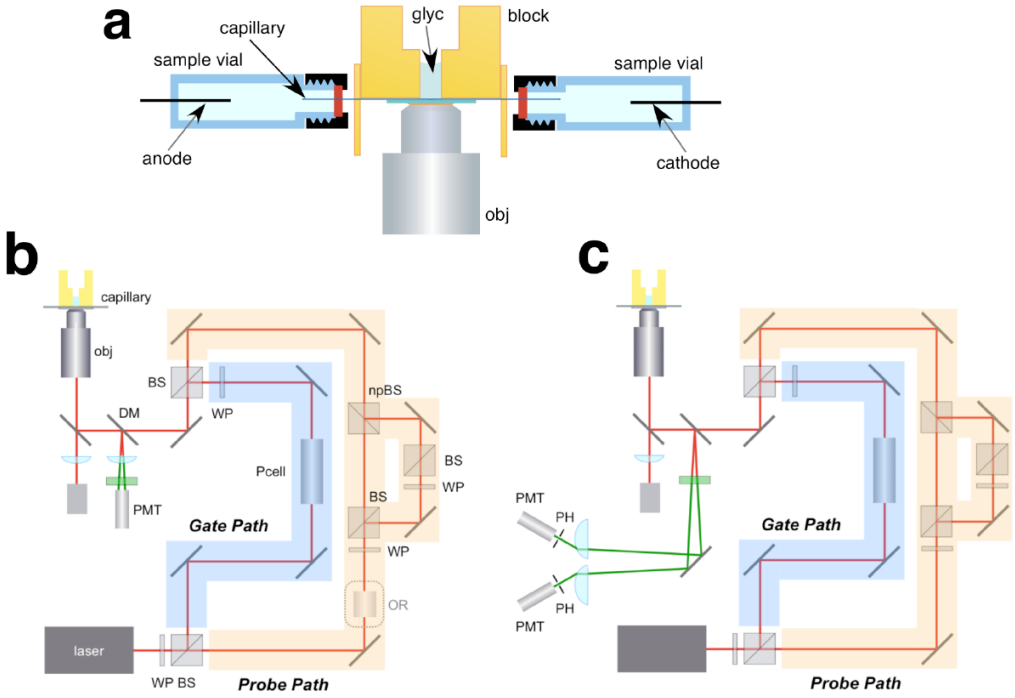

Figure 1.

Schematics showing optical configurations for bi-directional electrophoretic analysis on microsecond timescales. **(a)** Diagram of the electrophoresis assembly. Index-matching glycerin (glyc) fills small gaps between the capillary and its supporting coverslip. The microscope objective (obj) focuses two separate laser probe beams to positions in the capillary separated by less than 10 μm, with a microsecond-gated photoreaction beam sandwiched between these spots. **(b)** Diagram of an optical system based on a single detector. A mode-locked titanium:sapphire (Ti:S) beam passes through a half-wave plate (WP), with different polarization components directed to gate and probe paths using a polarizing beamsplitter (BS). The vertically polarized gate beam (blue path) passes through a Pockel's cell (Pcell), which is used to switch the beam to high intensities for periods of 0.7 – 10 μs (to generate fluorescent photoproduct) before passing through a second WP and being recombined into the main optical path using a second polarizing BS. The horizontally polarized probe beam (orange path) is split along two sub-paths using a WP/BS pair, with the vertically polarized split (right sub-path) passing through a WP/BS to equalize laser power and rotate the polarization to the horizontal plane. The two portions of the probe beam are recombined using a non-polarizing beamsplitter (npBS). In some studies, an optical rotator from a Pockel's cell is included in the probe path to allow high-intensity light to sequentially pass through or be reflected by the BS (i.e., alternating between the two sub-paths). After the gate and probe beams are recombined slightly off-axis from each other, they are directed through a dichroic mirror (DM) and reflected using a 90/10 beamsplitter into a high-NA microscope objective. Laser spots are aligned along the capillary axis using mirror adjustments and a three-axis micropositioning capillary mount. Reflected light is imaged using a video CCD and fluorescence from the two probe spots is split off from the beam path using the DM, passing through optical filters to a photomultiplier tube (PMT). **(c)** Diagram of the dual-detector configuration. This system is identical to the single-detector set-up, with the exception that the detection arm is extended ~2 m, allowing spatial separation of the fluorescence collected from the two probe sites. Fluorescence from these two paths is directed off-axis through separate 7.5-cm focal length plano-convex lenses, and is focused through ~3-mm diameter pinholes positioned in front of separate PMTs.

