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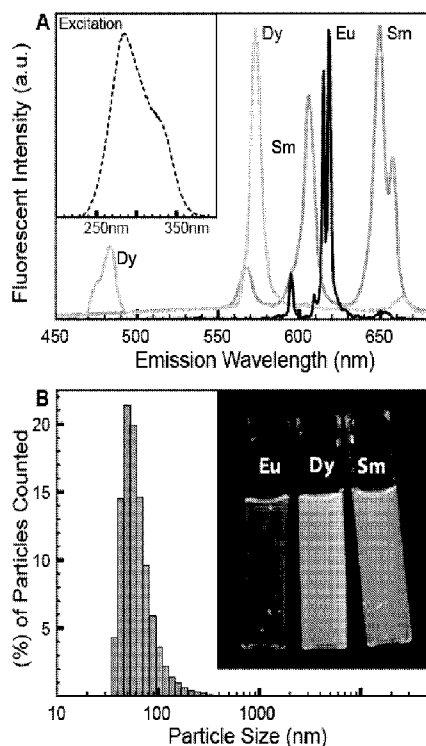
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(54) **Title:** SPECTRALLY ENCODED MICROBEADS AND METHODS AND DEVICES FOR MAKING AND USING SAME

FIGURE 1



(57) **Abstract:** Spectrally encoded microbeads and methods and devices for making and using spectrally encoded microbeads are provided. The disclosed methods and devices facilitate the preparation and use of microbeads containing multiple lanthanide nanoparticles, which microbeads have uniquely identifiable spectral codes. The disclosed microbeads, and the methods and devices for making and using same, find use in multiplexing and high-throughput biomarker analysis.



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**SPECTRALLY ENCODED MICROBEADS AND METHODS AND DEVICES FOR MAKING AND
USING SAME**

CROSS-REFERENCE TO RELATED APPLICATIONS

- [0001]** This application claims of benefit of U.S. Provisional Patent Application No. 61/692,618, filed August 23, 2012, which application is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

- [0002]** The invention was made with government support under Grant No. DE-AC02-05CH11231 awarded by the U.S. Department of Energy. The government has certain rights in the invention.

INTRODUCTION

- [0003]** Over the past several years, advances in biomedical research technology have driven an unprecedented explosion of genomic and proteomic data, yet the challenge of translating new biomarkers of disease into actionable diagnostics and therapeutics remains daunting. To both validate and deploy the vast numbers of recent discoveries into clinical practice requires new approaches to multiplexing and high-throughput biomarker analysis. Despite intense research, few practically available cost-effective assays for multiplexing exist, and new approaches are needed. Beyond diagnostics, advances in multiplexing may have impact on basic research and development systems, including combinatorial drug discovery.
- [0004]** Multiplexed assays require that individual probes be reliably identified and tracked throughout an experiment. This identification and tracking is often done using planar arrays, where the identity of each probe is encoded by its physical position. An alternative approach uses encoded beads, where each probe is attached to a separate bead that is uniquely identifiable.
- [0005]** Bead-based assays offer faster reaction kinetics, increased assay flexibility, and improved reproducibility and reduced costs due to the ability to attach probes to multiple particles at once. However, technical challenges in bead encoding have limited their practical application to date. Existing encoding methods generally fall into two categories: spatial encoding and spectral encoding. Spatial encoding schemes

create graphical patterns or bar codes in the particle material in a variety of ways. However, spatial methods face difficulties in cost-effective fabrication, often require large particles to generate large code sets, and have slower and more challenging code readout than existing spectral methods due to orientation requirements.

[0006] Spectral encoding schemes incorporate mixtures of photoluminescent materials such as lanthanides, quantum dots (QDs), or fluorescent dyes that emit light at different wavelengths to generate uniquely identifiable signatures. These schemes allow identification of codes in any orientation and are compatible with conventional bead synthesis procedures and standard detection optics, making them particularly attractive. Despite the promise of spectral encoding schemes, technical challenges have limited their practical code capacity. Organic dyes have broad emission spectra, narrow Stokes shifts, and limited photostability, making it difficult to deconvolve spectral signatures from multiple dyes and reducing the usable lifetime of the codes. Quantum dots offer relatively narrow and tunable excitation spectra, and have therefore been the subject of considerable recent interest for encoding schemes. However, QDs have complicated photophysics and can undergo energy transfer and re-absorption when tightly packed together. These effects limit the number of optical codes that can be created, due to re-absorption losses at higher concentrations in the beads. As a result, the largest experimentally produced spectral code sets from organic dyes or quantum dots have fallen far short of theoretical expectations. The best known commercial system, Luminex®, has been limited to 500 unique codes and code sets synthesized in the literature have been even smaller.

[0007] Accordingly, there exists a need in the art for improved multiplexing and high-throughput biomarker analysis techniques and tools. The present disclosure addresses this need and provides related advantages.

SUMMARY

[0008] Spectrally encoded microbeads and methods and devices for making and using spectrally encoded microbeads are provided. The disclosed methods and devices facilitate the preparation and use of microbeads containing multiple lanthanide nanoparticles, which microbeads have uniquely identifiable spectral codes. The disclosed microbeads, and the methods and devices for making and using same, find use in multiplexing and high-throughput biomarker analysis.

- [0009] The present disclosure provides a population of polymeric microbeads embedded with at least two different lanthanide nanoparticles, the population including: a plurality of polymeric microbeads, wherein each polymeric microbead of the plurality is embedded with at least two lanthanide nanoparticles having different luminescence spectra, and wherein the relative concentrations of the first and second lanthanide nanoparticles are substantially equal among the polymeric microbeads of the population.
- [0010] In some instances, the luminescence intensity level variation among all the members of the population is no greater than about 15 percent.
- [0011] Any of the aforementioned populations may have a luminescence intensity level variation among all the members of the population that is no greater than about 5 percent.
- [0012] Any of the aforementioned populations may have polymeric microbeads, wherein each polymeric microbead of the population has a diameter of less than 500 μm .
- [0013] Any of the aforementioned populations may have polymeric microbeads, wherein each polymeric microbead of the population has a diameter of less than 100 μm .
- [0014] Any of the aforementioned populations may have polymeric microbeads, wherein each polymeric microbead of the population has a diameter of less than 50 μm .
- [0015] Any of the aforementioned populations may have polymeric microbeads, wherein the diameter variation among all the members of the population is no greater than about 5 percent.
- [0016] Any of the aforementioned populations may have polymeric microbeads, wherein one or more of the lanthanide nanoparticles includes bismuth.
- [0017] The present disclosure also provides a set of populations of polymeric microbeads embedded with at least two different lanthanide nanoparticles, the set of populations of polymeric microbeads including: a first population of polymeric microbeads, wherein each polymeric microbead of the first population is embedded with at least a first lanthanide nanoparticle and a second lanthanide nanoparticle; wherein the first and second lanthanide nanoparticles comprise different lanthanides; and wherein the relative concentrations of the first and second lanthanide nanoparticles are substantially equal among the polymeric microbeads of the first

population; and a second population of polymeric microbeads, wherein each polymeric microbead in the second population is embedded with at least the first lanthanide nanoparticle and the second lanthanide nanoparticle; and wherein the relative concentrations of the first and second lanthanide nanoparticles are substantially equal among the polymeric microbeads of the second population; wherein the concentration of at least one of the first and second lanthanide nanoparticles is different between the polymeric microbeads of the first population and second population.

[0018] In some instances, the concentration of one of the first and second lanthanide nanoparticles is substantially equal for each polymeric microbead of the first population and second population of the set of populations.

[0019] In some instances, the concentration of the first lanthanide nanoparticle in the first population is a known percentage of the concentration of the first lanthanide nanoparticle in the second population of the set of populations, wherein the known percentage is other than 100 percent.

[0020] Any of the aforementioned sets of populations may have polymeric microbeads, wherein the luminescence intensity level variation among all the members of the first population is no greater than about 15 percent.

[0021] Any of the aforementioned sets of populations may have polymeric microbeads, wherein the luminescence intensity level variation among all the members of the first population is no greater than about 5 percent.

[0022] Any of the aforementioned sets of populations may have polymeric microbeads, wherein the luminescence intensity level variation among all the members of the second population is no greater than about 15 percent.

[0023] Any of the aforementioned sets of populations may have polymeric microbeads, wherein the luminescence intensity level variation among all the members of the second population is no greater than about 5 percent.

[0024] Any of the aforementioned sets of populations may have polymeric microbeads, wherein each polymeric microbead has a diameter of less than 500 μm .

[0025] Any of the aforementioned sets of populations may have polymeric microbeads, wherein each polymeric microbead has a diameter of less than 100 μm .

[0026] Any of the aforementioned sets of populations may have polymeric microbeads, wherein each polymeric microbead has a diameter of less than 50 μm .

- [0027] Any of the aforementioned sets of populations may have polymeric microbeads, wherein the diameter variation among all the members of the set of populations is no greater than about 5 percent.
- [0028] Any of the aforementioned sets of populations may have polymeric microbeads, wherein each polymeric microbead comprises 3 to 10 lanthanide nanoparticles, wherein each lanthanide nanoparticle has a different luminescence spectra.
- [0029] Any of the aforementioned sets of populations may have polymeric microbeads, wherein each polymeric microbead comprises an upconverting lanthanide nanoparticle.
- [0030] Any of the aforementioned sets of populations may have polymeric microbeads, wherein each polymeric microbead comprises a downconverting lanthanide nanoparticle.
- [0031] Any of the aforementioned sets of populations may include 24 or more different populations of polymeric microbeads.
- [0032] Any of the aforementioned sets of populations may include 64 or more different populations of polymeric microbeads.
- [0033] Any of the aforementioned sets of populations may have polymeric microbeads, wherein the lanthanide nanoparticles comprise bismuth.
- [0034] The present disclosure also provides a set of populations of polymeric microbeads embedded with at least two different lanthanide nanoparticles, the set of populations of polymeric microbeads being produced using a method including: mixing at least two fluids into a first solution, wherein each fluid includes a polymerizable component and a lanthanide nanoparticle having a different luminescence spectra; forming a first plurality of droplets from the first solution; subjecting the first plurality of droplets to polymerization conditions, thereby producing a first population of polymeric microbeads embedded with at least two different lanthanide nanoparticles; mixing the at least two fluids into a second solution, wherein the concentration of at least one of the different lanthanide nanoparticles is different between the first solution and the second solution; forming a second plurality of droplets from the second solution; and subjecting the second plurality of droplets to polymerization conditions, thereby producing a second population of polymeric microbeads embedded with the at least two different lanthanide nanoparticles, wherein the concentration of at least one of the at least two

different lanthanide nanoparticles is different between the polymeric microbeads of the first population and the polymeric microbeads of the second population.

[0035] In some instances, the concentration of one of the first and second lanthanide nanoparticles is substantially equal for each polymeric microbead of the first population and second population of the set of populations.

[0036] In some instances, the concentration of the first lanthanide nanoparticle in the first population is a known percentage of the concentration of the first lanthanide nanoparticle in the second population of the set of populations, wherein the known percentage is other than 100 percent.

[0037] The present disclosure also provides a microfluidic device, the device including: a flow channel having an inlet side and an outlet side; at least two inlets positioned toward the inlet side of the flow channel, wherein the inlets are configured to fluidly communicate with the flow channel; a mixing element positioned in the flow channel downstream of the at least two inlets; an input configured to hold a carrier fluid and fluidly communicate with the flow channel, wherein the input configured to hold a carrier fluid is configured to fluidly communicate with a portion of the flow channel downstream of the mixing element; and an outlet located at the outlet side of the flow channel, wherein the outlet is configured to fluidly communicate with the flow channel and is positioned downstream of the portion of the flow channel with which the input configured to hold a carrier fluid is configured to fluidly communicate.

[0038] In some instances, the microfluidic device includes a sample collection element in fluid communication with the outlet, and located downstream of the outlet.

[0039] Any of the aforementioned microfluidic devices may include a plurality of valves, including valves separating each of the at least two inlets from the flow channel respectively.

[0040] Any of the aforementioned microfluidic devices may include a valve between the mixing element and the portion of the flow channel with which the input configured to hold a carrier fluid is configured to fluidly communicate, wherein the valve between the mixing element and the portion of the flow channel with which the input configured to hold a carrier fluid is configured to fluidly communicate, when closed, prevents fluid contained in the mixing element from contacting fluid from the input configured to hold a hydrophobic carrier fluid. In some instances, such microfluidic devices may include a waste outlet located between the mixing element

and the valve between the mixing element and the portion of the flow channel with which the input configured to hold a carrier fluid is configured to fluidly communicate. In some instances, one or more of the plurality of valves is configured to be actuated pneumatically.

[0041] For any of the aforementioned microfluidic devices, the carrier fluid may be a hydrophilic carrier fluid or a hydrophobic carrier fluid. Where the carrier fluid is a hydrophilic carrier fluid, the microfluidic devices may also include an input which is configured to hold a hydrophobic carrier fluid and fluidly communicate with the flow channel and is located upstream of the mixing element. Where the carrier fluid is a hydrophilic carrier fluid, the input configured to hold a hydrophilic carrier fluid may include an on-chip resistor. Where the carrier fluid is a hydrophobic carrier fluid, the input configured to hold a hydrophobic carrier fluid may include an on-chip resistor. One or more of the above features may be combined in a single microfluidic device.

[0042] Where the carrier fluid is a hydrophobic carrier fluid, the microfluidic device may also include an input which is configured to hold a hydrophilic carrier fluid and fluidly communicate with the flow channel and is located upstream of the mixing element. Where the carrier fluid is a hydrophobic carrier fluid, the input configured to hold a hydrophilic carrier fluid may include an on-chip resistor. Where the carrier fluid is a hydrophobic carrier fluid, the input configured to hold a hydrophobic carrier fluid may include an on-chip resistor. One or more of the above features may be combined in a single microfluidic device.

[0043] For any of the aforementioned microfluidic devices the mixing element may include a staggered herringbone mixer.

[0044] Any of the aforementioned microfluidic devices may be fabricated by multi-layer soft lithography.

[0045] Any of the aforementioned microfluidic devices may be fully automated.

[0046] The present disclosure also provides a system including any of the aforementioned microfluidic devices, and a chamber, wherein the device is positioned in the chamber and exposed to nitrogen gas therein.

[0047] The present disclosure also provides a system including any of the aforementioned microfluidic devices, and a UV generating element positioned to expose a portion of the flow channel to UV radiation, wherein the portion to be exposed to UV radiation is downstream of the portion of the flow channel with which

the input configured to hold a carrier fluid is configured to fluidly communicate and upstream of the outlet.

[0048] The present disclosure also provides a system including any of the aforementioned microfluidic devices, and a plurality of inlet containers, wherein each inlet container is configured to fluidly communicate to a different one of the at least two inlets, and wherein each inlet container comprises a fluid comprising a different lanthanide nanoparticle and a polymerizable component. In some instances, the system includes a plurality of pumps, wherein the plurality of inlet containers is configured to fluidly communicate with the plurality of pumps. In some instances, the inlet containers include capillary tubing having a length that is at least 1000 times greater than the internal diameter of the capillary tubing. In some instances, the containers are positioned in a chamber and exposed to nitrogen gas therein. In some instances, the plurality of containers includes 2 to 10 lanthanide nanoparticles, wherein each lanthanide nanoparticle has a different luminescence spectra. In some instances, the lanthanide nanoparticle contained in each fluid is present at a concentration of 1 mg/mL to 250 mg/mL. One or more of the above features may be combined in a single system.

[0049] The present disclosure also provides a microfluidic device, the device including: a flow channel having an inlet side and an outlet side, wherein a portion of the flow channel is configured as a zig-zag mixer; at least two inlets positioned toward the inlet side of the flow channel, wherein the at least two inlets are configured to fluidly communicate with the flow channel; an input configured to hold a carrier fluid configured to fluidly communicate with the flow channel, wherein the input configured to hold a carrier fluid is configured to fluidly communicate with a portion of the flow channel downstream of the at least two inlets and upstream of the portion of the flow channel configured as a zig-zag mixture; and an outlet located at the outlet side of the flow channel, downstream of the portion of the flow channel configured as a zig-zag mixer. In some instances, the carrier fluid is a hydrophobic carrier fluid. In other cases, the carrier fluid is a hydrophilic carrier fluid.

[0050] The present disclosure also provides a method for producing a polymeric microbead comprising at least two different lanthanide nanoparticles, the method including: mixing at least two fluids into a solution, wherein each fluid comprises a polymerizable component and a different lanthanide nanoparticle; forming a droplet from the solution; and subjecting the droplet to polymerization conditions, thereby

producing a polymeric microbead comprising at least two different lanthanide nanoparticles, wherein the above steps are performed on any of the aforementioned microfluidic devices or with any of the aforementioned systems. In some instances the polymerization conditions include exposing the droplet to UV radiation. In some instances, the polymerization conditions include exposing the droplet to a temperature sufficient to initiate polymerization of the polymerizable component.

[0051] For any of the aforementioned methods, each fluid may have an approximately equivalent total concentration of lanthanide nanoparticles.

[0052] For any of the aforementioned methods, the relative concentration of each different lanthanide nanoparticle in the polymeric microbead may be controlled by adjusting the relative flow rates of the at least two fluids. The relative flow rates may be determined based at least in part upon solving the coupled flow equations:

$$\text{Eqn. 1} \quad Q_n = \frac{P_n - P_{mix}}{R_n}, \text{ and}$$

$$\text{Eqn. 2} \quad Q_{tot} = \frac{P_{mix}}{R_{mix}},$$

where

Q_n is the flow rate for each input;

P_n is the pressure for each input;

Q_{tot} is total flow rate;

P_{mix} is the pressure at the point where the fluids are mixed;

R_{mix} is the resistance at the point where the fluids are mixed; and

R_n is the resistance of each input.

[0053] For any of the aforementioned methods, the droplet size may be modulated by adjusting the pressure at a T-junction used to form the droplet.

[0054] The present disclosure also provides a method for producing a population of polymeric microbeads including a plurality of different lanthanide nanoparticles, the method including: mixing at least two fluids into a solution, wherein each fluid comprises a polymerizable component and a different lanthanide nanoparticle; forming a first plurality of droplets from the solution; and subjecting the first plurality

of droplets to polymerization conditions, thereby producing a first plurality of polymeric microbeads embedded with at least two different lanthanide nanoparticles, wherein the relative concentrations of the lanthanide nanoparticles are substantially equal among the polymeric microbeads of the first plurality of polymeric microbeads. In some instances, the method includes mixing the at least two fluids into a second solution, wherein the concentration of at least one of the different lanthanide nanoparticles in the second solution is different than in (i); forming a second plurality of droplets from the second solution; and subjecting the second plurality of droplets to polymerization conditions, thereby producing a second plurality of polymeric microbeads embedded with at least two different lanthanide nanoparticles, wherein the relative concentrations of the lanthanide nanoparticles are substantially equal among the polymeric microbeads of the second plurality of polymeric microbeads. In some instances, the concentration of at least one of the different lanthanide nanoparticles in the second solution is substantially equal to that in (i). In some instances, the concentration of one of the different lanthanide nanoparticles in the first plurality of polymeric microbeads is a known percentage of the concentration of the first lanthanide nanoparticle in the second plurality of polymeric microbeads, wherein the known percentage is other than 100 percent. In some instances, the polymerization conditions include exposing the first plurality of droplets to UV radiation. In some instances, the polymerization conditions include exposing the second plurality of droplets to UV radiation. In some instances, the polymerization conditions include exposing the first plurality of droplets to a temperature sufficient to initiate polymerization of the polymerizable component. In some instances, the polymerization conditions comprise exposing the second plurality of droplets to a temperature sufficient to initiate polymerization of the polymerizable component.

[0055] Any of the aforementioned methods for producing a population of polymeric microbeads may produce a population of polymeric microbeads such that the luminescence intensity level variation among all the members of the first plurality of polymeric microbeads is no greater than about 15 percent.

[0056] Any of the aforementioned methods for producing a population of polymeric microbeads may produce a population of polymeric microbeads such that the luminescence intensity level variation among all the members of the first plurality of polymeric microbeads is no greater than about 5 percent.

- [0057] Any of the aforementioned methods for producing a population of polymeric microbeads may produce a population of polymeric microbeads such that the luminescence intensity level variation among all the members of the second plurality of polymeric microbeads is no greater than about 15 percent.
- [0058] Any of the aforementioned methods for producing a population of polymeric microbeads may produce a population of polymeric microbeads such that the luminescence intensity level variation among all the members of the second plurality of polymeric microbeads is no greater than about 5 percent.
- [0059] The present disclosure also provides a method of imaging spectrally encoded microbeads, the method including: illuminating one or more microbeads selected from any of the aforementioned populations of microbeads with a light source; detecting luminescence emission from the microbead in a plurality of spectral bands; and determining the intensities of each different lanthanide nanoparticle present in the microbead using linear unmixing. In some instances, the spectral bands are defined by a plurality of emission filters that pass the characteristic emission peaks of each lanthanide nanoparticle. In some instances, the light source is a deep UV light source. In some instances, the light source is near IR light source.
- [0060] The present disclosure also provides a system, including: a microfluidic device including one or more inlet ports; a flow channel configured for fluid communication with the one or more inlet ports, wherein the flow channel is sized and shaped to provide a monolayer of polymeric microbeads in the flow channel; a sieve valve positioned in or downstream of the flow channel, wherein the sieve valve is configured to allow fluid flow through the flow channel while retaining the polymeric microbeads in the flow channel; and one or more outlet ports configured for fluid communication with the flow channel; and a light source configured to illuminate a portion of the flow channel. In some instances, the light source is a deep UV light source. In some instances, the light source is a near IR light source. Any of the above systems may include a camera configured to collect an image of the illuminated portion of the flow channel. Any of the above systems may include a display configured to display an image of the illuminated portion of the flow channel.

BRIEF DESCRIPTION OF THE DRAWINGS

- [0061] **FIG. 1** provides an emission spectra (Panel A) for each of nanoparticles (Dy-, Eu-, and Sm- doped $\text{Y}_{0.80}\text{Bi}_{0.15}\text{VO}_4$) when excited at 285 nm. Excitation spectra of all

three nanoparticles (inset) are nearly identical. FIG. 1 also provides a histogram (Panel B) showing particle size distribution for nanoparticles (as measured by dynamic light scattering), and a photograph of vials of nanoparticle suspensions illuminated with a UV lamp (inset).

[0062] **FIG. 2** provides schematics and related images of a microfluidic bead synthesizer according to some embodiments of the present disclosure and a method of using same. (Panel A) Stage 1 of bead synthesis. Mixtures of lanthanide nanoparticles (Eu alone, Eu/Sm, Eu/Dy) suspended in pre-polymer bead mixture flow into a microfluidic device at controlled ratios and are mixed on chip using a mixing element (an exemplary staggered herringbone mixer) is depicted. (Panel B) Stage 2 of bead synthesis. A hydrophilic carrier fluid (e.g., water pushes) the lanthanide mixture towards a T-junction containing a continuously flowing hydrophobic carrier fluid (e.g., oil) stream, producing droplets (shown in microscope image, inset). Droplets are polymerized into beads by exposing them to polymerization conditions (e.g., via illumination with UV light) and collected for later use. (Panel C) CAD drawing of the flow channels of the device showing the lanthanide inputs and the exemplary herringbone mixer, an input configured to hold a hydrophilic carrier fluid (an exemplary water input is depicted) and resistor, an input configured to hold a hydrophobic carrier fluid (e.g., an oil input) and bead output. (Panel D) Photograph of the bead synthesizer microfluidic device with food coloring in the channels and a dime for scale. Flow channels are colored as in panel C; control lines used to open and close on-chip valves are identified with “*”.

[0063] **FIG. 3** provides schematics and related images of a bead imaging setup according to some embodiments of the present disclosure. (Panel A) Photograph of microfluidic imaging device with flow channels shown in black and control channels shown in grey (and identified by “*”). Beads are injected into a 55 μm wide serpentine channel for imaging (photograph, inset); sieve valves at the end of the channel retain beads while permitting fluid flow to facilitate channel loading. Inputs (Injection 1 (Inj1), Bead In (BdIn), Injection 2 (Inj2)) and outputs (Waste 1 (W1), Waste 2 (W2), Bead Out (BdOut)) at either end of the device provide bidirectional flow. (Panel B) Schematic of a microscopy system used for imaging beads. Light from a suitable light source (e.g., a full-spectrum 300 W Xenon arc lamp) is collected (L1), reflected off a 400 nm long pass filter (M1) to reject visible light, and passed through a shutter (S1) and an excitation filter wheel (to switch between UV and

visible transillumination) before being focused (L2) into a deep UV liquid light guide. The other end of the liquid light guide is mounted on the condenser mount of a Nikon® Ti microscope, where the light is collimated by a fused silica lens (L3) and projected onto the sample. Emitted light from the sample is collected by a Plan Apo 4x/0.2 NA objective (L4), with a UV blocking filter placed between the sample and the objective. Emitted light is filtered through an emission filter wheel mounted beneath the objective before being focused onto the camera.

[0064] **FIG. 4** provides images and graphical data associated with an analysis procedure according to some embodiments of the present disclosure. (Panel A) Raw data in each of six luminescence channels. Data are scaled linearly. (Panel B) Reference spectra used for unmixing. (Panel C) Left: Linearly scaled black and white images of unmixed data from each channel (Dy, Eu, and Sm), with black set to the minimum intensity in the image and white set to the maximum intensity. Right: Bright field image of the same field of view. (Panel D) False color overlay of Dy, Eu, and Sm luminescence with scaling as in Panel C (shown here in greyscale).

[0065] **FIG. 5** provides a graphical representation of a 24 code matrix according to some embodiments of the present disclosure. (Panel A) Scatter plot of the median Dy/Eu and Sm/Eu luminescence ratios for 10 filled serpentines (1926 beads), with points false colored according to their Sm/Eu and Dy/Eu ratios (shown here in greyscale). Each point represents one bead. Grey ellipses around each code cluster illustrate three- and four-sigma contours derived from fitting a Gaussian mixture model to the data. Histograms of bead ratios in the Dy/Eu and Sm/Eu channels (black) and their corresponding Gaussian fits (grey fit lines) are shown along each axis; these histograms group all codes together. Inset: Measured cluster centroids and their corresponding programmed intensity ratios; the root mean square deviation between the programmed ratios and the measured ratios is 0.014. (Panel B) Standard deviations calculated from Gaussian fits to the bead ratio histograms in Panel A as a function of ratio (filled circles, identified with “*” for Dy and “+” for Sm). Square symbols illustrate the statistical standard deviation determined from replicated imaging of the same serpentine of beads.

[0066] **FIG. 6** provides a graph showing the reproducibility of lanthanide nanoparticle synthesis according to some embodiments of the present disclosure. Each individual batch of lanthanide nanoparticle suspensions were diluted 1:500 in DI water from the concentrated stock solutions. A luminescence emission spectrum (400-

800 nm) was obtained using a FluoroMax-3 spectrofluorometer for all of these diluted stock solutions. The excitation was the same for all solutions (285 nm through a 3-nm slit width excitation monochromator) and the emission parameters were also held constant for all emitters (3-nm emission slit width, 1-nm increment steps, and 0.1 sec integration time at each step) with the exception of the Europium lanthanide nanoparticles which, due to their brightness, had slit widths of 1 nm at both monochromators. For each emitter shown, the left column shows the emission spectra of each individual batch synthesized as a stacked plot: (Panel A) Sm, 4 batches, (Panel B) Dy, 3 batches, and (Panel C) Eu, 5 batches. In the right column, the normalized emission spectra for all batches are shown as an overlay for each emitter. Typically, only one color is observed in the overlaid spectra since the high reproducibility of the batches results in several spectra that are coincident.

[0067] **FIG. 7** provides images showing compatibility of exemplary spectrally encoded beads according to some embodiments of the present disclosure with commonly used visible fluorophores. A sample of the 24-code beads were imaged using a Chroma Sedat quad filter set (#89000), with a Lambda XL lamp, CoolSNAP™ HQ2 camera, 10x / 0.3 NA objective, and 1 sec exposure time for each luminescence channel. All four channel combinations were imaged, and the corresponding images are labeled with the excitation and emission centers of the filter sets. The 402/455 (DAPI channel) image shows weak luminescence; the other channels show negligible luminescence with the luminescence in the Cy5 channel being undetectable.

[0068] **FIG. 8** provides scatter plots of two different batches of synthesized beads (Bead Set 1 (grey) and Bead Set 2 (Black)) according to some embodiments of the present disclosure. The two batches were synthesized on different dates and imaged on the same date. The Set 1 batch of beads is missing one code due to a computer error.

[0069] **FIG. 9** provides a graph comparing programmed ratios (black) and measured code centroids and three sigma error ellipses for the Set 1 (“*”) and Set 2 (“+”) beads referenced in FIG. 8.

[0070] **FIG. 10** provides a schematic of peptide synthesis on spectrally encoded beads.

[0071] **FIG. 11** provides false color images demonstrating that embedded codes are robust to peptide synthesis conditions. (Panel A) Image showing beads containing

either Eu alone (blue) or a combination of Eu and Dy (yellow) after on-bead synthesis of either FLAG peptide (Eu beads, blue) or myc peptide (Eu/Dy beads, yellow).

(Panel B) Image showing binding of Alexa-647-labeled anti-FLAG antibody (red) and Alexa-555-labeled anti-myc antibody (green) to encoded beads.

[0072] **FIG. 12** provides a photograph showing the microfluidic bead reactor utilized for the immunoassays described in Example 4. The device features input and output channels (BeadIn, BeadOut) for loading beads into a single main reaction chamber, as well as 4 additional input channels (R1-R4) and a single output channel for introducing reagents into the reaction chamber. Flow is controlled by 4 valves that control reagent inputs, a pump (P1) that controls reagent flow rates, and 2 sieve valves (SvIn, SvOut) that retain beads during reagent exchanges.

[0073] **FIG. 13** provides a table showing the results of liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) analysis of peptides cleaved from spectrally encoded microbeads.

[0074] **FIG. 14** provides a graph showing relative Dy signal present within a 4-code bead set before and after peptide synthesis.

[0075] **FIG. 15** demonstrates single bead release through the use of a single constriction in a microfluidic channel. (Panel A) Photograph of a linear serpentine channel for imaging of spectrally encoded beads in a first-in first-out linear array. (Panel B) Montage of images from a movie showing release of a single bead. (Panel C) Graph showing changes in intensity at the exit of the serpentine channel as a function of time. Increases in intensity indicate the passage of individual beads at fairly regular intervals.

[0076] **FIG. 16** provides a luminescence spectra of Tm:YVO₄ with and without bismuth. Tm loading is 1% in both cases and bismuth is incorporated at ~15-20% replacement of Yttrium (Y).

[0077] **FIG. 17** provides a luminescence spectra of Er:YVO₄ with and without bismuth (Bi). Er loading is 5% in both cases and bismuth is incorporated at ~15-20% replacement of Yttrium (Y).

[0078] **FIG. 18** provides an emission scan of Tm:YVO₄ with varying Tm%. The emission scan shows adjustment of the Tm % loading to arrive at a preferred value of 1% Tm:YVO₄. Nanoparticle concentrations in these solutions are approximately constant (up to 5% variance in concentration).

- [0079] **FIG. 19** provides a graph showing a comparison of Er:YVO₄ luminescence (normalized to 1M counts). The graph shows adjustment of the Er % loading to arrive at a preferred value of 5% Er:YVO₄. Nanoparticle concentrations in these solutions are approximately constant (up to 5% variance in concentration).
- [0080] **FIG. 20** provides a luminescence spectra of the CeTb:LaPO₄ nanophosphor.
- [0081] **FIG. 21** provides a graph showing the results of a particle size analysis of the CeTb:LaPO₄ nanophosphor.
- [0082] **FIG. 22** provides emission spectra for commercially available PEG-diamine and the PEG-DAM synthesized from it. As a comparison, a commercially available PEG-600 is included to illustrate an acceptable level of autofluorescence for a PEG.
- [0083] **FIG. 23** provides emission spectra showing a comparison of the autofluorescence between the commercially available PEG-diamine and the PEG-diamine synthesized from PEG2K as described herein.
- [0084] **FIG. 24** provides a proton NMR readout showing the synthesis of PEG2K-monoacrylamide-monoBoc.

DEFINITIONS

- [0085] As used herein, the term “lanthanide nanoparticle” refers to a nanoparticle which includes a lanthanide and a host lattice.
- [0086] As used herein, the term “lanthanide” refers to Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La, combinations thereof, compounds containing Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La and combinations thereof, and ions of Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La and combinations thereof.
- [0087] As used herein, the term “nanoparticle” refers to a particle having one or more dimensions (e.g., diameter) of less than 1000 nm, e.g., about 500 nm or less, about 100 nm or less, about 50 nm or less, about 10 nm or less, about 5 nm or less, or about 1 nm or less. For example, a nanoparticle may have one or more dimensions (e.g., diameter) of from less than 1000 nm to about 500 nm, from about 500 nm to about 100 nm, from about 100 nm to about 10 nm, from about 50 nm to about 10 nm, from about 10 nm to about 5 nm, or from about 5 nm to about 1 nm. Nanoparticles may have a generally spherical shape or a non-spherical shape.
- [0088] As used herein, the term “microbead” refers to a particle having one or more dimensions (e.g., diameter) of about 1000 μm or less, e.g., about 500 μm or less, about 100 μm or less, about 50 μm or less, about 10 μm or less, or about 5 μm or less.

For example, a microbead may have one or more dimensions (e.g., diameter) of from about 1000 μm to about 1 μm , from about 500 μm to about 1 μm , from about 100 μm to about 1 μm , from about 50 μm to about 1 μm , from about 10 μm to about 1 μm , or from about 5 μm to about 1 μm . Microbeads may have a generally spherical shape or a non-spherical shape.

[0089] It will be appreciated that throughout this present disclosure reference is made to amino acids according to the single letter or three letter code. For the reader's convenience, the single and three letter amino acid code is provided below:

G	Glycine	Gly	P	Proline	Pro
A	Alanine	Ala	V	Valine	Val
L	Leucine	Leu	I	Isoleucine	Ile
M	Methionine	Met	C	Cysteine	Cys
F	Phenylalanine	Phe	Y	Tyrosine	Tyr
W	Tryptophan	Trp	H	Histidine	His
K	Lysine	Lys	R	Arginine	Arg
Q	Glutamine	Gln	N	Asparagine	Asn
E	Glutamic Acid	Glu	D	Aspartic Acid	Asp
S	Serine	Ser	T	Threonine	Thr

[0090] Reference to "peptide" herein is meant to encompass a polymer of amino acids linked by native amide bonds and/or non-native amide bonds.

[0091] It should be understood that as used throughout, and unless specifically indicated otherwise, the term "amino acid" is used herein in its broadest sense, and includes naturally occurring amino acids as well as non-naturally occurring amino acids, including amino acid analogs and derivatives. The latter includes molecules containing an amino acid moiety. One skilled in the art will recognize, in view of this broad definition, that reference herein to an amino acid includes, for example, naturally occurring proteogenic L-amino acids; D-amino acids; chemically modified amino acids such as amino acid analogs and derivatives; naturally occurring nonproteogenic amino acids such as norleucine, p-alanine, ornithine, etc.; and chemically synthesized compounds having properties known in the art to be characteristic of amino acids. As used herein, the term "proteogenic" indicates that the amino acid can be incorporated into a peptide, polypeptide, or protein in a cell through a metabolic pathway.

[0092] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0093] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0094] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0095] It must be noted that as used herein and in the appended claims, the singular forms “a,” “and,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a lanthanide nanoparticle” includes a plurality of such lanthanide nanoparticles and reference to “the microbead” includes reference to one or more microbeads and equivalents thereof known to those skilled in the art, and so forth.

[0096] It is further noted that the claims may be drafted to exclude any recited element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

[0097] To the extent any definition of a term defined herein conflicts with a definition of a term in an application or reference incorporated by reference herein, the instant application shall control.

[0098] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[0099] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible. This is intended to provide support for all such combinations.

DETAILED DESCRIPTION

[00100] The present disclosure provides spectrally encoded microbeads and methods and devices for making and using spectrally encoded microbeads. The disclosed methods and devices facilitate the preparation and use of microbeads containing multiple lanthanide nanoparticles, which microbeads have uniquely identifiable spectral codes. The disclosed microbeads, and the methods and devices for making and using same, find use in multiplexing and high-throughput biomarker analysis.

Lanthanide Nanoparticles for Use in Spectrally Encoded Microbeads

[00101] The spectrally encoded microbeads of the present disclosure generally include two or more different lanthanide nanoparticles. Suitable lanthanide nanoparticles for incorporation into the spectrally encoded microbeads include nanoparticles including a lanthanide and a host lattice.

[00102] Lanthanides which may be incorporated into the disclosed lanthanide nanoparticles include, for example, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La, combinations thereof, compounds containing Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La and combinations thereof, and ions of Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La and combinations thereof.

[00103] A variety of suitable nano-crystal host lattices which may be utilized in the disclosed lanthanide nanoparticles are known in the art. For example, lanthanide dopants may be incorporated into a host lattice to provide lanthanide-doped yttrium orthovanadate (YVO₄), lanthanide-doped oxide, lanthanide-doped fluoride, lanthanide-doped chloride, lanthanide-doped bromide, lanthanide-doped iodide, lanthanide-doped lanthanum phosphate, and lanthanide-doped strontium borates (e.g., SrB₄O₇, SrB₆O₁₀ and Sr₄B₁₄O₂₅), among others.

[00104] Lanthanide nanoparticles according to the present disclosure may be prepared using methods known in the art or as described herein. An exemplary lanthanide nanoparticle synthesis scheme utilizing yttrium orthovanadate (YVO₄) as the host lattice is described in Example 1 below. Additional lanthanide nanoparticle preparation methods and materials are described, for example, in Xu et al. (2004) *Solid State Communications*, 130:465-468; Choi et al. (2010) *Journal of Luminescence*, 130:549-553; and Wang et al. (2008) *Angewandte Chemie-International Edition*, 47:906-909; the disclosure of each of which is incorporated by reference herein.

[00105] Lanthanide nanoparticles according to the present disclosure may be configured as up-converting or down-converting lanthanide nanoparticles using methods known in the art. Suitable up-converting lanthanide nanoparticles may include, for example, NaGdF₄:Tm; NaGdF₄:Ln; NaGdF₄Yb; NaGdF₄Er; NaGdF₄Yb, Er; NaYF₄:Er; NaYF₄:Yb; NaYF₄:Er,Yb; NaYF₄:Tm,Yb; LaF₃:Yb,Tm; LaF₃:Yb,Er; and LaF₃:Yb,Ho nanoparticles. Suitable down-converting lanthanide nanoparticles may include, for example, YVO₄:Eu; YVO₄:Dy; and YVO₄:Sm nanoparticles. It should be noted that the above referenced lanthanides may be incorporated into the nanoparticles as their respective ions.

[00106] Materials may be added during preparation of the lanthanide nanoparticles to increase their UV absorption. For example, in some embodiments bismuth is incorporated into the lanthanide nanoparticles to increase their UV absorption.

[00107] In some embodiments, lanthanide nanoparticles as disclosed herein may be modified (e.g., covered or coated) in a suitable material to facilitate formation of a stable colloid suspension of the lanthanide nanoparticles in a carrier fluid. Suitable materials may include materials which prevent aggregation of the lanthanide nanoparticles in the carrier fluid (e.g., H₂O) and/or facilitate maintenance of a nanoparticle form of the lanthanide nanoparticles. For example, suitable materials which

may be used to cover or coat the lanthanide nanoparticles may include polyethyleneimine (PEI), polyacrylic acid (PAA), sodium citrate, or citric acid. Polyethyleneimine (PEI) may be suitable for use, e.g., as a coating material in order to make the nanophosphors more compatible with a monomer mixture bearing free amines.

[00108] The lanthanide nanoparticles described herein may be incorporated into microbeads, e.g., polymeric microbeads, to provide spectrally encoded microbeads as discussed in greater detail below.

Spectrally Encoded Microbeads

[00109] The spectrally encoded microbeads of the present disclosure generally include two or more different lanthanide nanoparticles as discussed herein and one or more polymers, copolymers or combinations thereof.

[00110] A variety of polymers may be utilized in the lanthanide nanoparticles described herein. Suitable polymers may be selected which can evenly and irreversibly entrap the lanthanide nanoparticle materials within a polymer matrix. Suitable polymers may include, for example, poly(ethylene glycol) (PEG), polystyrene, polyethylene, poly acrylic acid, poly(methyl methacrylate) (PMMA), polysaccharides, and copolymers or combinations thereof.

[00111] In some embodiments, suitable polymers are those which are capable of forming microbeads as a result of a polymerization process, e.g., a thermal- or photo-initiated polymerization process. Such polymers may include, for example, polyacrylate (e.g., poly (PEG-diacrylate)), polyacrylamide (e.g., PEG-diacrylamide), polymethacrylate, polymethacrylamide, polystyrene, polythiol-ene, polyurethane, epoxy resin, polysaccharide (e.g., agarose), as well as copolymers or combinations of two or more of the above. Suitable polymers may also include polyurethanes/polyureas, polysiloxanes, organosiloxanes, polyethers (e.g., polyethylene glycol (PEG)), polyvinylpyrrolidones (PVP), vinyl ethers, vinyl acetates, polyimides, polysulfones, polyamic acids, polyamides, polycarbonates, polyesters, and copolymers or combinations of two or more of the above.

[00112] It should be noted that the above polymers may be provided in monomer form during the microbead preparation process, and these monomers may be polymerized to form the above polymers, copolymers or combinations thereof in the spectrally encoded microbeads of the present disclosure. Suitable monomers may include those

which can be polymerized *in situ* alone or with a cross-linking agent to form a cross-linked resin. Additional monomers which may be utilized in the lanthanide nanoparticles described herein may include, e.g., monomers which are capable of participating in thiol-ene thiol-yne reactions, e.g., pentaerythritol tetrakis(3-mercaptopropionate) (TT); diallyl phthalate (DAP); 1,3,5-triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione (TTT); 1,7-octadiyne (OY); mercaptoacetic acid (MA); allylamine (AA), pentaerythritol triallyl ether (PTE) and propargylamine (PA). These monomers find use, for example, in photo-initiated polymerization processes. For additional discussion of thiol-ene thiol-yne reactions and monomers suitable for use therein, see, e.g., Prasath et al. (2010) *Polym. Chem.*, 1: 685-692, the disclosure of which is incorporated by reference herein.

[00113] In some embodiments, a suitable monomer for use in preparation of the disclosed microbeads is selected from a PEG diacrylamide (PEG-DAM), a PEG monoacrylamide-monoamine (PEG-AM) and a PEG-monoacrylamide-monoBoc. A PEG-monoacrylamide-monoBoc may find particular use when the microbead is to be used as a substrate in a downstream peptide synthesis reaction.

[00114] In some embodiments, the present disclosure is directed to specific populations of spectrally encoded microbeads, for example, a population of polymeric microbeads embedded with at least two different lanthanide nanoparticles, wherein the population includes a plurality of polymeric microbeads, wherein each polymeric microbead of the plurality is embedded with at least two lanthanide nanoparticles having different luminescence spectra, and wherein the relative concentrations of the first and second lanthanide nanoparticles are substantially equal (e.g., not significantly different) among the polymeric microbeads of the population.

[00115] In some embodiments, a set of populations of polymeric microbeads embedded with at least two different lanthanide nanoparticles is provided, the set of populations of polymeric microbeads including a first population of polymeric microbeads, wherein each polymeric microbead of the first population is embedded with at least a first lanthanide nanoparticle and a second lanthanide nanoparticle; wherein the first and second lanthanide nanoparticles comprise different lanthanides; and wherein the relative concentrations of the first and second lanthanide nanoparticles are substantially equal (e.g., not significantly different) among the polymeric microbeads of the first population; and a second population of polymeric microbeads, wherein each polymeric microbead in the second population is embedded

with at least the first lanthanide nanoparticle and the second lanthanide nanoparticle; and wherein the relative concentrations of the first and second lanthanide nanoparticles are substantially equal (e.g., not significantly different) among the polymeric microbeads of the second population; wherein the concentration of at least one of the first and second lanthanide nanoparticles is different between the polymeric microbeads of the first population and second population.

[00116] In some embodiments, the concentration of the first lanthanide nanoparticle is substantially equal (e.g., not significantly different) for each polymeric microbead of the first population and second population. By providing a set of populations of polymeric microbeads wherein the concentration of a first lanthanide nanoparticle is substantially equal (e.g., not significantly different) for each polymeric microbead of the first population and second population, an internal lanthanide nanoparticle standard may be provided. It should be noted that an internal lanthanide nanoparticle standard may be provided wherein the concentration of the lanthanide nanoparticle standard is not substantially equal across all populations of the set. For example, a lanthanide nanoparticle standard could have a concentration of approximately X for each member of a first population in a set and a concentration of approximately Y for each member of a second population in the set, wherein Y is a known percentage of X other than 100 percent, e.g., 10 percent, 50 percent, 150 percent or 200 percent.

[00117] The devices and methods disclosed herein allow for the precise control of the concentration of the lanthanide nanoparticles in the spectrally encoded polymeric microbeads of the present disclosure. Accordingly, a population of polymeric microbeads may be provided such that the relative concentrations of at least two different lanthanide nanoparticles are substantially equal (e.g., not significantly different) among the polymeric microbeads of the population. In other words, the population of polymeric microbeads may be provided such that each polymeric microbead in the population has at least substantially the same ratio of two or more lanthanide nanoparticles as the other polymeric microbeads in the population.

[00118] This precision allows a population of spectrally encoded microbeads to be provided such that the luminescence intensity level variation among all the members of the population is no greater than about 25 percent, e.g., no greater than about 20 percent, no greater than about 15 percent, no greater than about 10 percent, no greater than about 5 percent, no greater than about 4 percent, no greater than about 3 percent, no greater than about 2 percent, or no greater than about 1 percent. In some

embodiments, the luminescence intensity level variation among all the members of the population is from about 25 percent to about 1 percent, e.g., from about 20 percent to about 1 percent, from about 15 percent to about 1 percent, from about 10 percent to about 1 percent, from about 5 percent to about 1 percent, from about 4 percent to about 1 percent, from about 3 percent to about 1 percent, or from about 2 percent to about 1 percent.

[00119] By precisely providing unique, identifiable spectral codes using multiple lanthanide nanoparticles a number of uniquely identifiable polymeric microbead populations may be provided. In some embodiments, a set of populations of polymeric microbeads as described herein includes 2 or more different populations of polymeric microbeads, e.g., 5 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, 60 or more, 70 or more, 80 or more, 90 or more, or 100 or more different populations of polymeric microbeads, wherein the polymeric microbeads for each population include a different spectral code when compared with the polymeric microbeads of the other populations in the set. In some embodiments, a set of populations of polymeric microbeads as described herein includes 10^2 or more, 10^3 or more, 10^4 or more, 10^5 or more, 10^6 or more, or 10^7 or more different populations of polymeric microbeads, wherein the polymeric microbeads for each population include a different spectral code when compared with the polymeric microbeads of the other populations in the set. For example, in some embodiments, a set of populations of polymeric microbeads as described herein includes from about 2 to about 10, from about 10 to about 20, from about 20 to about 30, from about 30 to about 40, from about 40 to about 50, from about 50 to about 60, from about 60 to about 70, from about 70 to about 80, from about 80 to about 90, or from about 90 to about 100 different populations of polymeric microbeads, wherein the polymeric microbeads for each population include a different spectral code when compared with the polymeric microbeads of the other populations in the set. As a further example, in some embodiments, a set of populations of polymeric microbeads as described herein includes from about 10 to about 10^7 , from about 10^2 to about 10^7 , from about 10^3 to about 10^7 , from about 10^4 to about 10^7 , from about 10^5 to about 10^7 , or from about 10^6 to about 10^7 different populations of polymeric microbeads, wherein the polymeric microbeads for each population include a different spectral code when compared with the polymeric microbeads of the other populations in the set. The number of different identifiable populations may be calculated by taking the number of resolvable

luminescence levels to the power of the number of different lanthanide nanoparticles. For example, using 6 different lanthanide nanoparticles with 10 resolvable levels each, the number of different, uniquely identifiable populations is 10^6 or 1,000,000. This code space can be increased as discussed in greater detail below, by utilizing both up-converting and down-converting lanthanide nanoparticles.

[00120] The polymeric microbead populations described herein may be provided in a variety of population sizes. For example, a polymeric microbead population as described herein may include 5 or more, 10 or more, 100 or more, 500 or more, 1000 or more, 1500 or more or 2000 or more polymeric microbeads. In some embodiments, a polymeric microbead population as described herein includes from about 5 to about 2000, from about 10 to about 2000, from about 100 to about 2000, from about 500 to about 2000, from about 1000 to about 2000 or from about 1500 to about 2000 polymeric microbeads.

[00121] The polymeric microbeads disclosed herein may include two or more different lanthanide nanoparticles, e.g., 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, or 10 or more, wherein each lanthanide nanoparticle has a different luminescence spectra. For example, in some embodiments, the polymeric microbeads disclosed herein may include from 2 to 10, from 3 to 10, from 4 to 10, from 5 to 10, from 6 to 10, from 7 to 10, from 8 to 10, or from 9 to 10 lanthanide nanoparticles, wherein each lanthanide nanoparticle has a different luminescence spectra.

[00122] The spectrally encoded polymeric microbeads of the present disclosure have one or more dimensions (e.g., diameter) of about 1000 μm or less, e.g., about 500 μm or less, about 100 μm or less, about 50 μm or less, about 10 μm or less, or about 5 μm or less. For example, a spectrally encoded microbead may have one or more dimensions (e.g., diameter) of from about 1000 μm to about 1 μm , from about 500 μm to about 1 μm , from about 100 μm to about 1 μm , from about 50 μm to about 1 μm , from about 10 μm to about 1 μm , or from about 5 μm to about 1 μm . The spectrally encoded polymeric microbeads may have a generally spherical shape or a non-spherical shape.

[00123] For populations of spectrally encoded polymeric microbeads, each member of the population may have approximately the same one or more dimensions, e.g., one or more dimensions as listed above. In some embodiments, the members of a population of spectrally encoded polymeric microbeads have a diameter such that the diameter

variation among all the members of the population is no greater than about 10 percent, e.g., no greater than about 5 percent, no greater than about 1 percent, no greater than about 0.1 percent, or no greater than about 0.01 percent. In some embodiments, the diameter variation among all the members of the population is from about 10 percent to about 1 percent, e.g., from about 5 percent to about 1 percent, from about 4 percent to about 1 percent, from about 3 percent to about 1 percent, or from about 2 percent to about 1 percent. In some embodiments, the diameter variation among all the members of the population is from about 5 percent to about 0.01 percent, e.g., from about 4 percent to about 0.01 percent, from about 3 percent to about 0.01 percent, from about 2 percent to about 0.01 percent, from about 1 percent to about 0.01 percent, or from about 0.1 percent to about 0.01 percent.

[00124] Spectrally encoded polymeric microbeads according to the present disclosure may include or be modified to include one or more reactive functional groups for the attachment of a molecule or molecules to the spectrally encoded polymeric microbeads. For example, monomers containing a single acrylate group and a functional group (thiol, amine, hydroxyl, carboxylic acid) can be added before polymerization to yield a microbead with functionality suitable for the attachment of an additional molecule or molecules to the microbeads subsequent to polymerization. An exemplary molecule would be hydroxy-PEG-acrylate. As an additional example, in a thiol-ene polymerization system, such reactive functional groups may be provided when the spectrally encoded polymeric microbeads are formed using functional monomers containing carboxylate (mercaptoacetic acid), hydroxyl (pentaerythritol triallyl ether), or amine (allylamine) moieties. See, e.g., Prasath et al. (2010) *Polym. Chem.*, 1: 685-692, the disclosure of which is incorporated by reference herein. Spectrally encoded polymeric microbeads including one or more reactive functional groups, e.g., carboxyl groups, hydroxyl groups or amine groups, can be used for the attachment of one or more molecules, e.g., nucleic acids, peptides, or subunits thereof, to the spectrally encoded polymeric microbeads described herein.

Methods of Making Spectrally Encoded Microbeads

[00125] The present disclosure provides methods for producing spectrally encoded microbeads as described herein. These methods may be conducted using microfluidic devices as described in greater detail below. In some embodiments, a population of polymeric microbeads including two or more different lanthanide nanoparticles is

provided. The method may include, for example: (i) mixing at least two fluids into a first solution, wherein each fluid comprises a polymerizable component (e.g., a polymer or monomer) and a different lanthanide nanoparticle; (ii) forming droplets from the solution; and (iii) subjecting the droplets to polymerization conditions, thereby producing a first set of polymeric microbeads embedded with at least two different lanthanide nanoparticles, wherein the relative concentrations of the lanthanide nanoparticles are substantially equal (e.g., not significantly different) among the polymeric microbeads of the first set.

[00126] In some embodiments, the method may include the following additional steps (iv) mixing the at least two fluids into a second solution, wherein the concentration of at least one of the different lanthanide nanoparticles in the second solution is different than in (i) above; (v) forming droplets from the solution; and (vi) subjecting the droplets to polymerization conditions, thereby producing a second set of polymeric microbeads embedded with at least two different lanthanide nanoparticles, wherein the relative concentrations of the lanthanide nanoparticles are substantially equal (e.g., not significantly different) among the polymeric microbeads of the second set.

[00127] In some embodiments, the concentration of at least one of the different lanthanide nanoparticles in the second solution is substantially equal (e.g., not significantly different) to that in (i) above.