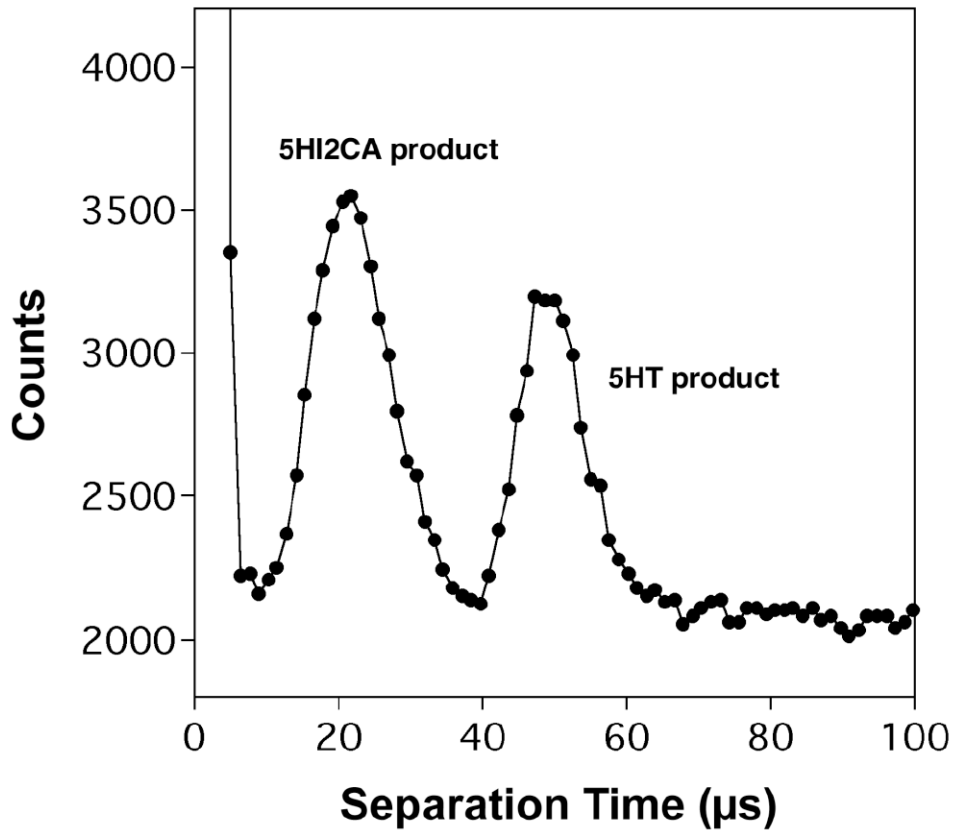

Figure 2.

Bi-directional separation of positively and negatively charged transient photochemical products. Photoproducts are generated from a sample containing 250 μM 5HT and 280 μM 5HI2CA in 25 mM pH 5.6 MES with 0.1% (w/v) Pluronic. Samples are created using a 4-μs gate period and components are separated using a potential of 15 kV. Data are collected in bins of 1.28 μs and the resultant trace represents the sum of 40,000 sequential records. Products of opposite charge migrate toward separate probe sites that sandwich the gate region, with separation distances for 5HI2CA and 5HT products of 1.5 μm and 6.8 μm, respectively.