[00128] The above mixing steps may be implemented using a variety of “on-chip” and “off-chip” mixing elements as described in greater detail below.

[00129] The step of forming droplets from the solution may include, for example, contacting the solution (which may be hydrophilic due to the presence of a hydrophilic carrier fluid, e.g., water) with a hydrophobic carrier fluid (e.g., mineral oil or water-immiscible organic solvent, e.g. octanol) such that droplets are formed. This may be accomplished, for example, by introducing the solution into a flowing stream including the hydrophobic carrier fluid. Alternatively, a hydrophobic carrier fluid (e.g., mineral oil or water-immiscible organic solvent, e.g. octanol) can be used to form the solution, and droplets can be formed by contacting the hydrophobic carrier fluid with a hydrophilic carrier fluid (e.g., water). This may be accomplished, for example, by introducing the hydrophobic carrier fluid into a flowing stream including the hydrophilic carrier fluid.

[00130] Any suitable device and/or method for droplet formation may be utilized to form droplets in the context of the present disclosure, including, e.g., the utilization of

flow focusing nozzles. See, e.g., Ward et al. (2005) *Electrophoresis*, 26:3716-3724, the disclosure of which is incorporated by reference herein.

[00131] The droplet size may be modulated by adjusting the pressure used to form the droplet, e.g., at the interface of the solution and the hydrophobic carrier fluid. In addition, droplet size may be modulated by adjusting the geometry, e.g., size and shape, of the microfluidic device channels.

[00132] One or more stabilizers or surfactants may be added to one or more of the carrier fluids to prevent droplet merging and sticking of droplets to the walls of the microfluidic device. Suitable surfactants may include, for example, Abil® EM90 (a silicon based emulsifier; CAS No. 144243-53-8) and Span™ 80 (CAS No. 1338-43-8), among others.

[00133] The step of subjecting the droplets to polymerization conditions may include, for example, exposing the droplets to UV radiation or elevated temperatures to initiate polymerization. Other suitable polymerization conditions are known in the art and may be selected provided that they are compatible with the polymers and/or monomer components to be polymerized, e.g., thiol-ene polymerization, redox-initiated polymerization, and controlled radical polymerization by Reversible Addition-Fragmentation chain Transfer (RAFT), Atom Transfer Radical Polymerization (ATRP) or Nitroxide-Mediated Polymerization (NMP). See also, e.g., Piskin E. et al. (1994) *J. of Biomaterials Science – Polymer Edition* 5:451-471; the disclosure of which is incorporated by reference herein.

[00134] In some embodiments, droplets are exposed to radiation (e.g., UV radiation) by localizing the radiation (e.g., UV radiation) exposure onto a microfluidic device (as discussed in greater detail below) such that the droplets are only irradiated after they have been formed on the microfluidic device and before they exit the microfluidic device. Radiation (e.g., UV radiation) localization may be achieved using an inverted microscope by mounting the microfluidic device on the microscope stage. For example, UV illumination may occur through the objective onto a very small area and an additional aperture within the microscope UV light path may further restrict the UV irradiation to a specific area of the microfluidic device.

[00135] Where a polymerization method is utilized to form the spectrally encoded polymeric microbeads, a suitable polymerization initiator (e.g., a photoinitiator or thermal initiator) may be utilized which is compatible with the polymerizable components and the polymerization conditions. For example, where a UV

polymerization process is utilized, a suitable initiator may include a compound that, when exposed to UV light, undergoes a photoreaction, producing reactive species that are capable of initiating polymerization. Exemplary photoinitiators may include, e.g., acetophenones, benzyl and benzoin compounds, benzophenone, cationic photoinitiators, and thioxanthenes. In some embodiments, a photoinitiator such as 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure® 2959) is utilized. Suitable thermal initiators may include, for example, azo compounds, peroxides or hydroperoxides, persulfates, and the like.

[00136] Methods which do not require polymerization may also be used to form the spectrally encoded polymeric microbeads. For example, a polymer precipitation method may be utilized in which a pre-formed polymer (e.g., a mid- to high-molecular weight polymer) is dissolved in a suitable solvent (e.g., water) along with dispersed lanthanide nanoparticles. Droplets of this solution can be formed by introducing the solution into an immiscible carrier fluid (e.g., a hydrophobic carrier fluid, e.g., mineral oil). The immiscible carrier fluid and polymer should be selected such that the polymer does not dissolve in the immiscible carrier fluid, and the immiscible carrier fluid is capable of accepting the solvent leaching from the droplet as the polymeric microbead is formed through precipitation. Additional solvent-immiscible carrier fluid combinations may include, e.g., dichloromethane as a solvent and poly(vinyl alcohol) (PVA) as an immiscible carrier fluid. Microbead preparation methods utilizing a dichloromethane-poly(vinyl alcohol) (PVA) combination are described, for example, in Berkland et al. (2002) *Journal of Controlled Release*, 73:59-74, and Berkland et al. (2004) *Journal of Controlled Release*, 94:129-141, the disclosure of each of which is incorporated by reference herein.

[00137] The steps of mixing at least two fluids can occur either before or after droplet formation depending on the particular microfluidic device architecture utilized. For example, where a herringbone type mixing architecture is utilized the two fluids may be mixed prior to droplet formation. Alternatively, where a zig-zag type mixing architecture is utilized droplets containing unmixed lanthanide nanoparticles may be formed and subsequently mixed to distribute the lanthanide nanoparticles within a droplet.

[00138] Accurate programming of spectral codes for the spectrally encoded microbeads may be facilitated by precisely controlling the flow from each of the lanthanide nanoparticle fluid inputs. This may be accomplished, in part, by solving

the coupled flow equations (1) and (2) to determine the pressure (P_n) from each input (n) to achieve the desired flow rates (Q_n):

$$\text{(Eqn. 1):} \quad Q_n = \frac{P_n - P_{mix}}{R_n}$$

$$\text{(Eqn. 2):} \quad Q_{tot} = \frac{P_{mix}}{R_{mix}}$$

where Q_{tot} is the total flow rate from all lanthanide inputs, P_{mix} is the pressure at the inlet to the mixing channel where all lanthanide input streams come together, R_{mix} is the resistance of the mixing channel, and (R_n) is the resistance of each input. The methods described herein may be implemented using one or more microfluidic devices as described in greater detail below, alone or in combination with one or more electronic control devices. The methods may be implemented via software stored in a computer readable medium and configured to run on the one or more electronic control devices.

Microfluidic Devices and Systems for Preparing Spectrally Encoded Microbeads

[00139] The present disclosure provides microfluidic devices and systems configured for the preparation of spectrally encoded microbeads as described herein. In some embodiments, a microfluidic device according to the present disclosure includes a flow channel having an inlet side and an outlet side; at least two inlets positioned toward the inlet side of the flow channel, wherein the inlets are configured to fluidly communicate with the flow channel; a mixing element positioned in the flow channel downstream of the at least two inlets; an input configured to hold a hydrophobic carrier fluid and fluidly communicate with the flow channel, wherein the input configured to hold a hydrophobic carrier fluid is configured to fluidly communicate with a portion of the flow channel downstream of the mixing element; and an outlet located at the outlet side of the flow channel, wherein the outlet is configured to fluidly communicate with the flow channel and is positioned downstream of the portion of the flow channel with which the input configured to hold a hydrophobic carrier fluid is configured to fluidly communicate. Generally, an input configured to hold a hydrophobic carrier fluid will be made of a material that is compatible with the

hydrophobic carrier fluid and which is sized and shaped to hold the hydrophobic carrier fluid.

[00140] The portion of the flow channel downstream of the mixing element with which the input configured to hold a hydrophobic carrier fluid is configured to fluidly communicate may be configured as a T-junction, e.g., as depicted in FIG. 2.

[00141] In other embodiments, a microfluidic device according to the present disclosure includes a flow channel having an inlet side and an outlet side; at least two inlets positioned toward the inlet side of the flow channel, wherein the inlets are configured to fluidly communicate with the flow channel; a mixing element positioned in the flow channel downstream of the at least two inlets; an input configured to hold a hydrophilic carrier fluid and fluidly communicate with the flow channel, wherein the input configured to hold a hydrophilic carrier fluid is configured to fluidly communicate with a portion of the flow channel downstream of the mixing element; and an outlet located at the outlet side of the flow channel, wherein the outlet is configured to fluidly communicate with the flow channel and is positioned downstream of the portion of the flow channel with which the input configured to hold a hydrophilic carrier fluid is configured to fluidly communicate. Generally, an input configured to hold a hydrophilic carrier fluid will be made of a material that is compatible with the hydrophilic carrier fluid and which is sized and shaped to hold the hydrophilic carrier fluid.

[00142] The portion of the flow channel downstream of the mixing element with which the input configured to hold a hydrophilic carrier fluid is configured to fluidly communicate may be configured as a T-junction.

[00143] It should be noted that while FIG. 2 depicts an embodiment wherein an input configured to hold a hydrophobic carrier fluid is positioned downstream from the inlets, such a depiction is not intended to be limiting and the downstream fluid input may be configured to hold either a hydrophilic carrier fluid or a hydrophobic carrier fluid so as to provide a hydrophilic in hydrophobic droplet (e.g., a water-in-oil droplet) or a hydrophobic in hydrophilic droplet (e.g., an oil-in-water droplet) accordingly.

[00144] As discussed previously herein, droplet size may be modulated by adjusting the pressure at the interface (e.g., at a T-junction) of the hydrophilic and hydrophobic carrier fluids. In addition, droplet size may be modulated by adjusting the geometry of the microfluidic device channels.

[00145] In other embodiments, a microfluidic device is provided which includes a flow channel having an inlet side and an outlet side, wherein a portion of the flow channel is configured as a zig-zag mixer; at least two inlets positioned toward the inlet side of the flow channel, wherein the at least two inlets are configured to fluidly communicate with the flow channel; an input configured to hold a hydrophobic (or hydrophilic) carrier fluid and fluidly communicate with the flow channel, wherein the input configured to hold a hydrophobic (or hydrophilic) carrier fluid input is configured to fluidly communicate with a portion of the flow channel downstream of the at least two inlets and upstream of the portion of the flow channel configured as a zig-zag mixer; and an outlet located at the outlet side of the flow channel, downstream of the portion of the flow channel configured as a zig-zag mixer.

[00146] While the above embodiments are described with respect to “on-chip” mixing elements, i.e., mixing elements incorporated on or in the microfluidic device itself, it should be noted that mixing of the lanthanide nanoparticle inputs can also occur “off-chip”, i.e., in a separate device (e.g., a separate microfluidic device). A variety of suitable mixing elements may be utilized as off-chip mixing elements, including, e.g., rotary pump mixers and the like. Once mixed the lanthanide nanoparticle inputs can be returned to a microfluidic device (e.g., via a single input) for droplet formation.

[00147] The functions of lanthanide nanoparticle input mixing and droplet formation may also be provided by connecting a plurality of microfluidic devices in series. For example, mixing of lanthanide nanoparticle inputs may occur in a first microfluidic device which is configured to communicate with a second microfluidic device configured to provide droplet formation and microbead synthesis.

[00148] In some embodiments, a microfluidic device as described herein includes an input which is located upstream of one or more mixing elements and is configured to hold a hydrophilic (or hydrophobic) carrier fluid (e.g., a water input or an oil input) and fluidly communicate with the flow channel.

[00149] The inputs described herein may be configured to include on-chip resistors which facilitate control of the inputs. These on-chip resistors may be optimized, for example, for stable droplet production at the interface between a hydrophilic carrier fluid and a hydrophobic carrier fluid as described herein.

[00150] In some embodiments, a microfluidic device as described herein may include one or more valves positioned between a flow channel of the microfluidic device and one or more inlets and/or outlets of the microfluidic device. By opening, closing or

modulating these one or more valves, fluid communication between the flow channel and the one or more inlets and/or outlets can be controlled. One or more valves may also be positioned between the flow channel and one or more of the inputs configured to hold a hydrophilic (or hydrophobic) carrier fluid and the input configured to hold a hydrophobic (or hydrophilic) carrier fluid as described herein. One or more of the valves may be configured for actuation via a variety of mechanisms, e.g., mechanical, pneumatic, hydraulic, or a combination thereof. In some embodiments, the opening, closing and/or modulation of the valves is automatically controlled by a suitable electronic control device known in the art, such as a computer.

[00151] It should be noted that valveless systems may also be utilized. For example, flow channels may be pressurized to control the flow of the various inputs and outputs without the use of valves.

[00152] A microfluidic device as described herein may include a sample collection element in fluid communication with the outlet, and located downstream of the outlet. In some embodiments, a microfluidic device as described includes a waste outlet located between the mixing element and the valve between the mixing element and the portion of the flow channel with which the input configured to hold a hydrophobic (or hydrophilic) carrier fluid is configured to fluidly communicate.

[00153] Where the mixing element is positioned in the flow channel it may be selected from a variety of suitable structures designed to cause turbulent flow and/or transverse flow across the flow channel and thereby mix fluid streams originating from the least two inlets positioned toward the inlet side of the flow channel. These structures may be positioned on and/or in one or more walls of the flow channel and in some embodiments may be etched or ablated into one or more walls of the flow channel. Suitable structures may include, for example, wells or trenches formed in one or more walls of the flow channel, or obstructions positioned in the flow channel. In some embodiments, the flow channel is configured to include a herringbone configuration of grooves or channels positioned in the flow channel and configured to mix fluid streams originating from the least two inlets positioned toward the inlet side of the flow channel. In some embodiments, the herringbone structure is a staggered herringbone structure such as that depicted in FIG. 2, Panels A and B. In addition and/or as an alternative to the above passive mixing elements, active mixing may be utilized. Such active mixing elements may be positioned in or external to the flow channel and/or the microfluidic device as appropriate. Exemplary mixing elements are

described in Lee et al. (2011) *Int. J. Mol. Sci.* 12:3263-3287, the disclosure of which is incorporated by reference herein.

[00154] A microfluidic device according to the present disclosure may be formed using a variety of fabrication methods known in the art, including, e.g., wet etching, reactive ion etching, machining, photolithography, soft lithography (e.g., multi-layer soft lithography), hot embossing, injection molding, laser ablation, *in situ* construction, and plasma etching. An example of a suitable fabrication method utilizing multi-layer soft lithography is provided in Example 2. Selection of a suitable fabrication method may also depend at least in part on the material substrate to be used in the fabrication.

[00155] Substrates which may find use in the fabrication of a microfluidic device according to the present disclosure include, for example, silicon, glass, quartz, polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), thermoset polyester (TPE), polycarbonate (PC), cyclic olefin copolymer (COC), polystyrene (PS), polyvinylchloride (PVC), and polyethyleneterephthalate glycol (PETG). See, e.g., Fiorini G. S. and Chiu D. T. (2005) *BioTechniques* 38:429-446, the disclosure of which is incorporated by reference herein.

[00156] Operation of a microfluidic device according to the present disclosure may be automated such that operation of the device itself as well as the various input sources and related system components are automatically controlled by a suitable electronic control device known in the art, such as a computer, configured to run a microbead fabrication software program configured to implement the methods described herein.

[00157] The microfluidic devices described herein may operate in conjunction with one or more additional components as part of one or more systems. For example, a system according to the present disclosure may include one or more microfluidics devices as described herein and a radiation generating element (e.g., a UV generating element) positioned to expose a portion of the flow channel to radiation (e.g., UV radiation) and thereby facilitate microbead polymerization, wherein the portion to be exposed to radiation is downstream of the portion of the flow channel with which the input configured to hold a hydrophobic (or hydrophilic) carrier fluid is configured to fluidly communicate and upstream of the outlet.

[00158] A system according to the present disclosure may also include a plurality of inlet containers, wherein each inlet container is configured to fluidly communicate with a different one of the at least two inlets, and wherein each inlet container

includes a fluid including a different lanthanide nanoparticle or lanthanide nanoparticle combination and a polymerizable component (e.g., a polymer or monomer). The inlet containers may include capillary tubing for fluidically communicating between the at least two inlets and the flow channel. In some embodiments, the capillary tubing has a length that is substantially greater than its internal diameter, e.g., a length that is at least 100 times greater, at least 500 times greater, or at least 1000 times greater than the internal diameter of the capillary tubing. A system according to the present disclosure may also include a plurality of pumps wherein the plurality of inlet containers is configured to fluidly communicate with the plurality of pumps.

[00159] In some embodiments, one or more microfluidics devices as described herein, including optionally the above inlet containers and/or pumps, are positioned in a chamber which is purged with an inert gas, e.g., nitrogen, for example, to reduce oxygen inhibition of the microbead formation (e.g., polymerization) process.

[00160] As discussed above, each inlet container may include a fluid including a different lanthanide nanoparticle or lanthanide nanoparticle combination. In some embodiments, the plurality of containers includes 2 to 10, e.g., 3 to 10, 4 to 10, 5 to 10, 6 to 10, 7 to 10, 8 to 10, or 9 to 10 lanthanide nanoparticles, wherein each lanthanide nanoparticle has a different luminescence spectra.

[00161] The lanthanide nanoparticle contained in each fluid may be present at a concentration of from about 1 mg/mL to about 250 mg/mL, e.g., from about 5 mg/mL to about 250 mg/mL, from about 10 mg/mL to about 250 mg/mL, from about 20 mg/mL to about 250 mg/mL, from about 30 mg/mL to about 250 mg/mL, from about 40 mg/mL to about 250 mg/mL, from about 50 mg/mL to about 250 mg/mL, from about 60 mg/mL to about 250 mg/mL, from about 70 mg/mL to about 250 mg/mL, from about 80 mg/mL to about 250 mg/mL, from about 90 mg/mL to about 250 mg/mL, from about 100 mg/mL to about 250 mg/mL, from about 150 mg/mL to about 250 mg/mL, or from about 200 mg/mL to about 250 mg/mL.

Methods and Devices for Imaging Spectrally Encoded Microbeads

[00162] Methods and devices for imaging spectrally encoded microbeads are also provided by the present disclosure. Generally, such methods include steps of illuminating a microbead including a plurality of different lanthanide nanoparticles with a suitable source of illumination; detecting luminescence emission from the

microbead in a plurality of spectral bands; and determining the intensities of each different lanthanide nanoparticle present in the microbead using linear unmixing. The spectral bands may be defined by a plurality of emission filters that pass the characteristic emission peaks of each lanthanide nanoparticle. In this regard, it should be noted that one or more filters may be configured to pass multiple emission peaks. Linear unmixing generally includes least squares fitting after background subtraction and flat-field correction of the microbead images. Linear unmixing and the associated image analysis are described in greater detail in Example 3.

[00163] The source of illumination should be selected such that it is compatible with the particular lanthanide nanoparticles present in the spectrally encoded microbeads. For example a deep ultraviolet (UV) light source may be selected for use with down-converting lanthanide nanoparticles while a near-infrared (IR) light source may be used with up-converting lanthanide nanoparticles.

[00164] The methods described herein may be implemented using one or more microfluidic devices as described in greater detail below, alone or in combination with one or more electronic control devices. The methods may be implemented via software stored in a computer readable medium and configured to run on the one or more electronic control devices.

[00165] Suitable microfluidic devices for use in imaging spectrally encoded microbeads include those which are capable of providing a monolayer of spectrally encoded microbeads for image acquisition by a microscope and/or camera. Suitable microfluidic devices may include, for example, one or more inlet ports; a flow channel configured for fluid communication with the one or more inlet ports; a sieve valve positioned in or downstream of the flow channel, wherein the sieve valve is configured to allow fluid flow through the flow channel while retaining the polymeric microbeads in the flow channel; and one or more outlet ports configured for fluid communication with the flow channel. In some embodiments, e.g., as depicted in FIG. 3, a flow channel including a serpentine portion is provided, wherein the serpentine portion is sized and shaped to provide an ordered, linear array of polymeric microbeads.

[00166] Generally, the microfluidic devices for use in imaging the spectrally encoded microbeads may be prepared and operated using materials and methods similar to those described above in the context of the microbead preparation devices, provided

that they are compatible with the source of illumination selected for use during the imaging process.

[00167] A bead imaging system according to the present disclosure may include one or more microfluidic devices as described above and a light source configured to illuminate the monolayer of beads, e.g., in a portion of the flow channel. As discussed above, the source of illumination should be selected such that it is compatible with the particular lanthanide nanoparticles present in the spectrally encoded microbeads. Such a system may also include a camera configured to collect an image of the illuminated portion of the flow channel.

[00168] A bead imaging microfluidic device and/or system according to the present disclosure may be automated, and in some embodiments may include a display unit configured to display an image of the spectrally encoded microbeads, e.g., as positioned in the flow channel. The displayed image may include a false color overlay of lanthanide luminescence which is scaled and adjusted for microfluidic device autofluorescence.

Peptide Synthesis Using Spectrally Encoded Microbeads

[00169] Peptides may be used for a variety of purposes including, e.g., the preparation of epitope-specific antibodies, mapping of antibody epitopes and enzyme binding sites and the design of novel enzymes, drugs and vaccines. Accordingly, methods which provide for low-cost synthesis relative to commercially available methods and which can be used to synthesize multiple peptides simultaneously are of interest. Methods and devices for the solid-phase synthesis of predetermined peptide sequences which utilize spectrally encoded microbeads as described herein are provided. Generally, the methods include coupling a spectrally encoded microbead, e.g., as described herein, functionalized with one or more functional groups (e.g., peptide-bond forming functional groups, such as amine functional groups), with a first amino acid (e.g., an N-terminal protected amino acid). Additional amino acids can then be added, e.g., using the synthesis scheme described herein, or using standard tert-butoxycarbonyl (Boc) or 9-fluorenylmethoxycarbonyl (Fmoc) synthesis reagents, procedures and reaction times, e.g., as described in Amblard et al., *Mol Biotechnol.* (2006) Jul. 33(3):239-54; Lloyd-Williams P. et al. (1997) Chemical approaches to the synthesis of peptides and proteins. Boca Raton: CRC Press. 278; Merrifield R. B. (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *Journal of the*

American Chemical Society. 85, 2149-54; Carpino L. A. (1957) Oxidative reactions of hydrazines. Iv. Elimination of nitrogen from 1, 1-disubstituted-2-arenesulfonhydrazides1-4. *Journal of the American Chemical Society*. 79, 4427-31; McKay F. C. and Albertson N. F. (1957) New amine-masking groups for peptide synthesis. *Journal of the American Chemical Society*. 79, 4686-90; Anderson G. W. and McGregor A. C. (1957) T-butyloxycarbonylamino acids and their use in peptide synthesis. *Journal of the American Chemical Society*. 79, 6180-3; and Carpino L. A. and Han G. Y. (1972) 9-fluorenylmethoxycarbonyl amino-protecting group. *The Journal of Organic Chemistry*. 37, 3404-9; the disclosure of each of which is incorporated by reference herein. In some embodiments, a peptide synthesis scheme according to the present disclosure includes the incorporation of a cleavable linker (e.g., an aspartic acid-proline dipeptide linker) that can be cleaved under orthogonal conditions to peptide synthesis reactions.

[00170] In some embodiments of the disclosed methods, one or more of the above steps may be performed in a microfluidic device.

[00171] In some embodiments a peptide synthesis method according to the present disclosure includes steps of imaging a plurality of microbeads, identifying a plurality of spectral codes based on the imaging, and sorting one or more of the microbeads based on the identified spectral codes.

[00172] While the present disclosure describes microbeads containing multiple lanthanide nanoparticles, which microbeads have uniquely identifiable spectral codes, it should be noted that the devices and methods described herein may also find use in the preparation, analysis, and use of microbeads prepared using other encoding techniques such as the incorporation of quantum dots, organic dyes, and the like. For example, while lanthanide nanoparticle containing microbeads may be preferred for various reasons as discussed herein, the peptide synthesis methods described herein are not limited to the use of such microbeads, and may instead utilize microbeads prepared using other encoding techniques such as the incorporation of quantum dots, organic dyes, and the like.

Single Bead Release for Microfluidic Sorting

[00173] In some embodiments of the disclosed methods and devices, it may be advantageous to release microbeads one-at-a-time from a microfluidic channel. For example, in order to automate the sorting of beads for programmable peptide

synthesis, it may be advantageous to image arrays of beads and then release the beads one-at-a-time for downstream sorting. To achieve this type of release from a microfluidic channel, the microfluidic channel may be sized and shaped to include a constriction at a terminal end of the microfluidic channel. In other words, the microfluidic channel may be tapered at a terminal end. In some embodiments, the channel begins to narrow at a distance of approximately 50 μm to 150 μm or more from the terminal end and continues to narrow until the terminal end, e.g., in some embodiments, the channel begins to narrow at a distance of from about 60 μm to about 140 μm , from about 70 μm to about 130 μm , from about 80 μm to about 120 μm , from about 90 μm to about 110 μm , or about 100 μm , from the terminal end and continues to narrow until the terminal end. In some embodiments, the terminal end of the microfluidic channel may be a T-junction or another type of junction wherein the microfluidic channel meets one or more additional microfluidic channels. In some embodiments, a first side and a second side of the microfluidic channel may each narrow between approximately 10 μm and 20 μm over any one of the above distances, e.g., between about 11 μm and 19 μm , about 12 μm and 18 μm , about 13 μm and 17 μm , about 14 μm and 16 μm , or about 15 μm .

[00174] The above constriction allows for the metering of beads one-by-one at a channel outlet. This ability to release beads one at a time may be beneficial for a variety of bead uses. Accordingly, this feature has broad applicability to microfluidic devices in general where bead sorting applications are of interest.

EXAMPLES

[00175] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., s or sec, second(s); min, minute(s); h or hr, hour(s); Mn, number average molecular weight; Mw, weight average molecular weight; and the like.

Example 1: Synthesis of Lanthanide Nanoparticles

[00176] Nanoparticles for use in preparation of the disclosed spectrally encoded microbeads were synthesized using a polymer-assisted hydrothermal approach combined with microwave irradiation. Chemical reagents and polymers [poly(ethylene glycol) (PEG) and poly(acrylic acid) (PAA)] for nanoparticle synthesis were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Microwave synthesis was performed using a Biotage Initiator (Biotage AB, Uppsala, Sweden). Purification of the synthesized nanoparticles was performed by ultrafiltration using Amicon Ultra-15 centrifugal filter units with a 50,000 Dalton Molecular Weight Cutoff (MWCO) (Millipore, Billerica, MA), resulting in suspensions with a nanoparticle concentration of ~50 mg/mL in water. Luminescence spectra were measured using a FluoroMax-3 (Horiba Scientific, Kyoto, Japan) spectrofluorometer and the nanoparticle particle size distributions were measured using a Zetasizer Nano (Malvern Instruments, Malvern, UK).

[00177] Solutions (0.1 M) of the rare-earth (RE) dopants [Sm(NO₃)₃, Dy(NO₃)₃, Eu(NO₃)₃], Y(NO₃)₃, and Na₃VO₄ were prepared in advance. 14.2 mg of Bi(NO₃)₃ was added into 3 mL of a 10 w/w% solution of PEG (Mn ~ 2,000). This solution was then rapidly dissolved through brief sonication before being heated to 70 °C in an oil bath under magnetic stirring. A solution of Y(NO₃)₃ (800 µL) and the RE solution (e.g., Eu(NO₃)₃) (50 µL) was premixed and then added drop-wise into the stirring PEG solution. The PEG solution instantly turned white upon addition of the Y+RE mixture. This solution was stirred for 30 minutes, followed by the drop-wise addition of the Na₃VO₄ solution (950 µL). The suspension turned yellowish at this stage and the mixture was again stirred for 30 min. The suspension was transferred into a glass vial suitable for microwave synthesis and was heated to 180 °C at 15 bar for 60 min. Upon removal from the microwave, the suspension was pure white. The material was pelleted in a 15-mL disposable centrifuge tube and the PEG supernatant was removed. The pellet was then re-suspended in 3 mL of deionized H₂O, to which was added 5 mL of a 10 w/w% PAA solution (Mn ~ 1,400). This mixture was heated back up to 70 °C and stirred for 10 min. The solution was pH adjusted to 7.5 using 5 N NaOH and stirred for an additional 30 min. The suspension was then diluted 1:10 with deionized H₂O and sonicated for 18 hours. After sonication, any larger phosphor particles were pelleted under centrifugation and the remaining translucent suspension was filtered

consecutively through a 1 μm and 0.45 μm Polytetrafluoroethylene (PTFE) filters before being added to an ultracentrifugation filter unit for concentration and the removal of excess salts and polymers. After the entire reaction volume (~ 100 mL) had been passed through the membrane, the retained nanoparticles were washed 4 times with 15 mL of deionized water to exchange out the remaining solution. The final nanoparticle (NP) suspensions were white and milky in appearance and had a nanoparticle concentration of about 50 mg/mL.

[00178] The product of the above synthesis is a crystalline YVO₄ nanoparticulate host containing one of the trivalent rare earth dopants (Eu³⁺, Sm³⁺, or Dy³⁺), resulting in materials with unique emission spectra (FIG. 1, Panel A) when excited with UV light (FIG. 1, Panel A, inset). The nanoparticles have a size distribution from 30-160 nm (FIG. 1, Panel B) and are coated with poly(acrylic acid) to create stable aqueous suspensions (FIG. 1, Panel B, inset, illuminated with a UV lamp). Bismuth (at a 5-15% atomic replacement of yttrium) has also been incorporated into these nanoparticles to increase their UV absorption. The nanoparticles may be referred to herein simply by the rare earth dopant present (e.g., Eu). To test the reproducibility of nanoparticle production, multiple batches of each nanoparticle were synthesized and their emission spectra were compared (FIG. 6). In all cases, these spectra were virtually identical, demonstrating the ability to consistently and reproducibly produce nanoparticles.

Example 2: Synthesis of Ratiometrically Encoded Polymeric Beads

Microfluidic Device Production

[00179] To incorporate the prepared lanthanide nanoparticles into solid beads at programmed ratios, a custom, fully-automated microfluidic device was designed and fabricated. Devices were fabricated in poly(dimethylsiloxane) (PDMS, RTV 615, Momentive Performance Materials, Albany, NY) by Multi-Layer Soft Lithography using 4" test-grade silicon wafers (University Wafer, South Boston, MA) coated with multiple layers of SU8 (Microchem Corp., Newton, MA) and AZ50 XT photoresists (Capitol Scientific, Austin, TX) patterned by standard photolithography processes.

[00180] All photolithography masks were designed using AutoCAD (Autodesk, San Rafael, CA) and printed onto transparency film with a resolution of 30,000 dpi (FineLine Imaging, Colorado Springs, CO). To improve adhesion of subsequent photoresist layers, all wafers were first coated with a 5 μm layer of SU-8 2005

negative photoresist (Microchem Corp.) according to the manufacturer's instructions. All spin-coating steps were performed on a G3P-8 programmable spin coater (Specialty Coating Systems, Indianapolis, IN). After each coating step wafers were set on a flat surface for 10 to 20 minutes to allow photoresist to relax completely and reduce surface irregularities, except for the initial 5 μm adhesion layer of SU-8. All photoresist baking steps were done on aluminum-top hot plates (HS40A, Torrey Pines Scientific, Carlsbad CA). Mask alignment and photoresist exposure were done on a Quintel Q2001CT i-line mask aligner (Neutronix-Quintel, Morgan Hill, CA).

[00181] Bead synthesizer control molds were fabricated using SU-8 2025 photoresist according to the manufacturer's instructions for creating $\sim 25 \mu\text{m}$ thick channels. Flow molds were constructed with five layers of photoresist, one using AZ50 XT positive photoresist (Capitol Scientific, Austin, TX), the other four using different types of SU-8. Layers 1 and 2 were developed separately, but layers 3-5 (all SU-8) were developed together, after they had all been exposed, as this was found to reduce bubble formation, improve height uniformity, and allow for significantly better staggered herringbone fabrication. After the 5th layer, layers 3-5 were developed for 6 min in SU-8 Developer, followed by hard baking for 2 hours at 165°C , with an initial ramp from 65°C to 165°C at 120°C/hr . The five layers were:

[00182] I) 5 μm thick SU-8 2005 layer for the high resistance push water input. Spin-coat: (1) 500rpm for 5s with 5s ramp (spread), (2) 2900rpm for 30s with 8s ramp (cast). Soft bake: 65°C 2 min/ 95°C 3 min/ 65°C 2 min. UV exposure: 7.4s at 18.4 mW/cm². Post exposure bake: 65°C 2 min/ 95°C 3 min/ 65°C 2 min. Develop: 2 min in SU-8 Developer (Microchem).

[00183] II) 45 μm thick AZ50 XT layer to create rounded channels at valve locations. Spin-coat: (1) 200rpm for 5s with 1s ramp (spread), (2) 1400rpm for 30s with 5s ramp (cast), (3) 3400rpm for 1s with 1s ramp (edge bead removal). Soft bake: 65°C - 112°C full speed ramp for 22 min. Rehydrate overnight. UV exposure: 20s x 4 with 20s pauses in between at 18.4 mW/cm². Develop: 1:3 solution of AZ Electronic Materials AZ400k developer (Capitol Scientific). Hard bake: ramp from 65°C to 190°C at 10°C/hr , remain at 190°C for 4 hrs.

[00184] III) 45 μm thick SU8-2025 layer for the lanthanide inputs, mixer channel, and oil channels. Spin-coat: (1) 500rpm for 10s with 5s ramp (spread), (2) 1600rpm for 30s with 3.6s ramp (cast). Soft bake: 65°C 2 min/ 95°C 10 min/ 65°C 2 min. UV exposure: 13.1s at 18.4 mW/cm². Post-exposure: 65°C 2 min/ 95°C 9 min/ 65°C 2 min.

[00185] IV) 30 μm thick SU8-2025 layer on top of layer 3 in the mixer channel and downstream of the T-junction. Spin-coat: (1) 500rpm for 10s with 5s ramp (spread), (2) 3500rpm for 30s with 10s ramp (cast). Soft bake: 65°C 2 min/95°C 7 min/65°C 2 min. UV exposure: 14.3s at 18.4 mW/cm². Post-exposure bake: 65°C 3 min/95°C 6 min/65°C 2 min.

[00186] V) 35 μm thick SU8-2025 layer on top of layer 4 on the mixing channel for the staggered herringbone grooves. Spin-coat: (1) 500rpm for 10s with 5s ramp (spread), (2) 2500rpm for 30s with 6.7s ramp (cast). Soft bake: 65°C 2 min/95°C 7 min/65°C 2 min. UV exposure: 7s at 18.8 mW/cm². Post-exposure bake: 65°C 2 min/95°C 6 min/65°C 2 min.

[00187] All molds were silanized by exposure to trichloromethylsilane (Sigma-Aldrich, St. Louis, MO) vapors for 60 minutes. Each flow mold was then coated with a 4 mm thick layer of Momentive Materials RTV 615 (R.S. Hughes, Oakland, CA) mixed at a ratio of 1:5 (cross-linker:elastomer) using a Thinky AR-250 planetary centrifugal mixer (Thinky USA Inc, Laguna Hills, CA). This 4 mm thick layer was subsequently degassed in a vacuum chamber for 60 minutes. All control molds and slides for mounting the devices were spin coated with a ~ 20 μm thick layer of RTV 615 mixed at a ratio of 1:20 via a 2 step spin process: (1) 500 rpm for 5 s with a 5 s ramp (spread), and (2) 1900 rpm for 60 s with a 15 s ramp (cast). Flow molds, control molds, and coated slides were baked at 80°C for 1 hour, 40 minutes, and 20 minutes, respectively. Following baking, PDMS flow layers were peeled from molds, cut to the appropriate size, punched with a drill press (Technical Innovations, Brazoria, TX) at inlet and outlet ports, and aligned to control layers (still remaining on the molds). The aligned devices were then baked for an additional hour before being cut from the molds, punched to create control access ports, and placed on the coated slides. The entire assembly was then baked at 80°C for 1-12 hours to finalize device bonding. Synthesis devices were mounted on regular microscope glass slides.

Microfluidic Device Operation

[00188] Valves in the microfluidic devices were actuated by 10 mm pneumatic solenoid valves (Festo Corp., Hauppauge, NY) driven by an ethernet-based, programmable fieldbus I/O system with digital output modules (750-841 Programmable Fieldbus Controller, 750-504 4-Channel Digital Output Module, Wago Corp., Germantown, WI). All fluids were injected into the microfluidic devices using

pressure-driven flow from custom-made containers. Pressurized air to operate the valves and push fluids into the chips was supplied by a set of manual precision pressure regulators connected to the house air supply through a series of high efficiency filters for oil and particulate removal. A custom software platform written in MATLAB® (The MathWorks Inc., Natick, MA), with a graphical user interface, allowed for real time control and script-driven automation of all aspects of the chip operation, for bead synthesis. The UV light source for droplet polymerization was a Leica® EL6000 fluorescence excitation light source with a metal halide bulb and liquid light guide filtered by an Omega® UV filter cube set #XF02-2 (80nm band around 330nm).

Bead Synthesis

[00189] Encoded beads were generated by varying ratios of three pre-polymer input solutions each containing different lanthanide nanoparticles. The three monomer input solutions used in the microfluidic bead synthesizer all contained purified water with 42.8% v/v 700 MW PEG-diacrylate (Sigma-Aldrich), 6% v/v 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (“Irgacure® 2959”, a photoinitiator, Sigma-Aldrich) dissolved in methanol at 0.33 g/mL, and 5% v/v YVO4:Eu (25 mg/mL). One of the input solutions also contained 21.3% v/v YVO4:Dy (10 mg/mL) and one of the others contained 21.3% v/v YVO4:Sm (10 mg/mL). Droplets were formed into a continuous flowing stream of light mineral oil (Sigma-Aldrich) that contained 2% v/v Abil® EM90 (Evonik Industries, Germany) and 0.05% v/v Span™ 80 (Sigma-Aldrich) as surfactants to eliminate droplet merging and sticking to the PDMS walls. On device UV illumination was used to polymerize the droplets into beads downstream of the T-junction.

[00190] All monomer-lanthanide mixtures were injected into the chip from custom-made containers using PEEK™ capillary tubing with an inner diameter of 65 µm and a length of 30.5 cm to provide high input resistance relative to the resistance of the staggered herringbone channel. This helps minimize any potential flow rate errors due to inaccuracies in measuring the resistance of the staggered herringbone channel or fluctuations in set pressures. On-chip resistors optimized for stable drop production at the T-junction with input pressures near the middle of the pressure regulator range were used on the oil and push water inputs. To reduce oxygen inhibition of the PEG-diacrylamide polymerization, these containers were pressurized with nitrogen (95-99% purity) supplied by a high-precision, high-speed, computer-controlled pressure

regulator with eight independent output channels (MFCS-FLEX, 8 channels, 0-1000 mBar range, Fluigent SA, Paris, France). For the same reason, the microfluidic device was surrounded by a 95-99% pure nitrogen atmosphere during operation. The high gas permeability of PDMS ensures that the interior of the microfluidic device will equilibrate with this nitrogen atmosphere. The compressed nitrogen for these purposes was supplied by a membrane-based nitrogen generator (Membrane Module 210, Generon IGS, Pittsburg, CA) fed from the building compressed air supply.

[00191] After polymerization, the beads were smaller than the droplets, mostly due to oxygen-driven cross-linking inhibition on the droplet surface, and this reduction in size was highly dependent on the UV dose delivered to the droplets (the lower the dose, the smaller the beads). For the experimental conditions described here, the typical diameter shrinkage was approximately 7 μm , resulting in beads of approximately 46 μm +/- 1 μm . The measured error corresponded to approximately half a pixel in the image and thus the actual size variation of the beads is likely smaller. Sizes of beads were measured by fitting a circle to 3 user-selected points on the perimeter of the bead in a brightfield image using NIS-Elements (Nikon Instruments, Melville, NY).

[00192] Precise flow control from each of the lanthanide inputs facilitates the accurate programming of spectral codes. This was accomplished by performing a calibration routine to directly measure relative hydraulic resistances and then solving the coupled flow equations (1) and (2) to determine the pressure (P_n) from each input (n) to achieve the desired flow rates (Q_n):

$$\text{(Eqn. 1):} \quad Q_n = \frac{P_n - P_{mix}}{R_n}$$

$$\text{(Eqn. 2):} \quad Q_{tot} = \frac{P_{mix}}{R_{mix}}$$

[00193] Where Q_{tot} is the total flow rate from all lanthanide inputs, P_{mix} is the pressure at the inlet to the mixing channel where all lanthanide input streams come together, and R_{mix} is the resistance of the mixing channel. The resistance of each input (R_n) was determined relative to a fixed reference standard, PEG-diacrylate with food coloring, flowed into one of the lanthanide inputs. The pressures at these two inputs were set to the same value and the flow rate ratio (Q_n/Q_{ref}) was determined by measuring the

width taken up by each fluid in the channel. When the input pressures are equal, equation (1) reduces to:

$$\text{(Eqn. 3): } \frac{Q_n}{Q_{ref}} = \frac{(P_n - P_{mix})R_n}{(P_{ref} - P_{mix})R_{ref}} = \frac{R_{ref}}{R_n}$$

[00194] R_{mix} was determined by flowing lanthanide in pre-polymer at one of the lanthanide inputs at a fixed pressure and then measuring the pressure (P_{mix}) where the inputs come together at the entrance to the mixing channel. Under these conditions $Q_{tot} = Q_n$ so from equations (1) and (2):

$$\text{(Eqn. 4): } \frac{R_n}{R_{mix}} = \frac{P_n - P_{mix}}{P_{mix}}$$

[00195] P_{mix} was measured by opening the valve to a second lanthanide input and adjusting the pressure at this second input until there was no flow at this second input. Once the relative resistances for each of the lanthanide inputs (R_n) and the resistance downstream of the inputs (R_{mix}) is determined, the system of equations (1) and (2) can be solved to obtain the pressures needed for the desired flow rates of each lanthanide in monomer for accurately hitting each targeted spectral code.

[00196] The microfluidic bead synthesizer depicted in FIG. 2 operates in two stages. In stage one, the different lanthanides, suspended in poly(ethylene glycol) diacrylate, flow into the device and mix in a staggered herringbone mixer. During this mixing, the relative flow rates from the different lanthanide inputs determine the relative abundance of each lanthanide in a bead. In stage two, droplets are generated by flowing the lanthanide mixture into an oil stream at a T-junction, and then polymerized into beads through on-chip UV illumination. After producing beads with each mixture, the mixing channel is flushed with high-pressure water to clear the channel, the pressures are adjusted to pre-programmed values automatically for the next code, and the process repeats. Precise control over lanthanide flow rates, which is achieved by setting pressures based on a set of coupled flow equations and calibration parameters (see above), facilitates the accurate development of targeted spectral codes. The device depicted in FIG. 2, Panels C and D, was prepared as described above and incorporates controls for up to 8 lanthanide inputs.

[00197] To test both the feasibility of using lanthanide nanoparticles to create uniquely identifiable spectral codes and the performance of the microfluidic bead synthesizer, a

set of ratiometrically-encoded beads including varying levels of Dy and Sm and a constant level of Eu was synthesized. Codes were determined by the relative ratios of Dy/Eu and Sm/Eu, with the constant level of Eu providing an internal normalization to correct for spatial and temporal variations in either excitation intensity or detection efficiency. A two-dimensional grid of 24 ratiometric codes was synthesized containing 6 distinct levels of both Dy and Sm based on preliminary measurements suggesting these codes would be distinguishable with high accuracy. In three hours of fully automated unattended device operation, a set of 24 spectral codes with each code including approximately 1500 beads was produced.

Example 3: Imaging of Spectrally Encoded Beads

Microfluidic Device Production

[00198] To measure the lanthanide luminescence ratios in the spectrally encoded beads, an additional custom microfluidic device was developed to create an ordered linear array of ~190 beads within a narrow serpentine channel (FIG. 3, Panel A) covering a ~1 mm² area. Beads in the serpentine can be loaded and unloaded using on-chip valves in the fluidic circuit, allowing for efficient imaging of large numbers of beads.

[00199] All photolithography masks were designed using AutoCAD (Autodesk, San Rafael, CA) and printed onto transparency film with a resolution of 30,000 dpi (FineLine Imaging, Colorado Springs, CO). To improve adhesion of subsequent photoresist layers, all wafers were first coated with a 5 µm layer of SU-8 2005 negative photoresist (Microchem Corp.) according to the manufacturer's instructions. All spin-coating steps were performed on a G3P-8 programmable spin coater (Specialty Coating Systems, Indianapolis, IN). After each coating step wafers were set on a flat surface for 10 to 20 minutes to allow photoresist to relax completely and reduce surface irregularities, except for the initial 5 µm adhesion layer of SU-8. All photoresist baking steps were done on aluminum-top hot plates (HS40A, Torrey Pines Scientific, Carlsbad CA). Mask alignment and photoresist exposure were done on a Quintel Q2001CT i-line mask aligner (Neutronix-Quintel, Morgan Hill, CA).

[00200] Imaging device control molds were fabricated using SU-8 2025 according to the manufacturer's instructions for creating ~ 25 µm thick channels. Imaging device flow molds had the following layers:

[00201] I) ~50 μm thick layer of AZ 50 XT photoresist to create rounded channels at valve locations. Spin-coat: (1) 200 rpm for 5 s with a 1 s ramp (spread), (2) 750 rpm for 30 s with a 5 s ramp (cast), and (3) 2750 rpm for 1 s with a 1 s ramp (edge bead removal). Soft bake: 25 minutes with ramp between 65°C and 112°C at full speed, and allowed to cool to room temperature. Rehydrate overnight. UV exposure: 25 s x 3 at ~18 mW/cm². Develop: 1:3 solution of AZ AZ400k developer in water. Hard bake: Ramp from 65°C to 190°C at 10°C/hour, remaining at 190°C for 4 hours.

[00202] II) ~50 μm thick layer of SU-8 2050 to create all flow channels. This layer was fabricated largely according to the manufacturer's instructions, although it was found that soft baking set to ramp between 65°C and 95°C (rather than simply transferring wafers between hot plates set to 65°C and 95°C) helped prevent formation of bubbles within the photoresist.

[00203] All molds were silanized by exposure to trichloromethylsilane (Sigma-Aldrich, St. Louis, MO) vapors for 60 minutes. Each flow mold was then coated with a 4 mm thick layer of Momentive Materials RTV 615 (R.S. Hughes, Oakland, CA) mixed at a ratio of 1:5 (cross-linker:elastomer) using a Thinky AR-250 planetary centrifugal mixer (Thinky USA Inc, Laguna Hills, CA). This 4 mm thick layer was subsequently degassed in a vacuum chamber for 60 minutes. All control molds and slides for mounting the devices were spin coated with a ~ 20 μm thick layer of RTV 615 mixed at a ratio of 1:20 via a 2 step spin process: (1) 500 rpm for 5 s with a 5 s ramp (spread), and (2) 1900 rpm for 60 s with a 15 s ramp (cast). Flow molds, control molds, and coated slides were baked at 80°C for 1 hour, 40 minutes, and 20 minutes, respectively. Following baking, PDMS flow layers were peeled from molds, cut to the appropriate size, punched with a drill press (Technical Innovations, Brazoria, TX) at inlet and outlet ports, and aligned to control layers (still remaining on the molds). The aligned devices were then baked for an additional hour before being cut from the molds, punched to create control access ports, and placed on the coated slides. The entire assembly was then baked at 80°C for 1-12 hours to finalize device bonding. Imaging devices were mounted on cyclic olefin copolymer slides (COP480R, Pure Slides LLC, Medford, MA) to minimize the fluorescence background.

Microfluidic Device Operation

[00204] Valves in the microfluidic devices were actuated by 10 mm pneumatic solenoid valves (Festo Corp., Hauppauge, NY) driven by an ethernet-based,

programmable fieldbus I/O system with digital output modules (750-841 Programmable Fieldbus Controller, 750-504 4-Channel Digital Output Module, Wago Corp., Germantown, WI). All fluids were injected into the microfluidic devices using pressure-driven flow from custom-made containers. Pressurized air to operate the valves and push fluids into the chips was supplied by a set of manual precision pressure regulators connected to the house air supply through a series of high efficiency filters for oil and particulate removal. A custom software platform written in MATLAB® (The MathWorks Inc., Natick, MA), with a graphical user interface, allowed for real time control and script-driven automation of all aspects of the chip operation, for the imaging chips.

[00205] “Sieve” valves positioned at the end of the serpentine channel of the microfluidic device permitted fluid flow while retaining beads, facilitating pressure-driven packing of beads within the channel and maximizing imaging throughput. Multiple output ports collected both buffer and bead wastes; fluid injection ports at either side of the device allowed flushing of the serpentine from either side to clear stuck particles. These devices are mounted on cyclic olefin polymer slides (COP480R; Pure Slides, LLC., Medford, MA) to minimize autofluorescence; the PDMS itself was not significantly autofluorescent. Device control lines were pressurized to 25 psi (for fully sealing valves) and 35 psi (for sieve valves).

[00206] Prior to loading, bead batches were washed ten times in a solution of 1x Phosphate Buffered Saline (PBS) with 0.5% Tween and twice in a solution of 1x PBS with 0.1% Tween before being diluted to a working concentration of 100-200 beads per μL in 1x PBS with 0.1% Tween. Bead solutions ($\sim 25 \mu\text{L}$) were loaded into Tygon® tubing (using a 1 mL syringe), and connected directly to the device. Buffers were stored in pressurized vials connected to the device via Tygon® tubing. During serpentine loading, both bead and buffer inputs were pressurized at 3-5 psi and excess buffer was directed to the waste port. During imaging, buffer solution pressure was reduced to ~ 1 psi to relax bead packing. After imaging, the output was directed to a separate bead waste port to collect imaged beads for further use.

Bead Imaging

[00207] Bead imaging was performed using a Nikon® Ti microscope (Nikon Instruments, Melville, NY) with a custom built illuminator. Because the microscope objectives are not transparent to the short wavelength UV illumination used to excite the lanthanides, a transillumination geometry as shown in FIG. 3 was used. Light

from a full-spectrum 300W Xenon arc lamp (Newport, Irvine, CA) was collected, reflected off a 400 nm long pass mirror (CVI Melles-Griot, Albuquerque, NM) to reject visible light, then passed through a shutter and an excitation filter wheel (Sutter Instrument Co., Novato, CA), before being focused into a 3 mm diameter deep UV liquid light guide (Newport). The excitation wheel allowed switching between UV illumination for lanthanide excitation and visible light illumination for finding beads and imaging the device during bead loading. For UV imaging, the illumination light was filtered with a 292/27 excitation filter (Semrock, Rochester, NY) paired with UG11 absorptive glass (Newport). The illumination intensity at the sample was ~ 12.5 mW/cm². For visible light imaging the residual visible light reflected by the 400 nm long pass mirror was used, which was further filtered with a 409 nm long pass filter (Semrock), infra-red reflective mirror (Edmund Optics), and an OD 1.0 neutral density filter.

[00208] The other end of the liquid light guide was mounted on the condenser mount of a Nikon Ti microscope, where the light was collimated by a fused silica lens (Newport) and projected onto the sample. Emitted light from the sample was collected by a Plan Apo 4x/0.2NA (Nikon Instruments, Melville, NY) objective, with a UV blocking filter (Edmund Optics, Barrington, NJ) placed between the objective and the sample. Emitted light was filtered through an emission filter wheel mounted beneath the objective before being focused onto the camera. An image stack was collected which included six different images acquired through the following filters (all from Semrock): 482/35, 510/84, 543/22, 572/15, 615/20, and 630/92. The filters used here were chosen using a Monte Carlo optimization procedure to select filters which minimize the unmixing error. Typical exposure times were 5 seconds for the first four channels and 1 second for the last two. The camera used was an Andor DU-888 (Andor Technology, Belfast, Northern Ireland) operated in conventional readout mode at 13MHz with 2x2 binning. The microscope and camera were controlled by Micro-Manager software (Edelstein A, Amodaj N, Hoover K, Vale R, Stuurman N (2010) in *Current Protocols in Molecular Biology* (John Wiley & Sons, Inc.).

[00209] Bead images for testing bead autofluorescence in conventional dye channels were acquired on a Nikon Ti microscope with a 10x / 0.3 Plan Fluor objective and a Coolsnap HQ2™ CCD camera (Photometrics, Tucson, AZ). Illumination was from a Sutter XL lamp (Sutter Instrument Company, Novato, CA) and a Chroma 89000 filter set (Chroma Technology, Bellows Falls, VA) was used to define the excitation and

emission channels. The lamp was operated at full power and the exposure time for each image was 1 second.

Linear Unmixing and Image Analysis

[00210] All data analysis was performed with custom software written in MATLAB®. Reference spectra for unmixing were acquired from beads doped with a single lanthanide. These beads were spotted on quartz coverslips (to minimize background fluorescence) and an image stack was acquired as described above. The background was subtracted using a local background estimation procedure and the mean luminescence of the beads in each channel was measured. The device background spectrum was measured from a region of the microfluidic device where no beads were present. These reference spectra were then normalized so that each spectrum summed to one.

[00211] Before linear unmixing, the images of the beads in the serpentine device were corrected for camera bias and dark current by subtracting a dark image. Dark images were acquired by averaging 100 frames acquired with the same exposure times as the fluorescent images, but with the camera shutter closed. The image stack was then flat-field corrected by dividing each image by a corresponding flat-field image. Flat-field images were acquired by averaging 100 frames captured through each emission filter with white-light transmitted illumination and no sample present on the microscope. While the use of an internal standard corrected for variations in excitation intensity across the field of view, wavelength-dependent pixel response nonuniformity was monitored. Linear unmixing was then performed using standard least squares analysis to fit the intensity of each pixel of the measured image stack to a sum of the reference spectra times the abundance of each lanthanide. This unmixing process reduced the six-channel raw data to a four-channel image stack including background fluorescence and Dy, Eu, and Sm luminescence.