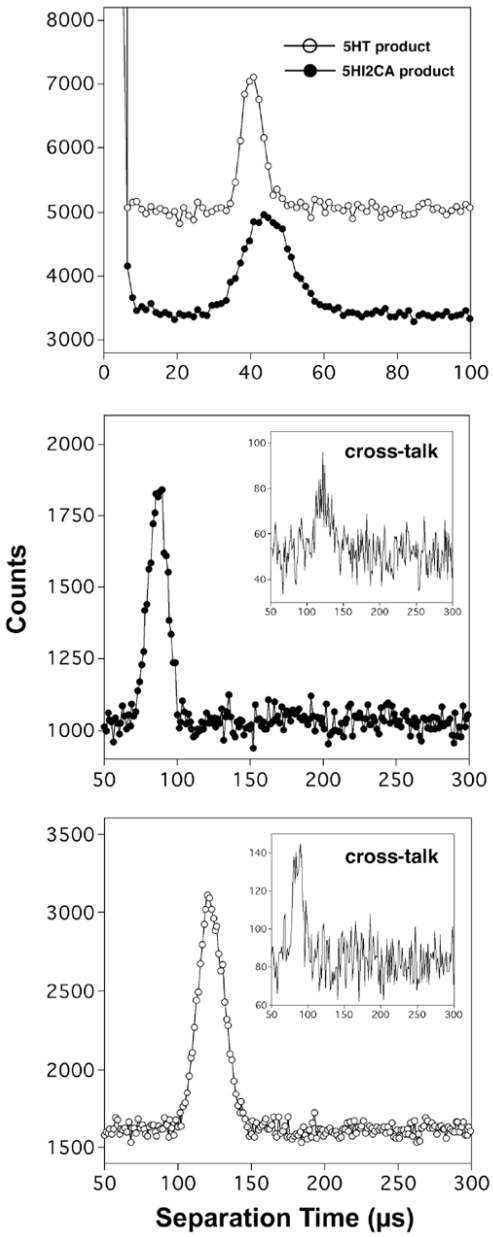

Figure 3.

Bi-directional electrophoresis using spatially isolated detection of probe site fluorescence. **Top plot:** Fractionation of 5HT and 5HI2CA photoproducts in less than 45 μs over migration distances of 11 μm and 3 μm, respectively. Samples contained 250 μM of both 5HT and 5HI2CA in 10 mM pH 5.6 MES with 0.5% (w/v) Pluronic. Photoproducts were generated using a 5-μs gate period and separated using a potential of 25 kV, and data were collected in bins of 1.28 μs. The traces represent a sum of 130,000 sequential records. **Middle and lower plots:** Electrophorograms produced from signal at the two separate detectors, with analyte arrival times purposefully displaced in time to reveal possible cross-talk between PMT channels. The middle plot shows PMT signal produced by imaging fluorescence from negatively charged compounds (e.g., the 5HI2CA photoproduct) while the lower plot is produced by a separate PMT responsible for imaging fluorescence from positive species (e.g., the 5HT photoproduct). Note that both channels reveal a cross-talk

between channels of 3 – 4%. A potential of 10 kV was used to fractionate photoproducts generated from a sample consisting of 250 μM of both 5HT and 5HI2CA in 10 mM MES (pH 5.6) containing 0.1% (w/v) Pluronic and 0.5mM $AlCl_3$ using a gate period of 10 μs. Both probe sites were positioned ~6 μm from the gate site. The data were collected using bins of 1.28 μs and summed to 15,000 records.

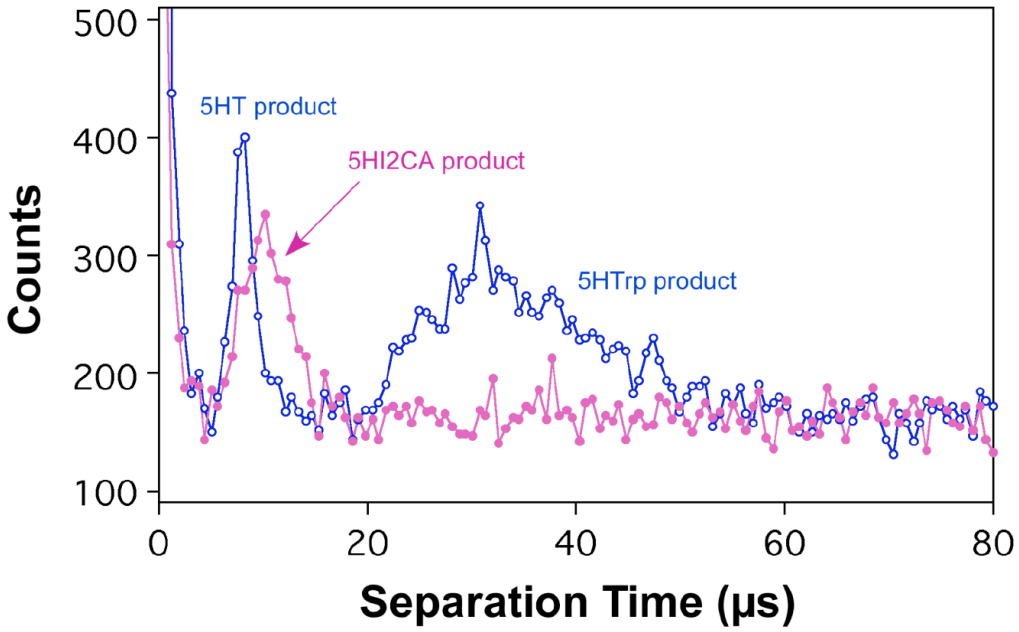

Figure 4.