[00212] Beads were then identified in the unmixed image by median filtering the Eu channel and performing adaptive local thresholding. The threshold parameters were adjusted to include as many pixels as possible in each bead while maintaining separation between them. For each bead identified, the pixel by pixel ratio of Dy to Eu and Sm to Eu luminescence was calculated and the median Dy/Eu and Sm/Eu luminescence ratio was recorded. To minimize the effect of wavelength-dependent pixel response nonuniformity on the CCD, only beads within the central 300 x 300 pixels on the CCD were analyzed. Because the data returned by linear unmixing were

on an arbitrary scale, a variation of Iterative Closest Point matching was used to determine overall scaling factors along the Dy/Eu and Sm/Eu axes to best map the observed data to the programmed codes (Besl PJ, McKay ND (1992) A Method for Registration of 3-D Shapes. *IEEE Trans Pattern Anal Mach Intell* 14:239–256; and Segal AV, Haehnel D, Thrun S (2009) in *Proceedings of Robotics: Science and Systems (RSS)*). Briefly, the algorithm works as follows: an initial transformation is determined that maps the brightest bead along each axis to the highest programmed level of that lanthanide. This transformation is applied to the data and the closest programmed level to each measured bead is determined. The transformation that best matches the measured beads to their closest programmed levels is determined, and the process is iterated until convergence. To account for small systematic errors between different serpentines, these scaling factors were determined separately for each serpentine. This systematic variation was largest along the Sm/Eu dimension, and correcting it reduced the overall CV by ~0.6%. This correction was statistically significant as compared to rescaling an equal number of subsets of the data without regard to which serpentines they originated from.

[00213] The Gaussian mixture model (GMM) was fit in MATLAB® and standard deviation ellipses and numbers of standard deviations between points and cluster centroids were determined using the Cholesky decomposition of the covariance matrix (Press WH, Teukolsky SA, Vetterling WT, Flannery BP (2007) *Numerical Recipes 3rd Edition: The Art of Scientific Computing* (Cambridge University Press). 3rd Ed.). Cross-validation was performed by splitting the bead data into ten disjoint sets, training the GMM on nine, and then testing the classification accuracy on the remaining test set. This was repeated for each of the ten test sets in turn. Measurement errors were determined by replicate imaging of two different serpentines of beads. For each bead, the mean and standard deviation of five repeated measurements were calculated. The standard deviations were then grouped by lanthanide ratio and averaged to give the statistical error plotted in FIG. 5, Panel B.

Imaging Results

[00214] Initial images of the beads showed that they are highly monodisperse, with a mean diameter of $46.4 \pm 1.0 \mu\text{m}$. Images of these beads at commonly used fluorescence wavelengths revealed minimal luminescence of the lanthanides in the

fluorescein, Cy3, and Cy5 emission channels, indicating that these beads are compatible with assays using these dyes for detection (FIG. 7).

[00215] Luminescence emission from the beads was detected in six spectral bands defined by emission filters chosen to pass the characteristic emission peaks of each lanthanide (Fig. 1, Panel A and FIG. 4, Panel A). The intensities of individual lanthanides were then determined by linear unmixing, which expresses the measured images as a sum of component images multiplied by each component's characteristic spectrum. Here, the three lanthanides Dy, Eu, and Sm were used, as well as the autofluorescence of the microfluidic device within which the beads are held (FIG. 4, Panel B). The unmixing error (the difference between the measured images and the component images times their spectra) was <2% for a typical image set. A typical set of unmixed images is shown in FIG. 4, Panel C. To identify the lanthanide ratios in each bead, beads in the image were first identified by adaptive local thresholding of the Eu channel. For each identified bead (spanning ~90 pixels), the Dy/Eu and Sm/Eu ratios were then calculated on a pixel by pixel basis, and median ratios for each bead were recorded.

Identification of Spectral Codes

[00216] To be practically useful, each code within an encoded bead set should cluster tightly around the predetermined, programmed ratios. The results of imaging 10 bead-filled serpentine containing a representative sample of 1926 beads from the 24 code set are shown in FIG. 5, Panel A. To determine cluster centroids for each code, k-means clustering was performed on the data, using the programmed values as the starting cluster centroids. The synthesized beads for each code cluster tightly, with the measured values for each code agreeing very well with the targeted values (inset FIG. 5, Panel A): the mean distance between the programmed ratios (0, 0.12, 0.27, 0.46, 0.70, and 1) and the measured ratios is only 0.014. Discounting the 0,0 code, the mean fractional error between the measured and programmed levels (distance from programmed to measured divided by distance of programmed to origin) is 2.9%. A second independently-generated code set shows similarly precise agreement with the programmed levels (FIGs. 8 and 9), indicating that the disclosed synthesizer produces spectrally-encoded beads with both high accuracy and repeatability. These code sets were synthesized independently with a three-week gap between the syntheses, and both code sets were imaged several additional weeks after the syntheses, demonstrating bead and reagent stability.

[00217] Another important consideration for a robust encoding scheme is that beads from different codes cluster tightly together and far from other codes, preventing misidentification of beads. The root mean square (RMS) deviation of individual beads from their code centroid, calculated as above, is 4%. To quantify how accurately beads can be assigned to a code, a two-dimensional Gaussian was fit to each code cluster.

[00218] Understanding and minimizing errors is important in order to maximize the code space that can be achieved with a given encoding scheme. The distribution of measured bead ratios around their programmed values can result from both errors in bead synthesis and bead imaging. These synthesis and imaging errors, in turn, are composed of both a systematic, instrumental component as well as a statistical, shot noise (either photon or lanthanide nanoparticle number) component. To probe the relative contributions of these sources of error in the measurements, the mean error for each Dy/Eu level and each Sm/Eu level was calculated, independent of the concentration of the other lanthanide. The histograms of the Dy/Eu and Sm/Eu ratios for all beads are shown at the edges of the scatter plot in FIG. 5, Panel A, along with Gaussian fits to the data. The widths (standard deviations) from these Gaussian fits are plotted (Fig. 5, Panel B), along with the statistical measurement error (determined by repeated imaging of the same serpentine of beads). The statistical measurement error accounts for roughly one half of the total error in the Dy/Eu channel and between one half and one third of the total error in the Sm/Eu channel. While these other sources of error can be further reduced, the fact that the results are within 2-3 fold of the measurement shot noise limit indicates that these other errors are relatively small.

[00219] The described experiments have demonstrated a system designed to precisely generate beads containing ratiometric spectral codes using a microfluidic device and luminescent lanthanide nanoparticles. 24 uniquely identifiable codes have been created containing a single reference level of Eu and 6 levels each of both Sm and Dy. Measurements of ~2,000 beads from this code set establish both that the measured ratios closely match the desired programmed ratios and that these codes are easily distinguished from one another, validating the accuracy and precision of this technique. Importantly, both this scheme and the device used to produce these codes can be extended to significantly larger code sets.

[00220] Exemplary embodiments of the disclosed devices are unique in incorporating automated on-chip mixing with multiple input streams while accurately achieving programmed ratiometric codes (error <3%), with low variation within a code (4%), and precise control over bead size (CV < 2.5%). An exemplary bead synthesizer described herein incorporates eight lanthanide inputs and is scalable with respect to both the number of lanthanide inputs and the rate of bead synthesis.

[00221] The ultimate performance of a spectral encoding scheme depends both on the number of encoding species and the number of intensity levels of each that can be reliably distinguished. The number of distinguishable intensity levels is inversely proportional to deviations from the programmed error level; therefore, minimization of synthesis and measurement errors is necessary to maximize the code space. These results establish that beads can be synthesized with a mean deviation from the programmed ratio of 2.9%. This number is significantly smaller than deviations from programmed intensities seen for QDs indicating that lanthanide nanoparticles suffer much less from energy transfer and re-absorption between particles.

[00222] The code set demonstrated above can be expanded through a minimization of code variation and the addition of other lanthanides. If, for example, it is assumed that the errors in intensity ratios are normally distributed, and require that the midpoint between any two programmed codes is at least four standard deviations from each other (corresponding to a misidentification probability of less than 10^{-4}), then the current intra-code variation of 4% should allow the resolution of seven intensity levels for Dy/Eu and Sm/Eu. By reducing the total error to the statistical measurement error in FIG. 5, the number of resolvable levels would increase to 12 per lanthanide while maintaining a code-calling accuracy rate greater than 99.99%.

[00223] A number of lanthanide nanoparticles with different dopants and distinct emission spectra have been synthesized, including erbium, thulium, holmium, and cerium/terbium. By incorporating these lanthanide nanoparticles, the code space size can be increased to $\sim 7^6 = 117,649$. In addition to the discussed downconverting (UV-excited) YVO₄ nanophosphors, there are also upconverting lanthanide nanophosphors that emit in the visible region upon excitation in the near-IR. By utilizing upconverting nanoparticles with the emitting species Dy, Er, Eu, Ho, Sm, Tb, and Tm an additional six ratiometric channels may be provided. By alternating excitation between UV and near-IR sources, it is therefore possible to separate the spectra of upconverting and downconverting nanocrystals. Such a system combining

upconverting and downconverting nanoparticles could have a code space as large as 7^{12} , approximately 14 billion.

[00224] The methodology and device described here allows for efficient and accurate synthesis of spectrally encoded beads using microfluidics and lanthanide nanoparticles. Given an expanded code space with additional lanthanide nanoparticles, this platform enables a multitude of diverse assays, including immuno-diagnostics, small molecule library screening, and combinatorial synthesis approaches.

[00225] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

Example 4: Peptide Synthesis Using Lanthanide Nanoparticle Encoded Microbeads

Materials and Methods

[00226] *Bead Production:* Amine-functionalized polyacrylamide beads were produced using a microfluidic device as described previously herein. Beads containing Europium alone were composed of purified water containing 27% v/v PEG-diacrylamide, 27% v/v amine-functionalized PEG-diacrylamide, 8% v/v lithium acylphosphinate (LAP) photoinitiator, and 5% v/v $\text{YVO}_4\text{:Eu}$ (25 mg/mL). Beads containing a mixture of Europium and Dysprosium were composed of the same reagent mixture, with the addition of 16% v/v $\text{YVO}_4\text{:Dy}$.

[00227] *Peptide Synthesis:* Each peptide synthesis reaction started with ~ 200 μL of packed encoded polymer beads suspended in a solution of 1x PBS with 0.1% Tween. The first amino acid (Proline) was added to beads manually to optimize amino acid loading. For this process, amine-functionalized polyacrylamide beads were loaded into 10 mL polypropylene syringes for manual peptide synthesis (New England Peptide; Gardner, MA). Beads were washed 3x with ~ 5 mL of methanol, an additional 2x with ~ 5 mL of dimethylformamide (DMF), and allowed to swell in dimethylformamide for 30 minutes. After swelling (and ejection of remaining DMF), beads were mixed with 5 mL of 0.1 M Fmoc-Pro-OH and 2.5 mL of 0.1 M 2-(1H-

Benzotriazole-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate (HBTU) and 0.4 M 4-methylmorpholine in DMF and incubated for an additional 30 minutes.

Following coupling, beads were washed an additional 3x in DMF and the Fmoc protecting group on the nascent peptide chain was removed via exposure to 5 mL of 20% 4-methylpiperidine in DMF for 2 minutes. Following deprotection, beads were again washed 3x in DMF, resuspended in DMF, and loaded into Symphony™ peptide synthesizer (Protein Technologies, Inc.) reaction vessels for automated addition of the remaining amino acids. For the amino acids in positions 2-7 within the peptide chain relative to the C-terminus, beads were first incubated with 3 volumes of 20% piperidine in DMF and mixed for 2 minutes and 30 seconds. Following this incubation, beads were washed 3x with DMF and then exposed to 2 volumes of 0.1 M Fmoc-protected amino acid in DMF and 1 volume of 0.4 M 4-methylmorpholine in DMF with mixing for 10 minutes. This washing and incubation process was repeated once more (referred to as “double coupling”), followed by a final 3x wash in DMF.

[00228] For amino acids in positions 8-14, the coupling followed the double coupling procedure outlined in the previous paragraph with the exception that each coupling step was incubated for 20 minutes.

[00229] For the final amino acid, the 20 minute double coupling procedure was followed by a 5 minute incubation with a solution of 20% 4-methylpiperidine in DMF (to remove the final Fmoc protecting group), 3 30 second washes with methanol, and drying under nitrogen for 10 minutes.

[00230] *Side Chain Deprotection:* Dried beads were transferred from the Symphony™ reaction vessels to 10 mL polypropylene syringes using a metal spatula. Beads were then incubated in 5 mL of a 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% water solution for 2 hours at room temperature on a shaking block to cleave off side chain protecting groups. At the end of this incubation, the TFA/TIS/water solution was ejected to waste and beads were washed ~5x with deionized water and an additional 3-4x with a solution of 1x PBS with 0.1% Tween.

[00231] *Peptide Cleavage from Beads:* Beads for peptide cleavage and peptide quality control via mass spectrometry were transferred to a 1.5 mL polypropylene Eppendorf tube. Beads were then pelleted via centrifugation at maximum speed in a benchtop centrifuge for 1 minute and washed 2-3x in a solution of 1% formic acid, 50% acetonitrile, and 50% water. Following the final wash, beads were resuspended in between 20 µL (for single bead cleavage experiments) and 1 mL of 1% formic acid,

50% acetonitrile, and 50% water and incubated in a heat block at 95°C for 1 hour. Beads were again pelleted via centrifugation at maximum speed and the supernatant (containing the cleaved peptides) was analyzed via LC-MS/MS mass spectrometry. To assess the minimum number of beads required to generate sufficient samples for LC-MS/MS analysis, we prepared a suspension of beads in 1% formic acid, 50% acetonitrile, and 50% water (as described above), generated a 1:2 dilution series to create samples with progressively smaller numbers of beads, and then counted the number of beads within each Eppendorf tube. Figures used to illustrate peptide quality and cleavage sites were generated using WebLogo software, Crooks et al., *Genome Research*, 14:1188-1190 (2004).

[00232] *Manual Immunoassays:* Peptide-conjugated beads suspended in 1x PBS with 0.1% Tween were further diluted via addition of 1x PBS with 0.1% Tween to a final concentration of about a 5-10 μ L pellet of packed beads within a total volume of 50 μ L. 1 μ L of either Alexa-555 conjugated anti-myc antibody, Alexa-647 conjugated anti-FLAG antibody, or both antibodies were then added to each reaction, and this final mixture was protected from light and incubated on a nutating platform at 4°C overnight (more than 12 hours). Following this incubation, beads were washed 3x with 1x PBS with 0.1% Tween via pelleting and resuspension, pipetted onto a quartz slide and covered with a quartz coverslip for imaging on an inverted microscope. To determine bead code identity, beads were imaged and analyzed as described previously herein. To determine antibody loading, beads were imaged using standard Cy3 and Cy5 filter cube sets with a 100 ms exposure time in each channel.

[00233] *On-chip immunoassays:* On-chip immunoassays were conducted using the same reagent solutions and incubation times as described above for the standard immunoassays.

[00234] *Microfluidic Devices:* PDMS molding masters and devices for the microfluidic bead reactor and bead imaging and release device were produced as described previously herein.

Peptide Synthesis and Immunoassays

[00235] To demonstrate the feasibility of using spectrally encoded beads as a substrate for solid phase peptide synthesis, two different well-characterized epitope tag peptides (FLAG and myc) (EKQLISEEDL and DYKDDDDK respectively) were synthesized on amine-functionalized polyacrylamide beads containing different combinations of

lanthanide nanoparticles. The FLAG peptide was synthesized on beads containing only Europium (Eu) nanoparticles, and the myc peptide was synthesized on beads containing both Eu and Dysprosium (Dy) nanoparticles (FIG. 10). In both cases, peptides were attached to the beads via an aspartic acid-proline dipeptide linker that can be cleaved under orthogonal conditions to peptide synthesis reactions. This linker remains conjugated to the beads during all peptide synthesis steps (facilitating downstream on-bead immunoassays) but can be cleaved to release peptides from beads for quality control via mass spectrometry.

[00236] Following synthesis, the Eu beads coated with FLAG peptides and the Eu/Dy beads coated with myc peptides were mixed with Alexa-647-labeled anti-FLAG antibodies and Alexa-555-labeled anti-myc antibodies, incubated overnight, washed to remove all unbound antibody, and then imaged at multiple wavelengths to identify the embedded codes (as described above) and with Cy3 and Cy5 filter sets to image bound labeled anti-FLAG and anti-myc antibodies. Both codes were easily identified post peptide synthesis, demonstrating that the embedded codes are robust to the harsh conditions required for peptide synthesis (FIG. 11, Panel A). In addition, the images demonstrated strong binding of labeled antibodies to the peptide-conjugated beads, with anti-FLAG antibodies binding only to the FLAG-conjugated Eu beads, and anti-myc antibodies binding only to the myc-conjugated Eu/Dy beads (FIG. 11, Panel B). Reactions took place both in standard reaction tubes and in a custom-made microfluidic bead reactor fabricated from polydimethylsiloxane (PDMS) (FIG. 12).

[00237] In addition to the immunoassays described above, the peptides were released from the beads by boiling them in a solution of 1% formic acid, 50% acetonitrile, and 50% water (which cleaves the aspartic acid-proline dipeptide linker) and then evaluated for quality via mass spectrometry. The peptides synthesized on spectrally encoded beads were of comparable quality to peptides synthesized on commercially available TentaGel™ beads (FIG. 13). In addition, a dilution series was performed and it was determined that peptides could be detected from single beads, with an estimated peptide loading level of 50 fmol per bead.

[00238] Finally, a 4-code set of beads containing an identical amount of Eu nanocrystals and varying amounts of Dy nanocrystals (0%, 33%, 66%, and 100%) was synthesized and imaged before and after peptide synthesis to further probe code stability following synthesis. The relative amounts of Dy nanocrystals remained unchanged before and after synthesis (FIG. 14).

Single bead release for microfluidic sorting

[00239] In order to automate the sorting of beads for programmable peptide synthesis, it may be advantageous to image arrays of beads and then release the beads one-at-a-time for downstream sorting. This capability was demonstrated by including a small constriction (wherein each side of the channel narrows between 10 and 20 μm over a 100 μm distance) at the outlet of a microfluidic, serpentine channel (FIG. 15). Without intending to be bound by any particular theory, this small constriction creates a pressure instability (known as the Haine's jump instability) at the beads pass through the constriction, which leads to metering of beads one-by-one at the channel outlet. The ability to release beads one at a time may also be beneficial for a variety of bead uses outside of the peptide synthesis field. Accordingly, this feature has broad applicability to microfluidic devices in general.

Example 5: Synthesis and Characterization of Er:YVO₄ and Tm:YVO₄*Materials and Methods*

[00240] *Materials for Tm and Er:YVO₄ Synthesis:* All chemical reagents and polymers [poly(ethylene glycol) (PEG) and poly(acrylic acid) (PAA)] for nanophosphor synthesis were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Microwave synthesis was performed using a Biotage Initiator (Biotage AB, Uppsala, Sweden). Purification of the synthesized nanophosphors was performed by ultrafiltration using Amicon Ultra-15 centrifugal filter units with a 30,000 MWCO (Millipore, Billerica, MA), resulting in suspensions with a nanophosphor concentration of ~50 mg/mL in water. Luminescence spectra were measured using a FluoroMax-3 (Horiba Scientific, Kyoto, Japan) spectrofluorometer.

[00241] *Typical synthesis of Tm:YVO₄ and Er:YVO₄:* Solutions (0.1 M) of the rare-earth (RE) dopants [Tm(NO₃)₃, Er(NO₃)₃], Y(NO₃)₃, and Na₃VO₄ were prepared beforehand. A solution of Y(NO₃)₃ and the rare earth solution was premixed (950 μL Y and 50 μL Tm for Tm:YVO₄, or 950 μL Y and 50 μL Er for Er:YVO₄) and added rapidly to 2 mL of a 10 w/w% solution of PEG (Mn ~ 2,000) being stirred at 70 °C in an oil bath under magnetic stirring. This solution was stirred for 20 minutes, followed by the drop-wise addition of the Na₃VO₄ solution (950 μL). The suspension turned yellowish at this stage and the mixture was again stirred for 30 min. The suspension was transferred into a glass vial suitable for microwave synthesis and was heated to 180 °C at 15 bar for 90 min. Upon removal from the microwave, the suspension was

pure white. The material was pelleted in a 15-mL disposable centrifuge tube and the PEG supernatant was removed. The pellet was then re-suspended in 3 mL of deionized H₂O, to which was added 5 mL of a 10 w/w% PAA solution (Mn ~ 1,400). This mixture was heated back up to 70 °C and stirred for 10 min. The solution was pH adjusted to 7.5 using 5 N NaOH and stirred for an additional 30 min. The suspension was then diluted 1:10 with deionized H₂O and sonicated for 18 hours. After sonication, any larger phosphor particles were pelleted under centrifugation and the remaining translucent suspension was filtered consecutively through 1 µm and 0.45 µm PTFE filters before being added to an ultracentrifugation filter unit for concentration and the removal of excess salts and polymers. After the entire reaction volume (~100 mL) had been passed through the membrane, the retained nanophosphors were washed 4 times with 15 mL of deionized water to exchange out the remaining solution. The final NP suspensions were white and milky in appearance and had a nanophosphor concentration of about 50 mg/mL.

Synthesis and characterization of Er:YVO₄ and Tm:YVO₄

[00242] Following on the successful synthesis and incorporation of Eu, Sm, and Dy phosphors into beads as discussed previously herein, a similar process was sought for additional rare earths Er and Tm, both of which can also be doped into a YVO₄ crystalline matrix to emit visible light when excited with ultraviolet light. To varying extents depending on the rare earth dopant (emitter) being used, the incorporation of bismuth into the lattice in addition to the rare earth dopant has a tendency to diminish the luminescence of that emitter. This was found to be the case for both Tm and Er, where their counterparts with bismuth synthesized using the protocols disclosed herein demonstrated relatively low luminescence. The non-bismuth material, however, showed sufficient luminescence for both emitters (FIGs. 16 and 17) to warrant further investigation. Originally, bismuth was incorporated to shift the excitation wavelength maximum from 280 nm to roughly 320 nm when bismuth is sufficiently incorporated into the matrix. However, it was determined that by using the illumination optics disclosed herein to read the codes, the excitation region around 280-300 nm was sufficiently covered such that the non-Bi-containing nanophosphors could be examined.

[00243] The loading levels of both Er and Tm were adjusted by synthesizing small batches of YVO₄ with increasing Tm or Er doping concentration in order to observe a doping level that maximized the luminescence. FIG. 18 shows this progression for Tm

and FIG. 19 for Er. A preferred stoichiometry for Tm was 1% loading, or $\text{Tm}_{0.01}\text{Y}_{0.99}\text{VO}_4$. Tm:YVO₄ suffers from autoquenching of the luminescence when the Tm loading gets too high, as was observed in going from 1% to 5% Tm doping.

[00244] FIG. 19 demonstrates that the inherent brightness or luminescence of a nanophosphor solution may be compared by examining the peak luminescent intensity against the background luminescence of the YVO₄ matrix. When higher Er concentrations were plotted as non-normalized spectra, there is enough variance in solution concentration, particle size, etc. to show significant differences between the excitation spectra (not shown here). However, plotted as normalized spectra as in FIG. 19, it is easier to see that the luminescence of the Er peaks at 524 and 554 nm increase as the Er doping is increased from 0.1% up to 5% and then additional loading (10 and 15 % dopant) has a negligible effect on the luminescence. For this reason, 5% loading was chosen as the preferred loading level for Er, or $\text{Er}_{0.05}\text{Y}_{0.95}\text{VO}_4$.

Example 6: Synthesis and Characterization of CeTb:LaPO₄

Materials and Methods

[00245] To a 20 mL microwave synthesis vial containing 10 mL H₂O was added 155 mg LaCl₃•7H₂O, 165 mg CeCl₃•7H₂O, and 57.6 mg TbCl₃•6H₂O. 370 mg of Na₃P₃O₁₀ was dissolved separately in 4 mL of H₂O and then added dropwise to the chlorides in the microwave vial. The vial was capped and heated to 180 °C for 60 min. The material was pelleted in a 15-mL disposable centrifuge tube and the supernatant was removed. The pellet was then re-suspended in 5 mL of deionized H₂O, to which was added 10 mL of a 10 w/w% PAA solution (Mn ~ 1,400). This mixture was heated back up to 70 °C and stirred for 10 min. The solution was pH adjusted to 7.5 using 5 N NaOH and stirred for an additional 30 min. The suspension was then diluted 1:10 with deionized H₂O and sonicated for 18 hours. After sonication, any larger phosphor particles were pelleted under centrifugation and the remaining translucent suspension was filtered consecutively through 1 µm and 0.45 µm PTFE filters before being added to an ultracentrifugation filter unit for concentration and the removal of excess salts and polymers. After the entire reaction volume (~100 mL) had been passed through the membrane, the retained nanophosphors were washed 4 times with 15 mL of deionized water to exchange out the remaining solution. The final NP suspensions were white and milky in appearance and had a nanophosphor concentration of about 200 mg/mL.

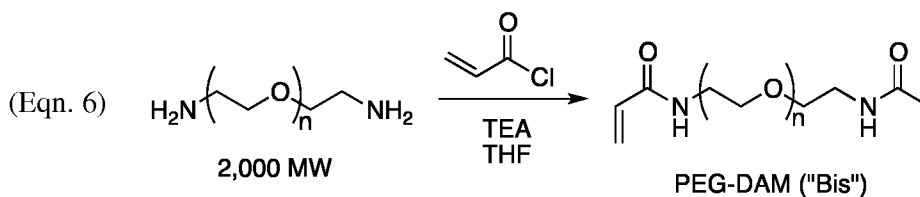
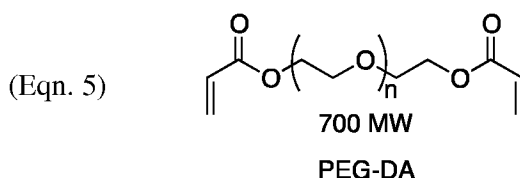
Characterization of CeTb:LaPO₄

[00246] Figure 20 shows the excitation and emission spectra for the CeTb:LaPO₄ nanophosphor suspension with an excitation maximum of 275 nm and excitation peaks at 490, 545 (most intense), 585, and 620 nm. Figure 21 shows the particle size distribution as measured by dynamic light scattering with an average particle size of 34 nm.

Example 7: Monomer Development and Synthesis

[00247] As previously described herein poly(ethylene glycol) diacrylate (PEG-DA) was used as a monomer in the preparation of microbeads according to the present disclosure. While beads made from this monomer may work when evaluated for start-to-finish feasibility and stability with respect to peptide synthesis and the subsequent biological assays to be performed, it is possible that the ester bonds of PEG-DA may be too acid- and base-sensitive for such applications. For this reason, acrylamide bond formation was investigated as a possible alternative bead chemistry for use in peptide synthesis applications.

[00248] PEG diacrylamide (PEG-DAM) was initially prepared from a commercially-available PEG-diamine source. The formula for the original PEG diacrylate (PEG-DA) and the initial synthesis scheme for the PEG diacrylamide (PEG-DAM) are shown below:

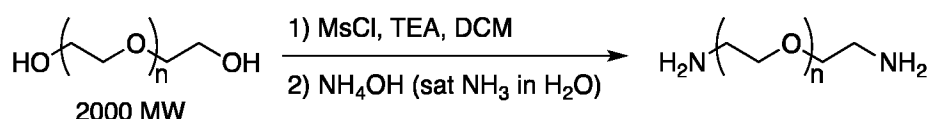


[00249] The above synthesis of PEG-DAM from a commercially-available PEG-diamine source was readily achieved. However, when tested with the beads, the PEG-

DAM exhibited an unacceptably high autofluorescence when excited with ultraviolet light. The autofluorescence was observed for both the starting material (PEG-diamine) and the final PEG-DAM product (FIG. 22). FIG. 22 provides emission spectra for commercially available PEG-diamine and the PEG-DAM synthesized from it. As a comparison, a commercially available PEG-600 is included to illustrate an acceptable level of autofluorescence for a PEG.

[00250] In view of the above results, it was determined that a new source for this monomer was needed. The following reaction scheme and synthesis procedure was tested:

(Eqn. 7)

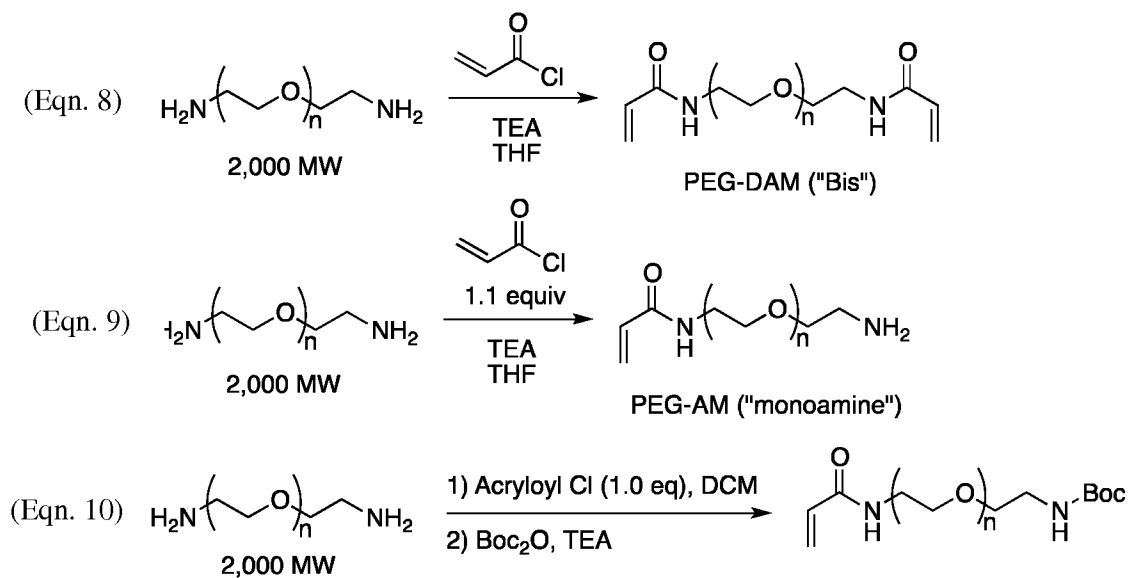


[00251] *Synthesis of PEG2K-diamine from PEG2K:* 20 g of PEG2K (2000 MW PEG) was added to a 500-mL round bottom flask containing 200 mL dichloromethane. 8.37 mL (3 equiv) of triethylamine was added and the flask was chilled to 0 °C while being stirred. 3.6 mL (2.2 equiv) of mesyl chloride was added dropwise and the solution was stirred overnight. About half of the dichloromethane was removed the next morning by rotary evaporation and the solids (triethylamine hydrochloride salt) were filtered out using a Buchner funnel. Next, a small portion of diethyl ether was added to further precipitate more triethylamine salt and this was again filtered. This filtrate was then slowly poured into a 1-L Erlenmeyer flask with 600 mL diethyl ether to precipitate the PEG-dimesylate product. This was used in the next step without further purification. Using an aliquot of the crude dimesylate, 7.2 g of PEG-dimesylate was added to a 500-mL glass Nalgene serum bottle with a stir bar and 150 mL NH₄OH (conc). This was stirred in the sealed bottle for 4 days. The NH₃(g) was removed under partial vacuum followed by the removal of ~50 mL H₂O after the NH₃ had been removed. This solution was made basic (pH 12-13) with 5 N NaOH and was transferred to a 250-mL separatory funnel where the aqueous layer was extracted 8 times with 40 mL dichloromethane. The dichloromethane extracts were pooled and dried over K₂CO₃ and the solution was transferred to a round bottom flask where the solvent was removed to yield ~10-15 g of a yellowish liquid. This was added dropwise into 350 mL of stirring diethyl ether to precipitate out the final product. The

product was removed by filtration through a Buchner funnel and was dried under vacuum until no ether was present. The final product was 6.0 g of a white powder (80% yield).

[00252] The PEG-diamine synthesized as described above exhibited lower absorption in solution by UV-vis as well as significantly less autofluorescence as observed by the spectrofluorometer. The comparison of autofluorescence is shown in FIG. 23.

[00253] The synthetic schemes for PEG-diamine to PEG-DAM (Eqn. 8), PEG-AM (Eqn. 9), and PEG-monoacrylamide-monoBoc (Eqn. 10) are provided below:



[00254] To obtain the PEG-DAM, PEG-diamine was reacted with acryloyl chloride (Eqn. 8). 2.0 g of PEG2K-diamine (with 2 equivalents of amine groups present per molecule of PEG) was added to a 250 mL round bottom flask along with 100 mL tetrahydrofuran (THF). 1120 μ L triethylamine was added and the solution was chilled to 0 $^{\circ}$ C. 243 μ L acryloyl chloride in 2 mL of THF was added dropwise to the flask and the mixture was stirred in the dark for 90 minutes with the flask returning to room temperature after the first 15 minutes. The mixture was then filtered to remove the triethylamine hydrochloride salt and the THF was removed using the rotary evaporator. The residue was taken up into 75 mL dichloromethane and transferred to a separatory funnel. The organic layer was washed once with 100 mL of saturated NaCl. The organic layer was saved and the brine layer was washed 4 times with 50 mL dichloromethane. All of the dichloromethane extracts were pooled and dried over K_2CO_3 . The dichloromethane was removed by rotary evaporator until $\sim$ 10 mL of

residue remained in the flask. This was added dropwise into 300 mL of stirring diethyl ether to precipitate the product. The product was isolated using a Buchner funnel and was dried under vacuum until no diethyl ether was detectable.

[00255] PEG-AM (Eqn. 9) was synthesized the same way as PEG-DAM except that no triethylamine is needed since the remaining amine on the PEG-diamine catalyzes the reaction. There is also a simpler work-up with no extraction for PEG-AM.

[00256] Some initial testing has indicated that there are times when the nanoparticle solutions tend to aggregate and become incompatible with the monomer mixture before polymerization. This was observed occasionally with PEG-DA, but the issues were always resolved. This aggregation has also been observed with PEG-DAM and to a greater extent with PEG-AM. The aggregation also appears to be more prevalent when PEG-AM is introduced with amines present. To address this issue, a monomer with a functional amine was suggested and successfully synthesized to test the nanoparticle compatibility: one of these types of molecules is shown in (Eqn. 10) and is called PEG-monoacrylamide-monoBoc where the Boc carbamate is protecting the amine that may be responsible for the nanoparticle aggregation issues.

[00257] The synthesis of the PEG-monoacrylamide-monoBoc is the same as for the PEG-AM, but while still in solution, 2 equivalents of Boc_2O are added to the solution and the solution is stirred overnight in the dark. This solution is extracted with brine to remove the triethylamine salt and then ultimately precipitated into diethyl ether as with all the other PEGs in this series of compounds. The NMR (FIG. 24) shows a clean product with no free amines. Future testing will be performed to determine compatibility with the nanophosphors and the ability to produce polymerized beads.

CLAIMS

What is claimed is:

1. A population of polymeric microbeads embedded with at least two different lanthanide nanoparticles, the population comprising:
 - a plurality of polymeric microbeads,
 - wherein each polymeric microbead of the plurality is embedded with at least two lanthanide nanoparticles having different luminescence spectra, and
 - wherein the relative concentrations of the first and second lanthanide nanoparticles are substantially equal among the polymeric microbeads of the population.
2. The population of polymeric microbeads as set forth in claim 1, wherein the luminescence intensity level variation among all the members of the population is no greater than about 15 percent.
3. The population of polymeric microbeads as set forth in claim 1 or 2, wherein the luminescence intensity level variation among all the members of the population is no greater than about 5 percent.
4. The population of polymeric microbeads as set forth in any one of claims 1-3, wherein each polymeric microbead has a diameter of less than 500 μm .
5. The population of polymeric microbeads as set forth in any one of claims 1-4, wherein each polymeric microbead has a diameter of less than 100 μm .
6. The population of polymeric microbeads as set forth in any one of claims 1-5, wherein each polymeric microbead has a diameter of less than 50 μm .
7. The population of polymeric microbeads as set forth in any one of claims 1-6, wherein the diameter variation among all the members of the population is no greater than about 5 percent.

8. The population of polymeric microbeads as set forth in any one of claims 1-7, wherein one or more of the lanthanide nanoparticles comprises bismuth.
9. A set of populations of polymeric microbeads embedded with at least two different lanthanide nanoparticles, the set of populations of polymeric microbeads comprising:
 - a first population of polymeric microbeads,
 - wherein each polymeric microbead of the first population is embedded with at least a first lanthanide nanoparticle and a second lanthanide nanoparticle;
 - wherein the first and second lanthanide nanoparticles comprise different lanthanides; and
 - wherein the relative concentrations of the first and second lanthanide nanoparticles are substantially equal among the polymeric microbeads of the first population; and
 - a second population of polymeric microbeads,
 - wherein each polymeric microbead in the second population is embedded with at least the first lanthanide nanoparticle and the second lanthanide nanoparticle; and
 - wherein the relative concentrations of the first and second lanthanide nanoparticles are substantially equal among the polymeric microbeads of the second population;
 - wherein the concentration of at least one of the first and second lanthanide nanoparticles is different between the polymeric microbeads of the first population and second population.
10. The set of populations of polymeric microbeads as set forth in claim 9, wherein the concentration of one of the first and second lanthanide nanoparticles is substantially equal for each polymeric microbead of the first population and second population.
11. The set of populations of polymeric microbeads as set forth in claim 9, wherein the concentration of the first lanthanide nanoparticle in the first population is a known percentage of the concentration of the first lanthanide nanoparticle in the second population, wherein the known percentage is other than 100 percent.

12. The set of populations of polymeric microbeads as set forth in any one of claims 9-11, wherein the luminescence intensity level variation among all the members of the first population is no greater than about 15 percent.
13. The set of populations of polymeric microbeads as set forth in any one of claims 9-12, wherein the luminescence intensity level variation among all the members of the first population is no greater than about 5 percent.
14. The set of populations of polymeric microbeads as set forth in any one of claims 9-13, wherein the luminescence intensity level variation among all the members of the second population is no greater than about 15 percent.
15. The set of populations of polymeric microbeads as set forth in any one of claim 9-14, wherein the luminescence intensity level variation among all the members of the second population is no greater than about 5 percent.
16. The set of populations of polymeric microbeads as set forth in any one of claims 9-15, wherein each polymeric microbead has a diameter of less than 500 μm .
17. The set of populations of polymeric microbeads as set forth in any one of claims 9-16, wherein each polymeric microbead has a diameter of less than 100 μm .
18. The set of populations of polymeric microbeads as set forth in any one of claims 9-17, wherein each polymeric microbead has a diameter of less than 50 μm .
19. The set of populations of polymeric microbeads as set forth in any one of claims 9-18, wherein the diameter variation among all the members of the set of populations is no greater than about 5 percent.
20. The set of populations of polymeric microbeads as set forth in any one of claims 9-19, wherein each polymeric microbead comprises 3 to 10 lanthanide nanoparticles, wherein each lanthanide nanoparticle has a different luminescence spectra.

21. The set of populations of polymeric microbeads as set forth in any one of claims 9-20, wherein each polymeric microbead comprises an upconverting lanthanide nanoparticle.
22. The set of populations of polymeric microbeads as set forth in any one of claims 9-21, wherein each polymeric microbead comprises a downconverting lanthanide nanoparticle.
23. The set of populations of polymeric microbeads as set forth in any one of claims 9-22, wherein the set comprises 24 or more different populations of polymeric microbeads.
24. The set of populations of polymeric microbeads as set forth in any one of claims 9-23, wherein the set comprises 64 or more different populations of polymeric microbeads.
25. The set of populations of polymeric microbeads as set forth in any one of claims 9-24, wherein the lanthanide nanoparticles comprise bismuth.
26. A set of populations of polymeric microbeads embedded with at least two different lanthanide nanoparticles, the set of populations of polymeric microbeads being produced using a method comprising:
 - mixing at least two fluids into a first solution, wherein each fluid comprises a polymerizable component and a lanthanide nanoparticle having a different luminescence spectra;
 - forming a first plurality of droplets from the first solution;
 - subjecting the first plurality of droplets to polymerization conditions, thereby producing a first population of polymeric microbeads embedded with at least two different lanthanide nanoparticles;
 - mixing the at least two fluids into a second solution, wherein the concentration of at least one of the different lanthanide nanoparticles is different between the first solution and the second solution;
 - forming a second plurality of droplets from the second solution; and
 - subjecting the second plurality of droplets to polymerization conditions, thereby producing a second population of polymeric microbeads embedded with the at least two different lanthanide nanoparticles,

wherein the concentration of at least one of the at least two different lanthanide nanoparticles is different between the polymeric microbeads of the first population and the polymeric microbeads of the second population.

27. The set of populations of polymeric microbeads as set forth in claim 26, wherein the concentration of one of the first and second lanthanide nanoparticles is substantially equal for each polymeric microbead of the first population and second population.

28. The set of populations of polymeric microbeads as set forth in claim 26, wherein the concentration of the first lanthanide nanoparticle in the first population is a known percentage of the concentration of the first lanthanide nanoparticle in the second population, wherein the known percentage is other than 100 percent.

29. A microfluidic device, the device comprising:

a flow channel having an inlet side and an outlet side;

at least two inlets positioned toward the inlet side of the flow channel,

wherein the inlets are configured to fluidly communicate with the flow channel;

a mixing element positioned in the flow channel downstream of the at least two inlets;

an input configured to hold a carrier fluid and fluidly communicate with the flow channel, wherein the input configured to hold a carrier fluid is configured to fluidly communicate with a portion of the flow channel downstream of the mixing element; and

an outlet located at the outlet side of the flow channel, wherein the outlet is configured to fluidly communicate with the flow channel and is positioned downstream of the portion of the flow channel with which the input configured to hold a carrier fluid is configured to fluidly communicate.

30. The microfluidic device as set forth in claim 29, wherein the microfluidic device comprises a sample collection element in fluid communication with the outlet, and located downstream of the outlet.

31. The microfluidic device as set forth in claim 29 or 30, wherein the microfluidic device comprises a plurality of valves, including valves separating each of the at least two inlets from the flow channel respectively.
32. The microfluidic device as set forth in any one of claims 29-31, wherein the microfluidic device comprises a valve between the mixing element and the portion of the flow channel with which the input configured to hold a carrier fluid is configured to fluidly communicate, wherein the valve between the mixing element and the portion of the flow channel with which the input configured to hold a carrier fluid is configured to fluidly communicate, when closed, prevents fluid contained in the mixing element from contacting fluid from the input configured to hold a hydrophobic carrier fluid.
33. The microfluidic device as set forth in claim 32, wherein the microfluidic device comprises a waste outlet located between the mixing element and the valve between the mixing element and the portion of the flow channel with which the input configured to hold a carrier fluid is configured to fluidly communicate.
34. The microfluidic device as set forth in any one of claims 31-33, wherein one or more of the plurality of valves is configured to be actuated pneumatically.
35. The microfluidic device as set forth in any one of claims 29-34, wherein the carrier fluid is a hydrophilic carrier fluid.
36. The microfluidic device as set forth in claim 35, wherein the microfluidic device comprises an input which is configured to hold a hydrophobic carrier fluid and fluidly communicate with the flow channel and is located upstream of the mixing element.
37. The microfluidic device as set forth in claim 35 or 36, wherein the input configured to hold a hydrophilic carrier fluid comprises an on-chip resistor.
38. The microfluidic device as set forth in 35 or 36, wherein the input configured to hold a hydrophobic carrier fluid comprises an on-chip resistor.
39. The microfluidic device as set forth in any one of claims 29-34, wherein the carrier fluid is a hydrophobic carrier fluid.

40. The microfluidic device as set forth in claim 39, wherein the microfluidic device comprises an input which is configured to hold a hydrophilic carrier fluid and fluidly communicate with the flow channel and is located upstream of the mixing element.
41. The microfluidic device as set forth in claim 39 or 40, wherein the input configured to hold a hydrophilic carrier fluid comprises an on-chip resistor.
42. The microfluidic device as set forth in any one of claims 39 or 40, wherein the input configured to hold a hydrophobic carrier fluid comprises an on-chip resistor.
43. The microfluidic device as set forth in any one of claims 29-42, wherein the mixing element is a staggered herringbone mixer.
44. The microfluidic device as set forth in any of claims 29-43, wherein the device is fabricated by multi-layer soft lithography.
45. The microfluidic device as set forth in any of claims 29-44, wherein the device is fully automated.
46. A system comprising the microfluidic device as set forth in any one of claims 29-45, and a chamber, wherein the device is positioned in the chamber and exposed to nitrogen gas therein.
47. A system comprising the microfluidic device as set forth in any one of claims 29-45, and a UV generating element positioned to expose a portion of the flow channel to UV radiation, wherein the portion to be exposed to UV radiation is downstream of the portion of the flow channel with which the input configured to hold a carrier fluid is configured to fluidly communicate and upstream of the outlet.
48. A system comprising a microfluidic device as set forth in any one of claims 29-45, and a plurality of inlet containers, wherein each inlet container is configured to fluidly communicate to a different one of the at least two inlets, and wherein each inlet container comprises a fluid comprising a different lanthanide nanoparticle and a polymerizable component.

49. The system as set forth in claim 48, comprising a plurality of pumps, wherein the plurality of inlet containers is configured to fluidly communicate with the plurality of pumps.
50. The system as set forth in claim 48 or 49, wherein the inlet containers comprise capillary tubing having a length that is at least 1000 times greater than the internal diameter of the capillary tubing.
51. The system as set forth in any one of claims 48-50, wherein the containers are positioned in a chamber and exposed to nitrogen gas therein.
52. The system as set forth in any one of claims 48-51, wherein the plurality of containers comprises 2 to 10 lanthanide nanoparticles, wherein each lanthanide nanoparticle has a different luminescence spectra.
53. The system as set forth in any one of claims 48-52, wherein the lanthanide nanoparticle contained in each fluid is present at a concentration of 1 mg/mL to 250 mg/mL.
54. A microfluidic device, the device comprising:
- a flow channel having an inlet side and an outlet side, wherein a portion of the flow channel is configured as a zig-zag mixer;
 - at least two inlets positioned toward the inlet side of the flow channel, wherein the at least two inlets are configured to fluidly communicate with the flow channel;
 - an input configured to hold a carrier fluid configured to fluidly communicate with the flow channel, wherein the input configured to hold a carrier fluid is configured to fluidly communicate with a portion of the flow channel downstream of the at least two inlets and upstream of the portion of the flow channel configured as a zig-zag mixture; and
 - an outlet located at the outlet side of the flow channel, downstream of the portion of the flow channel configured as a zig-zag mixer.

55. The microfluidic device as set forth in claim 54, wherein the carrier fluid is a hydrophobic carrier fluid.
56. The microfluidic device as set forth in claim 54, wherein the carrier fluid is a hydrophilic carrier fluid.
57. A method for producing a polymeric microbead comprising at least two different lanthanide nanoparticles, the method comprising:
- mixing at least two fluids into a solution, wherein each fluid comprises a polymerizable component and a different lanthanide nanoparticle;
 - forming a droplet from the solution; and
 - subjecting the droplet to polymerization conditions, thereby producing a polymeric microbead comprising at least two different lanthanide nanoparticles, wherein the above steps are performed on a microfluidic device as set forth in any one of claims 29-45 and 54 or with a system as set forth in any one of claims 46-53.
58. The method as set forth in claim 57, wherein the polymerization conditions comprise exposing the droplet to UV radiation.
59. The method as set forth in claim 57, wherein the polymerization conditions comprise exposing the droplet to a temperature sufficient to initiate polymerization of the polymerizable component.
60. The method as set forth in any one of claims 57-59, wherein each fluid has an approximately equivalent total concentration of lanthanide nanoparticles.
61. The method as set forth in any one of claims 57-60, wherein the relative concentration of each different lanthanide nanoparticle in the polymeric microbead is controlled by adjusting the relative flow rates of the at least two fluids.
62. The method as set forth in claim 61, wherein the relative flow rates are determined based at least in part upon solving the coupled flow equations:

$$Q_n = \frac{P_n - P_{mix}}{R_n}, \text{ and}$$

$$Q_{tot} = \frac{P_{mix}}{R_{mix}},$$

where

Q_n is the flow rate for each input;

P_n is the pressure for each input;

Q_{tot} is total flow rate;

P_{mix} is the pressure at the point where the fluids are mixed;

R_{mix} is the resistance at the point where the fluids are mixed; and

R_n is the resistance of each input.

63. The method as set forth in any one of claims 57-62, wherein the droplet size is modulated by adjusting the pressure at a T-junction used to form the droplet.
64. A method for producing a population of polymeric microbeads comprising a plurality of different lanthanide nanoparticles, the method comprising:
- (i) mixing at least two fluids into a solution, wherein each fluid comprises a polymerizable component and a different lanthanide nanoparticle;
 - (ii) forming a first plurality of droplets from the solution; and
 - (iii) subjecting the first plurality of droplets to polymerization conditions, thereby producing a first plurality of polymeric microbeads embedded with at least two different lanthanide nanoparticles, wherein the relative concentrations of the lanthanide nanoparticles are substantially equal among the polymeric microbeads of the first plurality of polymeric microbeads.
65. The method as set forth in claim 64, comprising:
- (iv) mixing the at least two fluids into a second solution, wherein the concentration of at least one of the different lanthanide nanoparticles in the second solution is different than in (i);
 - (v) forming a second plurality of droplets from the second solution; and

- (vi) subjecting the second plurality of droplets to polymerization conditions,
thereby producing a second plurality of polymeric microbeads embedded with at least two different lanthanide nanoparticles, wherein the relative concentrations of the lanthanide nanoparticles are substantially equal among the polymeric microbeads of the second plurality of polymeric microbeads.
66. The method as set forth in claim 65, wherein the concentration of at least one of the different lanthanide nanoparticles in the second solution is substantially equal to that in (i).
67. The method as set forth in claim 65, wherein the concentration of one of the different lanthanide nanoparticles in the first plurality of polymeric microbeads is a known percentage of the concentration of the first lanthanide nanoparticle in the second plurality of polymeric microbeads, wherein the known percentage is other than 100 percent.
68. The method as set forth in claim 64, wherein the polymerization conditions comprise exposing the first plurality of droplets to UV radiation.
69. The method as set forth in claim 65, wherein the polymerization conditions comprise exposing the second plurality of droplets to UV radiation.
70. The method as set forth in claim 64, wherein the polymerization conditions comprise exposing the first plurality of droplets to a temperature sufficient to initiate polymerization of the polymerizable component.
71. The method as set forth in claim 65, wherein the polymerization conditions comprise exposing the second plurality of droplets to a temperature sufficient to initiate polymerization of the polymerizable component.
72. The method as set forth in any one of claims 64-71, wherein the luminescence intensity level variation among all the members of the first plurality of polymeric microbeads is no greater than about 15 percent.

73. The method as set forth in claim 64-72, wherein the luminescence intensity level variation among all the members of the first plurality of polymeric microbeads is no greater than about 5 percent.
74. The method as set forth in any one of claims 65-73, wherein the luminescence intensity level variation among all the members of the second plurality of polymeric microbeads is no greater than about 15 percent.
75. The method as set forth in any one of claims 65-74, wherein the luminescence intensity level variation among all the members of the second plurality of polymeric microbeads is no greater than about 5 percent.
76. A method of imaging spectrally encoded microbeads, the method comprising:
 illuminating one or more microbeads selected from a population of microbeads as set forth in one of claims 1-28 with a light source;
 detecting luminescence emission from the microbead in a plurality of spectral bands; and
 determining the intensities of each different lanthanide nanoparticle present in the microbead using linear unmixing.
77. The method as set forth in claim 76, wherein the spectral bands are defined by a plurality of emission filters that pass the characteristic emission peaks of each lanthanide nanoparticle.
78. The method as set forth in claim 76, wherein the light source is a deep UV light source.
79. The method as set forth in claim 76, wherein the light source is near IR light source.
80. A system, comprising:
 a microfluidic device comprising
 one or more inlet ports;

a flow channel configured for fluid communication with the one or more inlet ports, wherein the flow channel is sized and shaped to provide a monolayer of polymeric microbeads in the flow channel;

a sieve valve positioned in or downstream of the flow channel, wherein the sieve valve is configured to allow fluid flow through the flow channel while retaining the polymeric microbeads in the flow channel; and

one or more outlet ports configured for fluid communication with the flow channel; and

a light source configured to illuminate a portion of the flow channel.

81. The system as set forth in claim 80, wherein the light source is a deep UV light source.
82. The system as set forth in claim 80, wherein the light source is a near IR light source.
83. The system as set forth in any one of claims 80-82, comprising a camera configured to collect an image of the illuminated portion of the flow channel.
84. The system as set forth in any one of claims 80-83, comprising a display configured to display an image of the illuminated portion of the flow channel.