Bi-directional electrophoresis using alternating probe beams. In this detection strategy, the two probe spots were alternately focused into the channel on the anodic and cathodic side of the gate spot at a switching frequency of 1 kHz. A sample containing 5HT, 5HTrp, and 5HI2CA, each at 250 μM in 20 mM MES (pH 5.5) with 0.1% (w/v) Pluronic was fractionated following photochemical gating using a potential of 39 kV. Migration distances were 3.0 μm for positive and neutral components and 2.0 μm for negative species. Photochemical products of each analyte were generated using 0.7-μs gate periods and data were collected in bin sizes of 640 ns. Plots represent a sum of 19,000 sequential electrophoretic traces. Positive (5HT) and negative (5HI2CA) photoproducts are analyzed in distinct channels, with an analysis time of 10 μs required to characterize mobilities of both compounds. A neutral 5HTrp photoproduct is observed in the “positive” product channel due to a residual electroosmotic flow component.

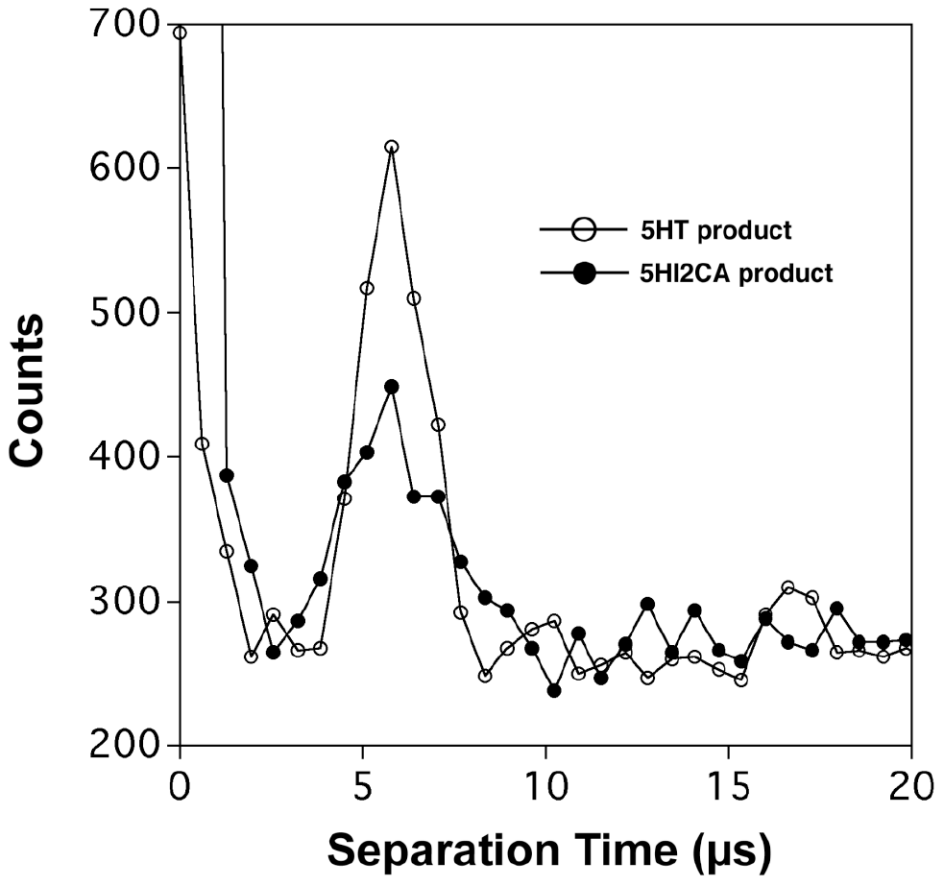

Figure 5.

Bi-directional electrophoretic analysis of oppositely charged transient molecules in ca. 6 μs using alternating (1 kHz) probe beams. Sample contained 500 μM of both 5HT and 5HI2CA in 20 mM MES (pH 5.5) with 0.1% Pluronic; photoproducts were generated using a 0.7-μs gate period and separated using a potential of 46 kV over distances of 2.2 μm (5HT product) and 2.0 μm (5HI2CA product). Data were collected in bins of 640 ns and electropherograms represent a sum of 6000 sequential electrophoretic traces.