FIGURE 1

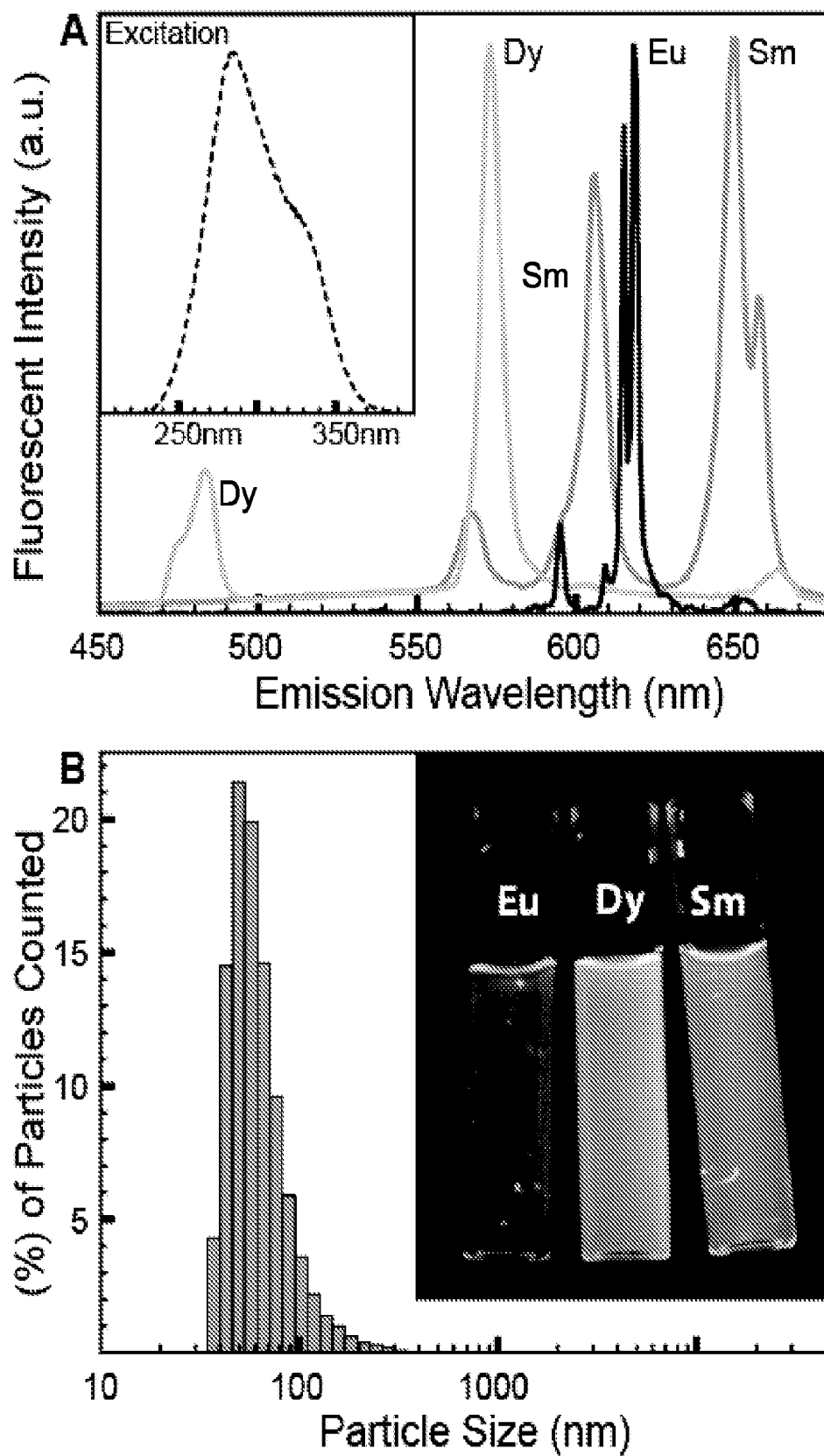


FIGURE 2

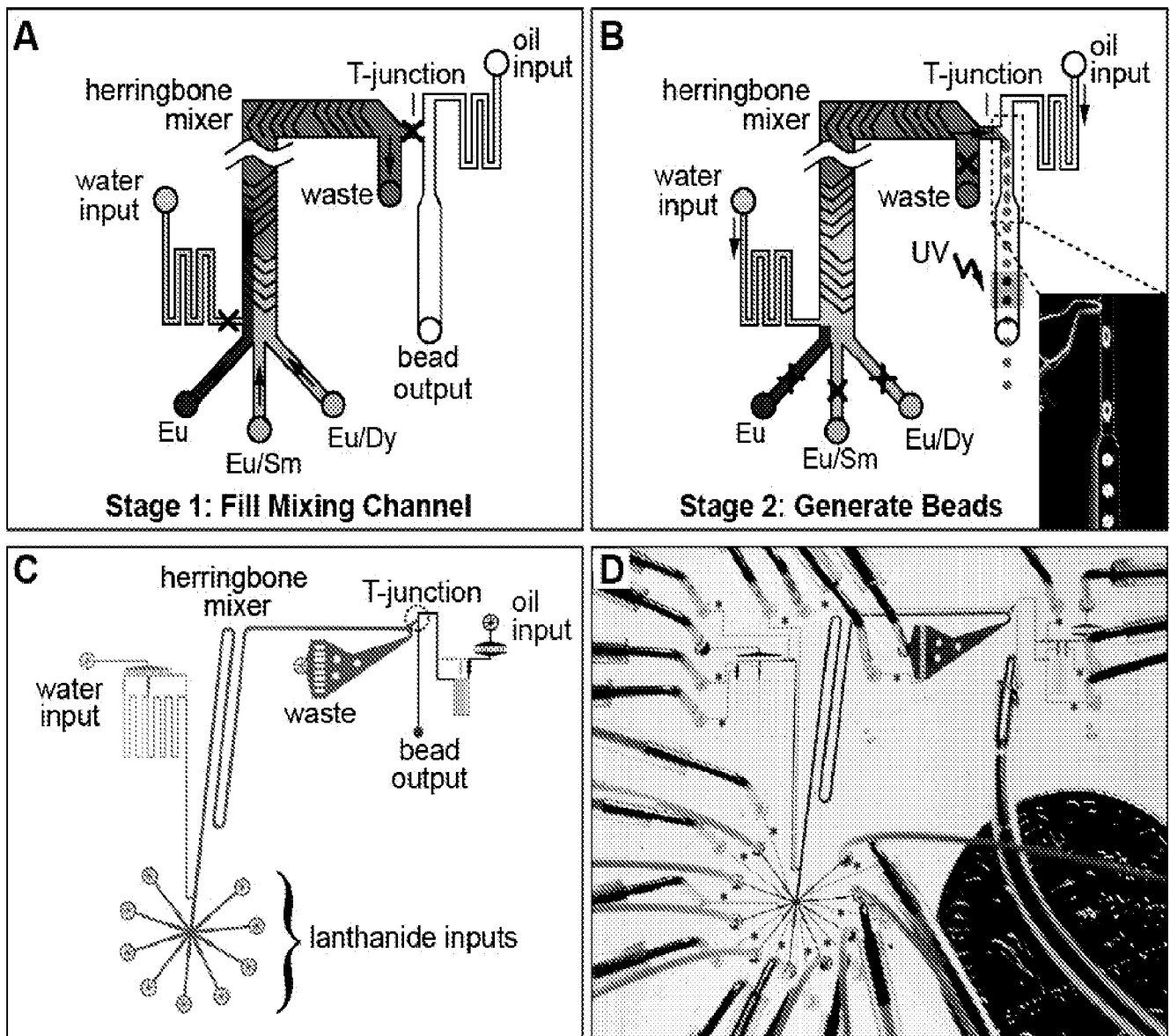
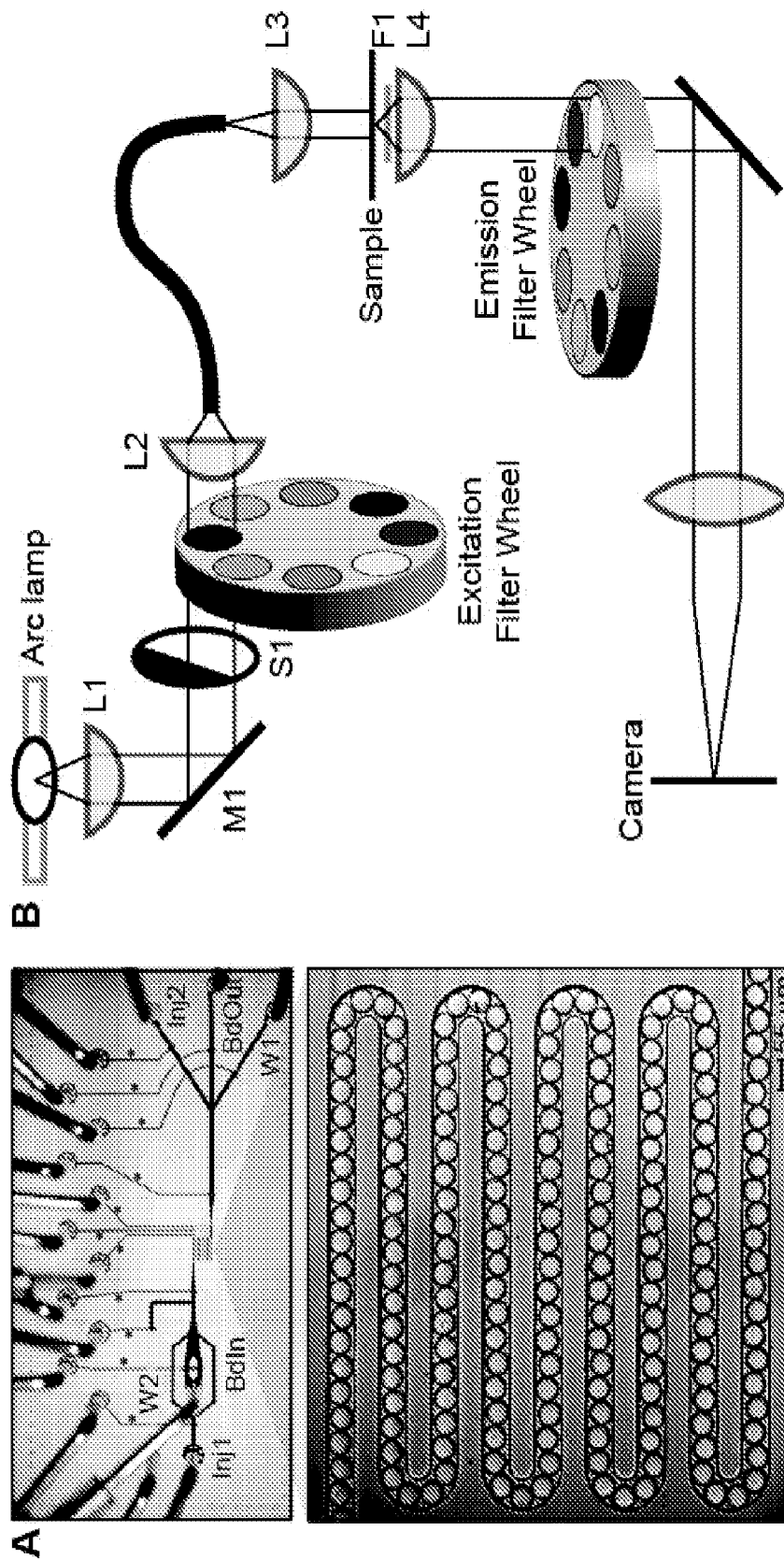


FIGURE 3



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FIGURE 4

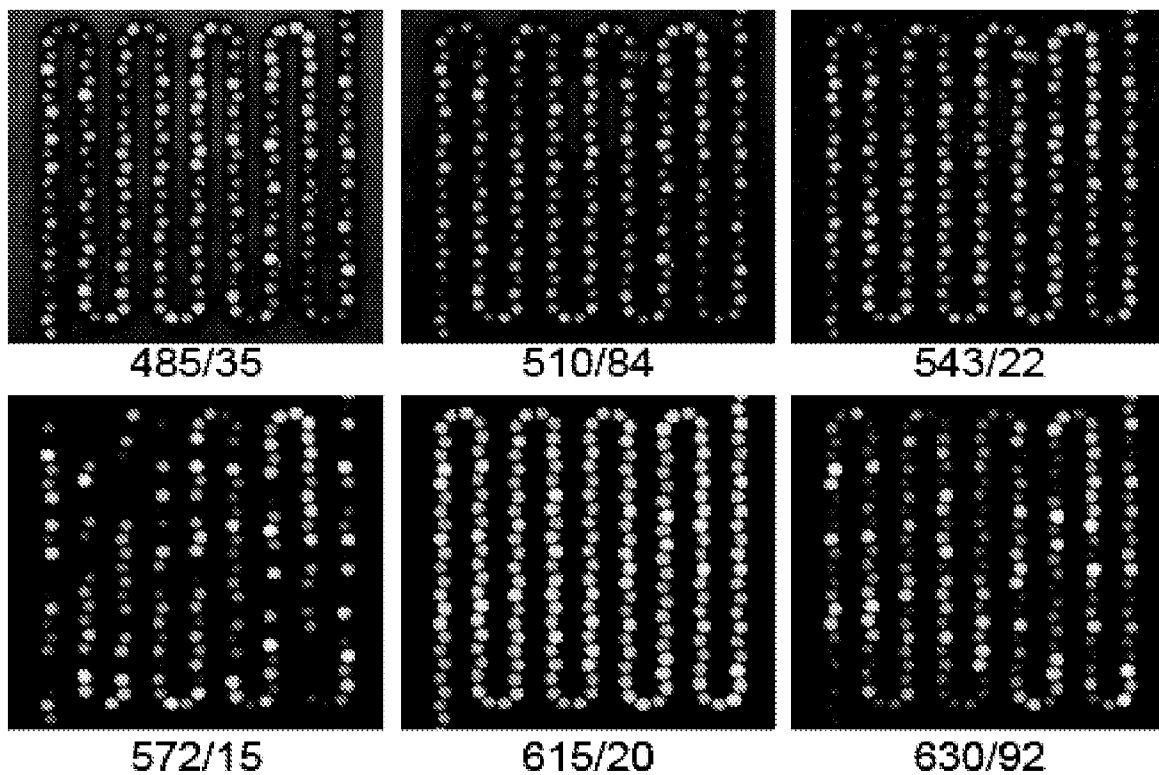
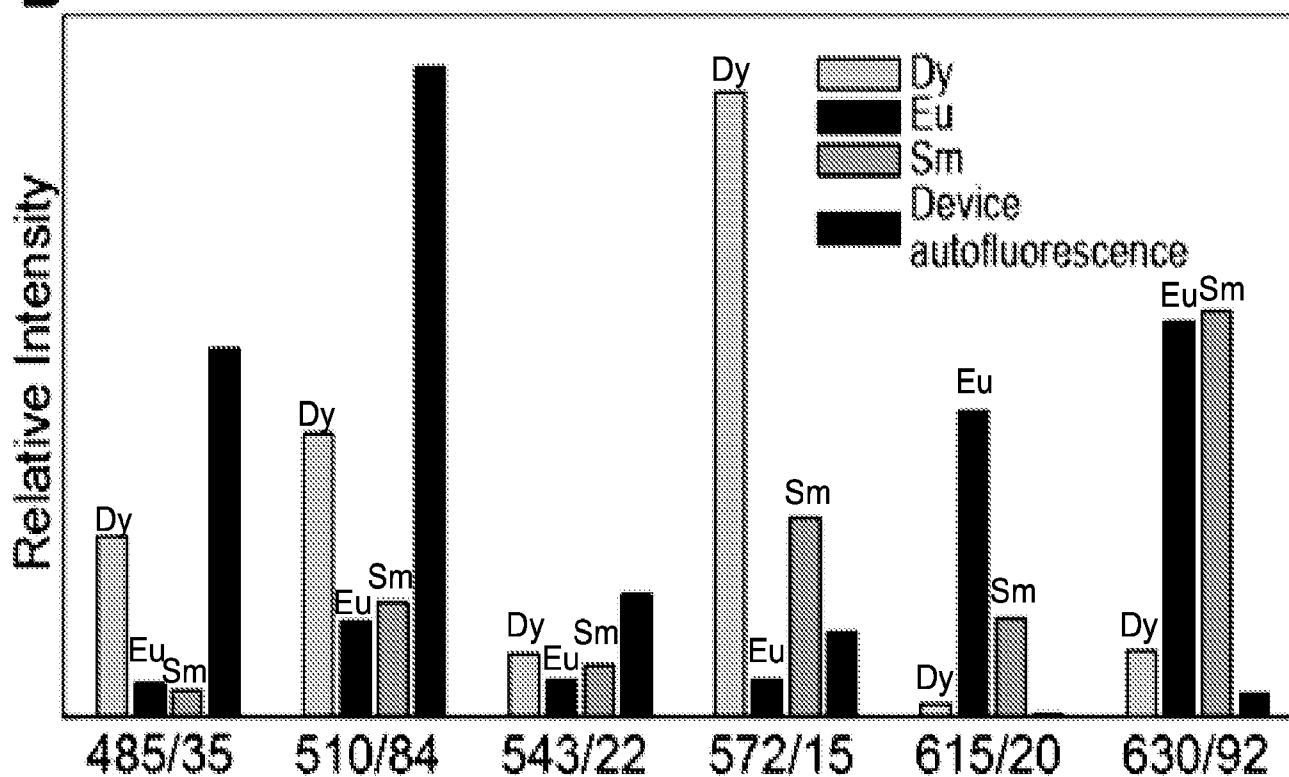
A**B**

FIGURE 4 (Cont.)

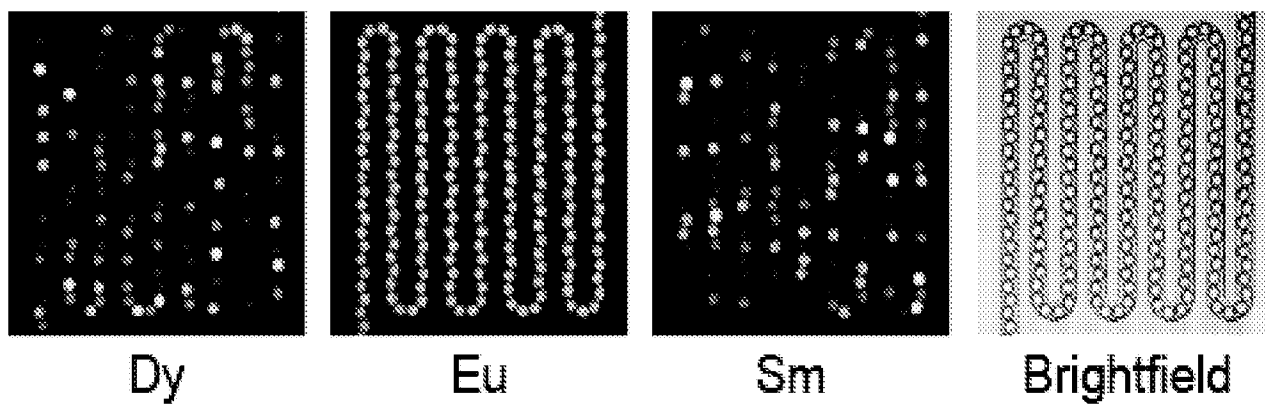
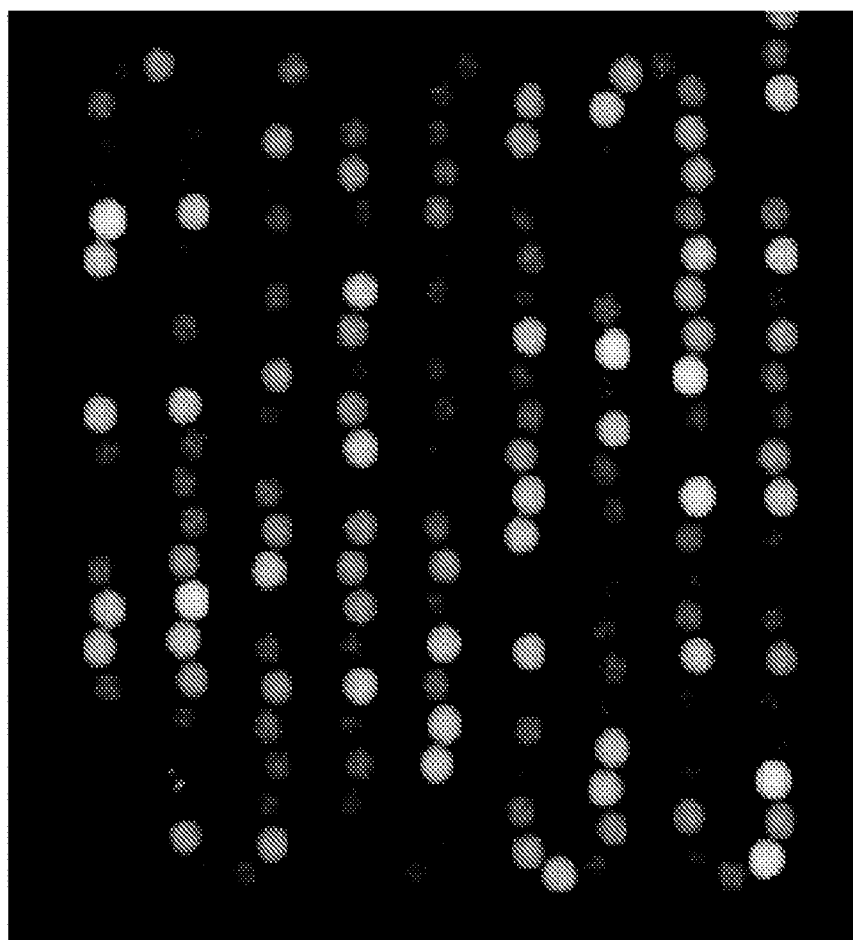
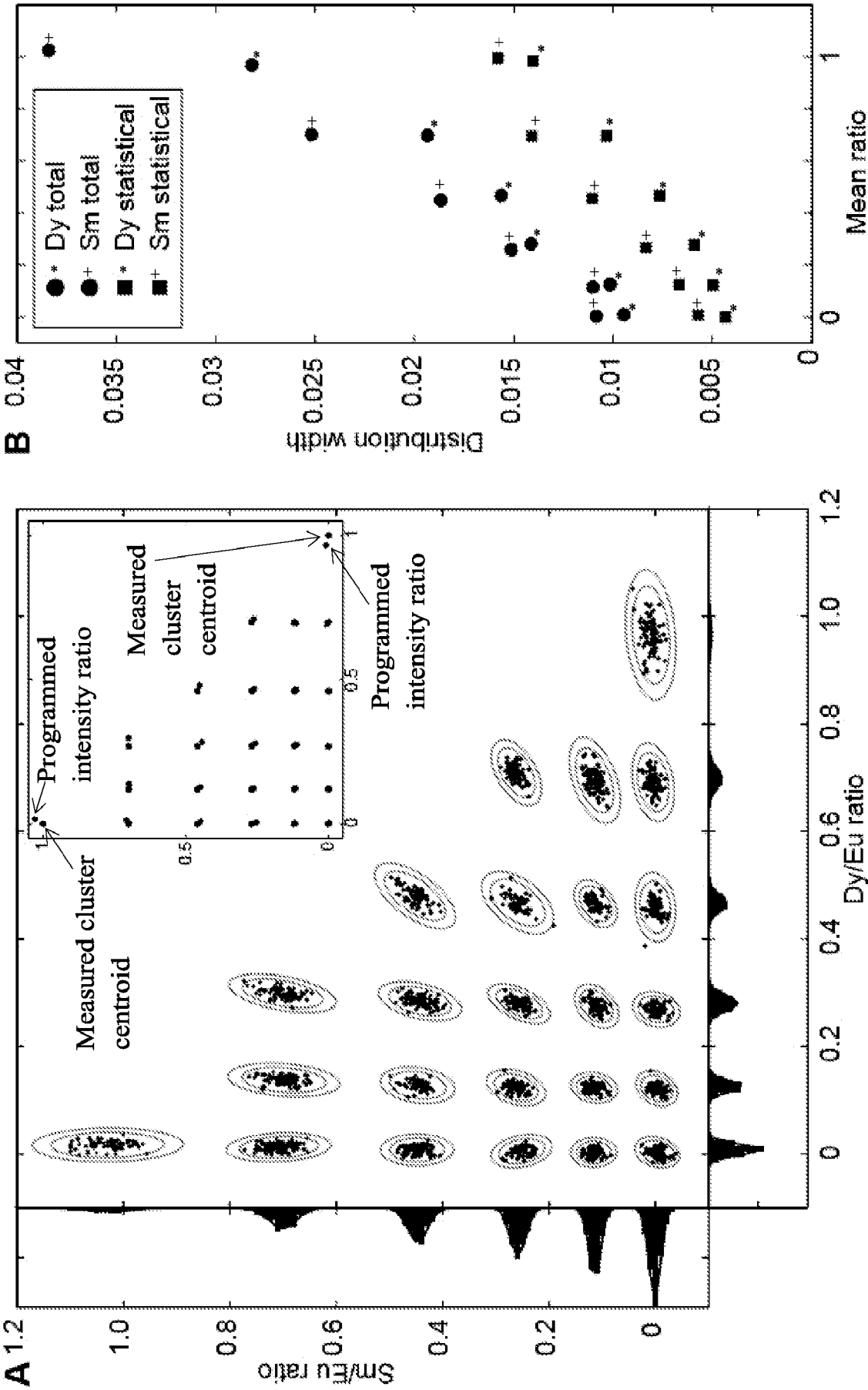
C**D**

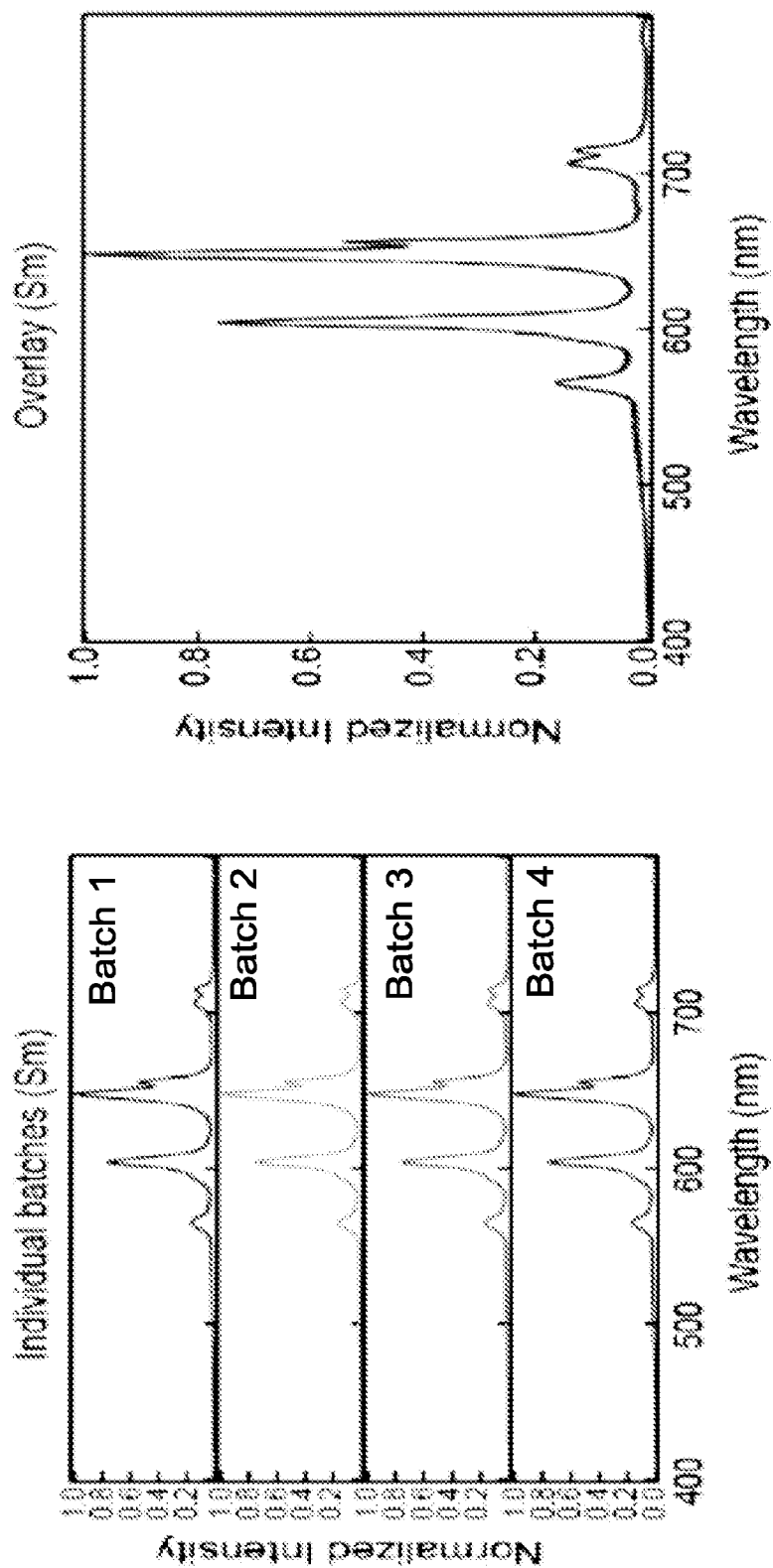
FIGURE 5



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FIGURE 6

A



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FIGURE 6 (Cont.)

B

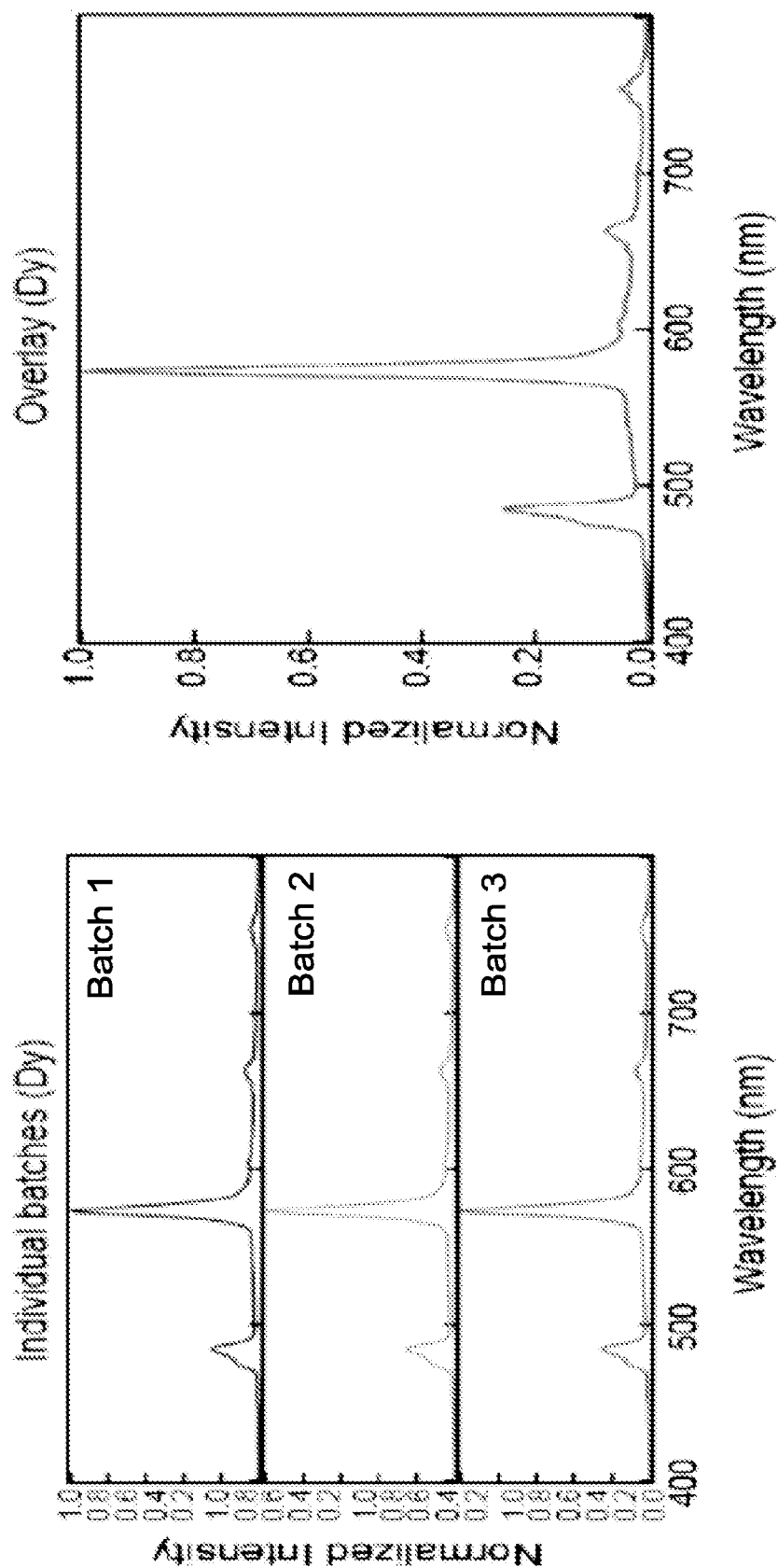
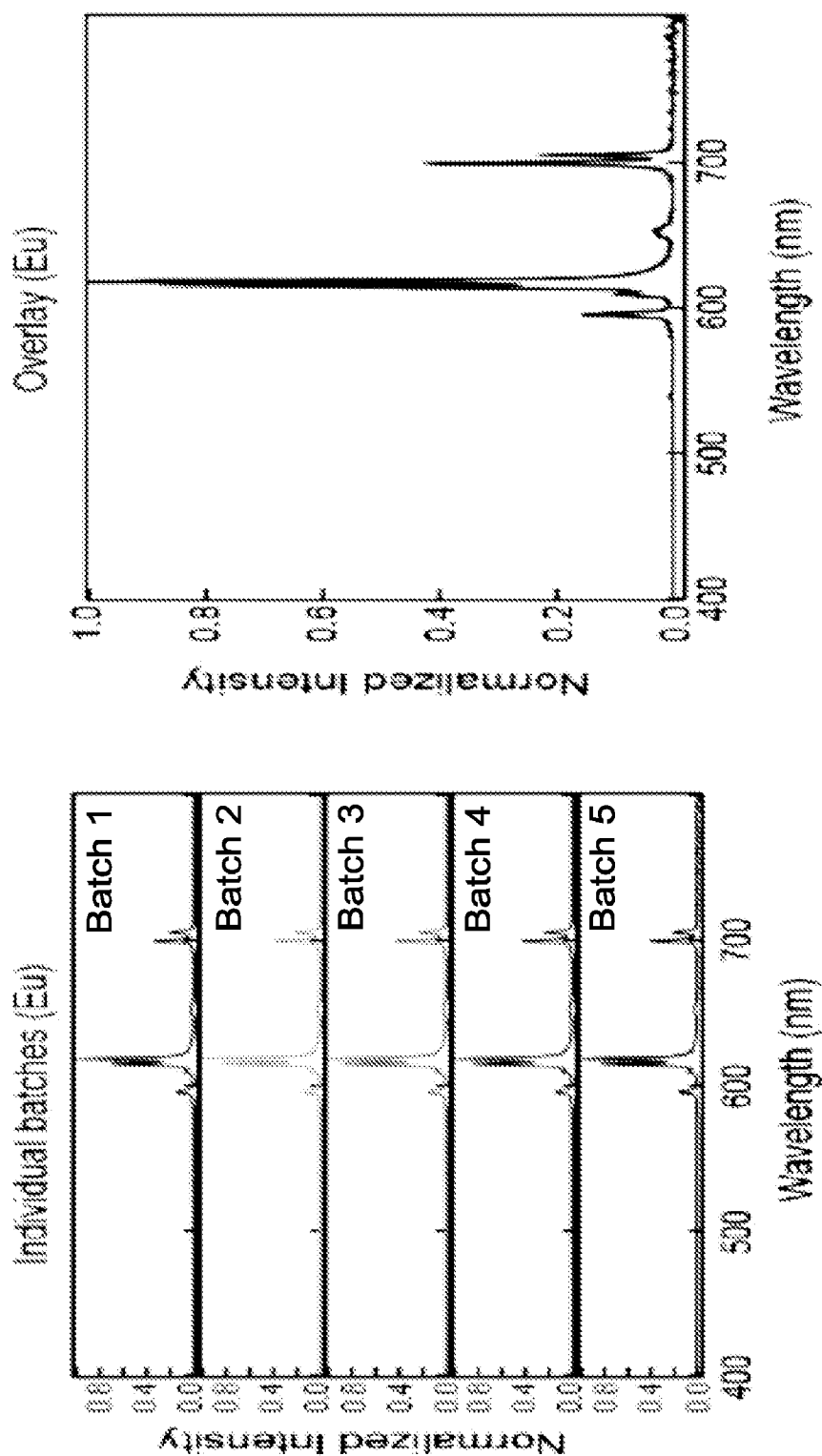


FIGURE 6 (Cont.)

C



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FIGURE 7

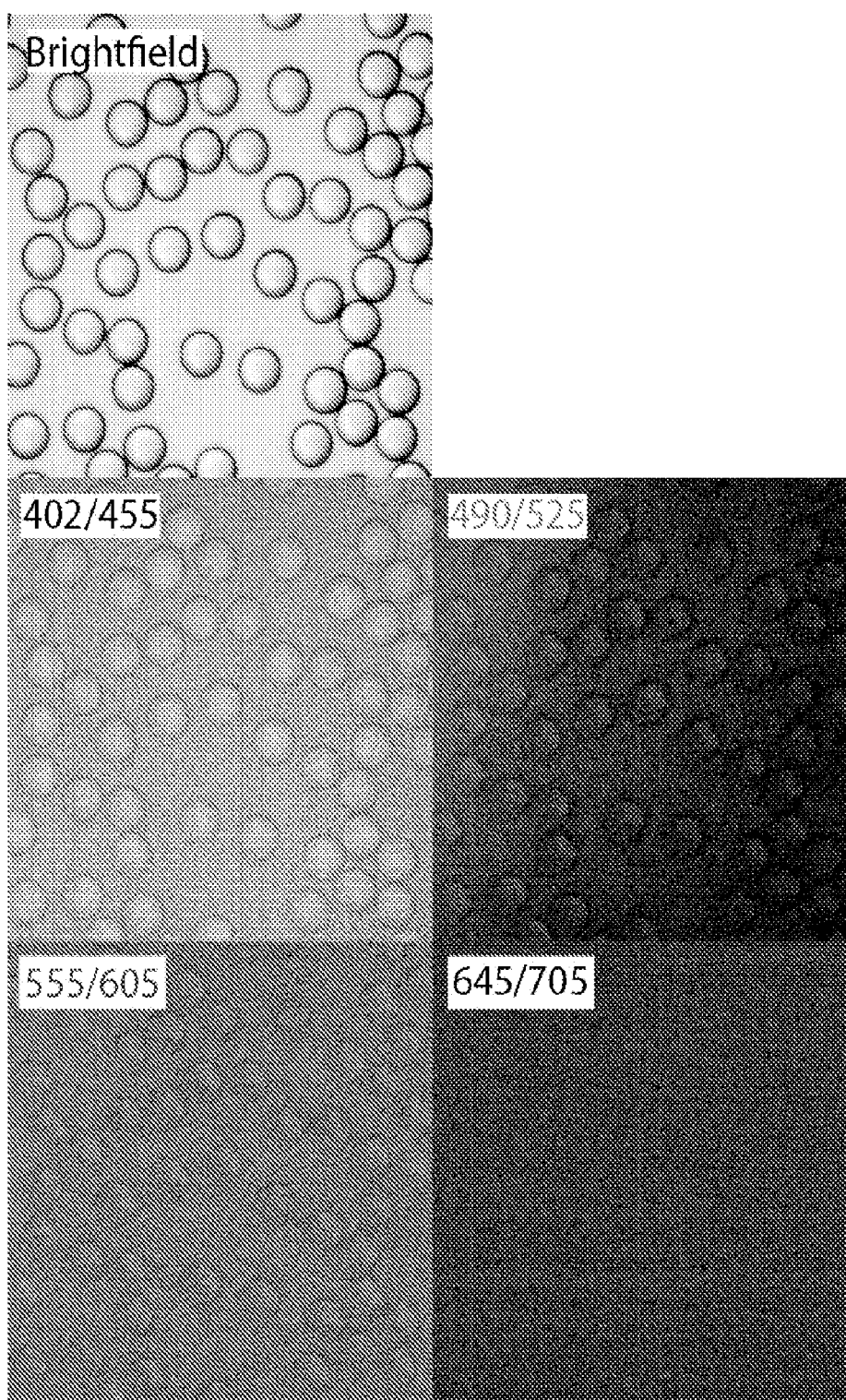


FIGURE 8

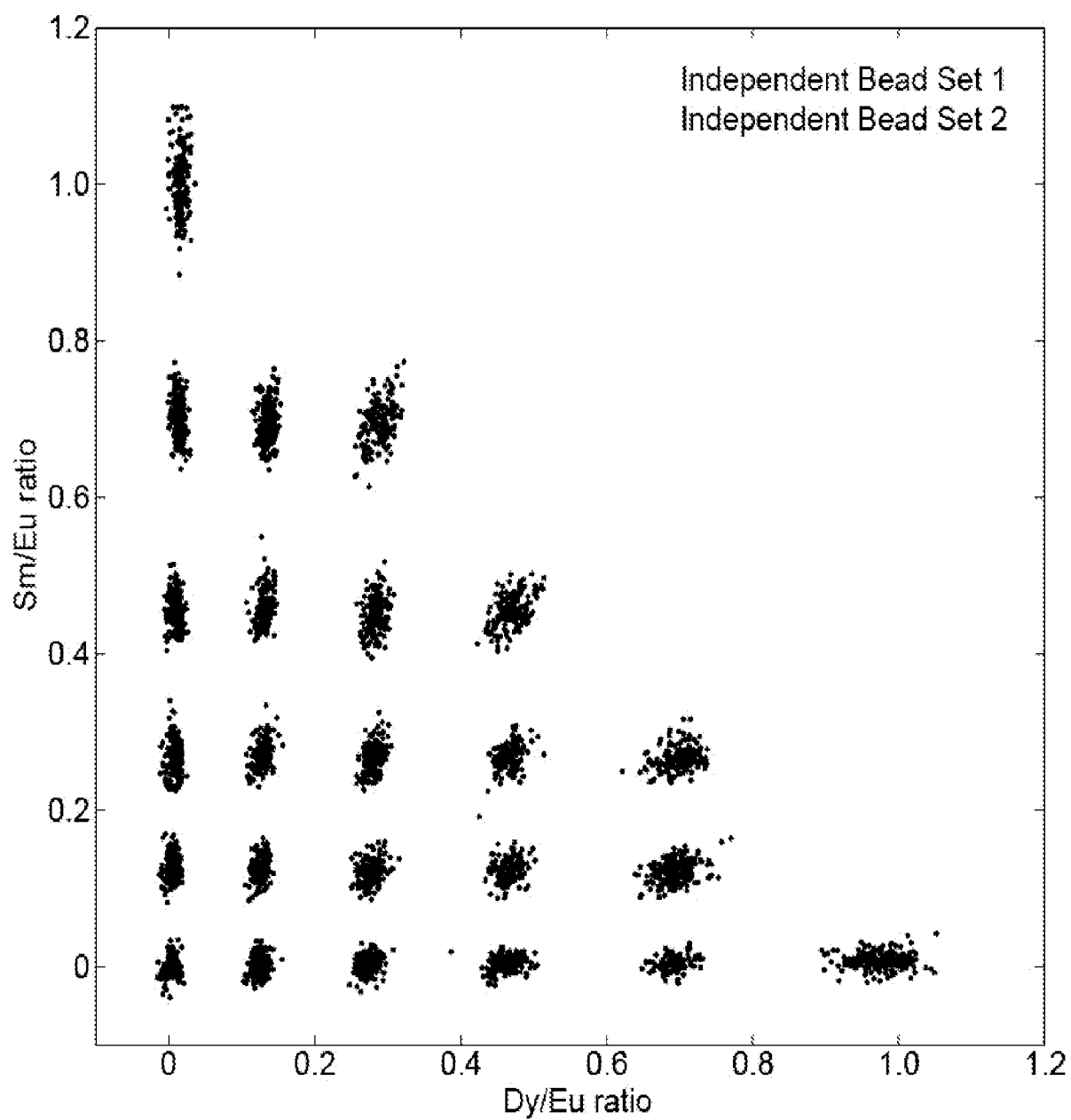


FIGURE 9

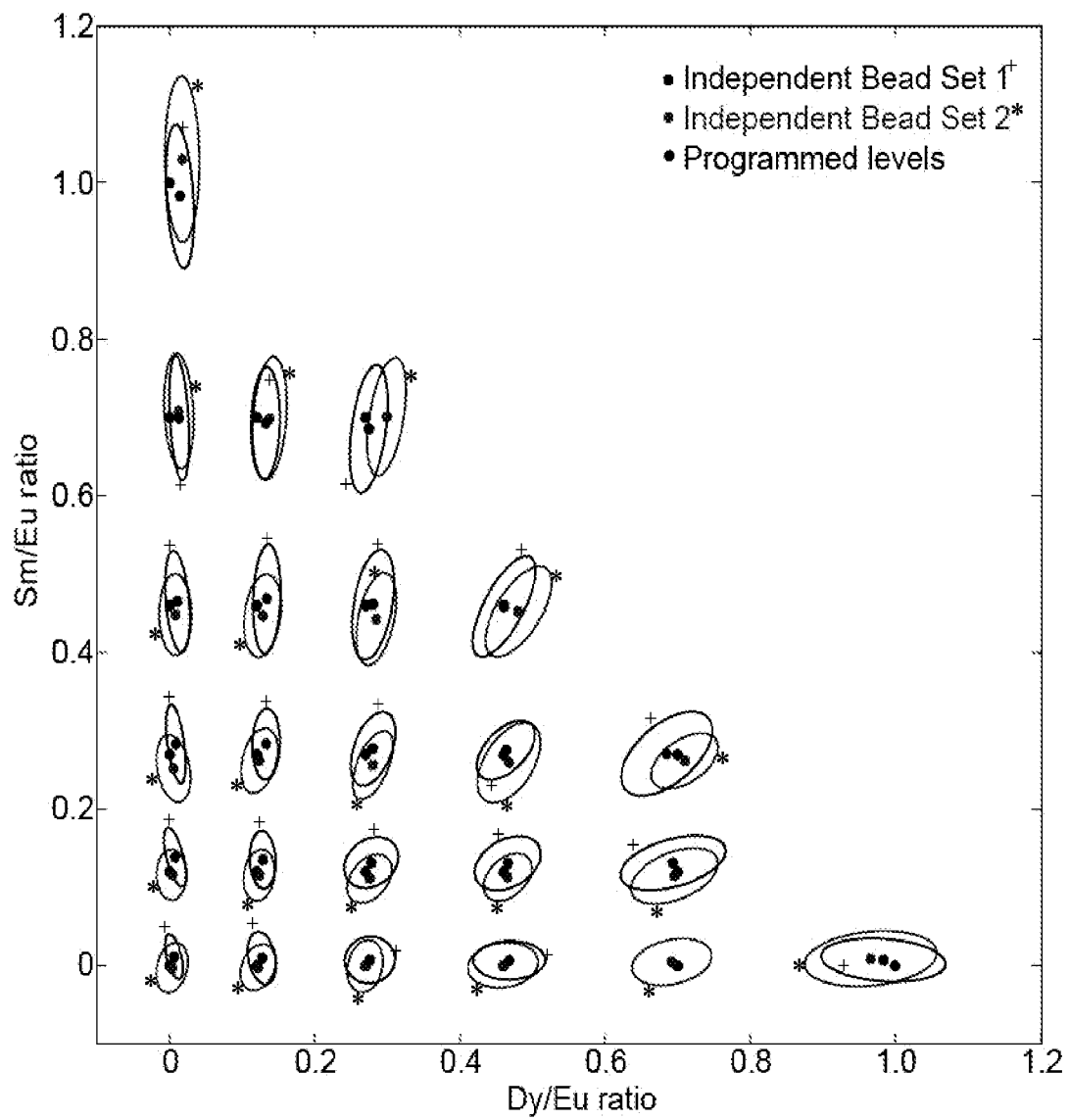


FIGURE 10

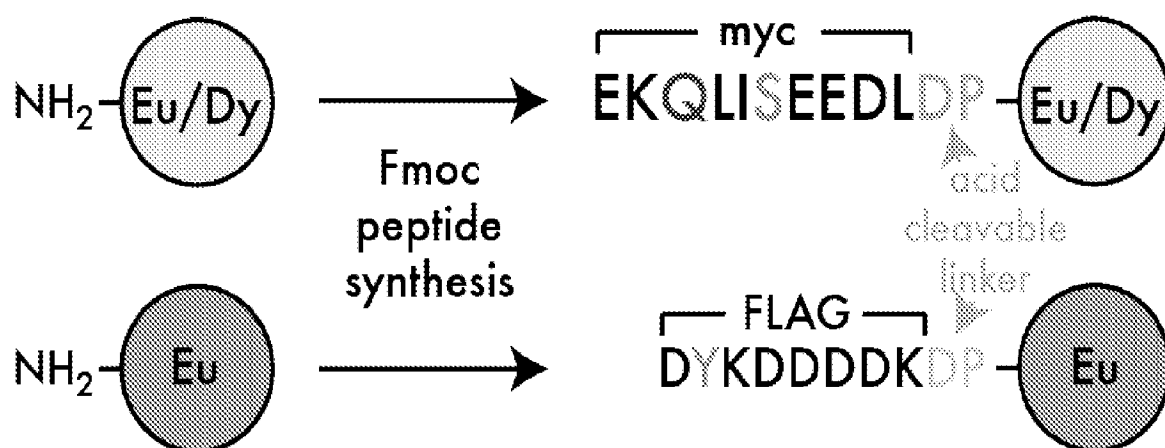


FIGURE 11

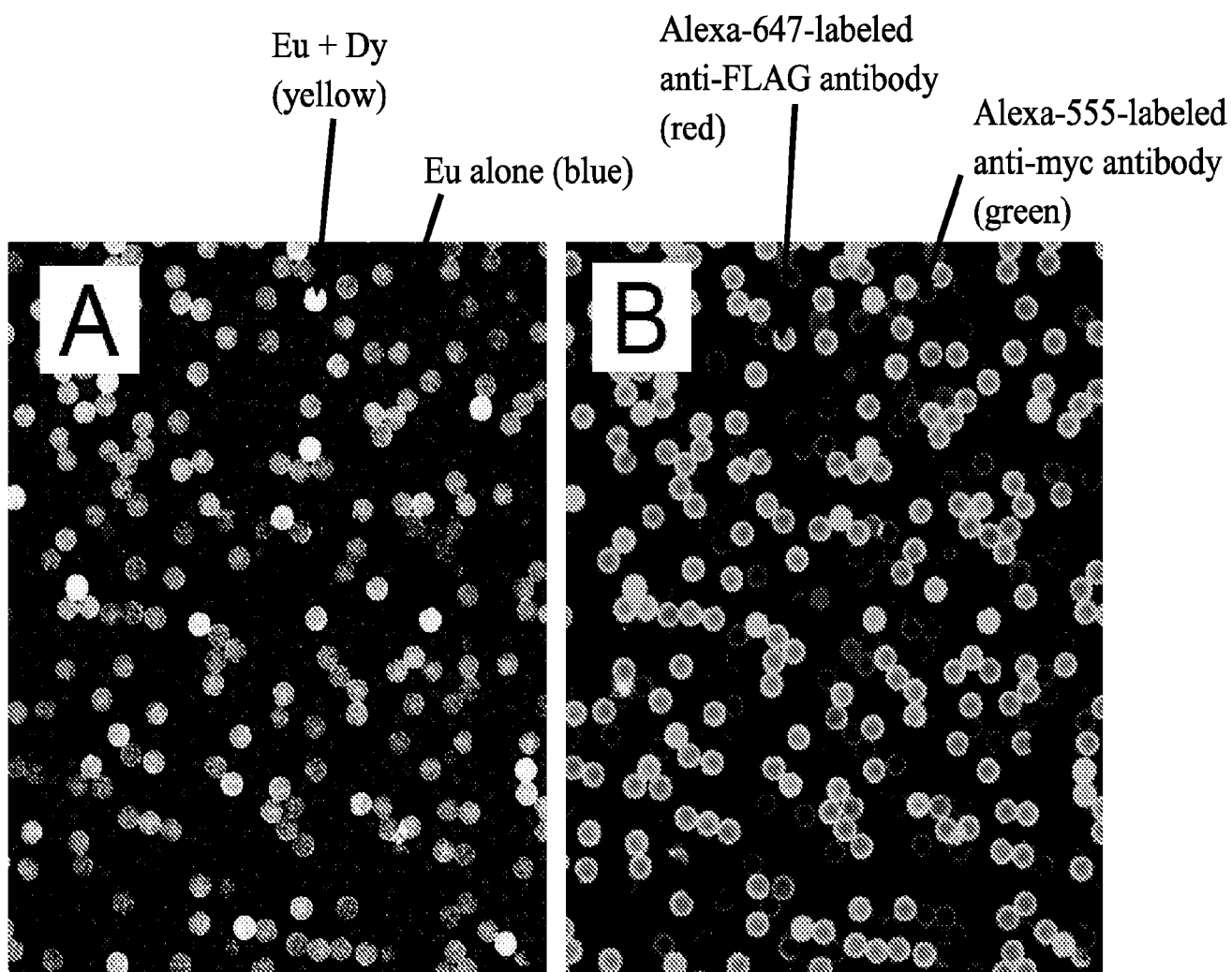


FIGURE 12

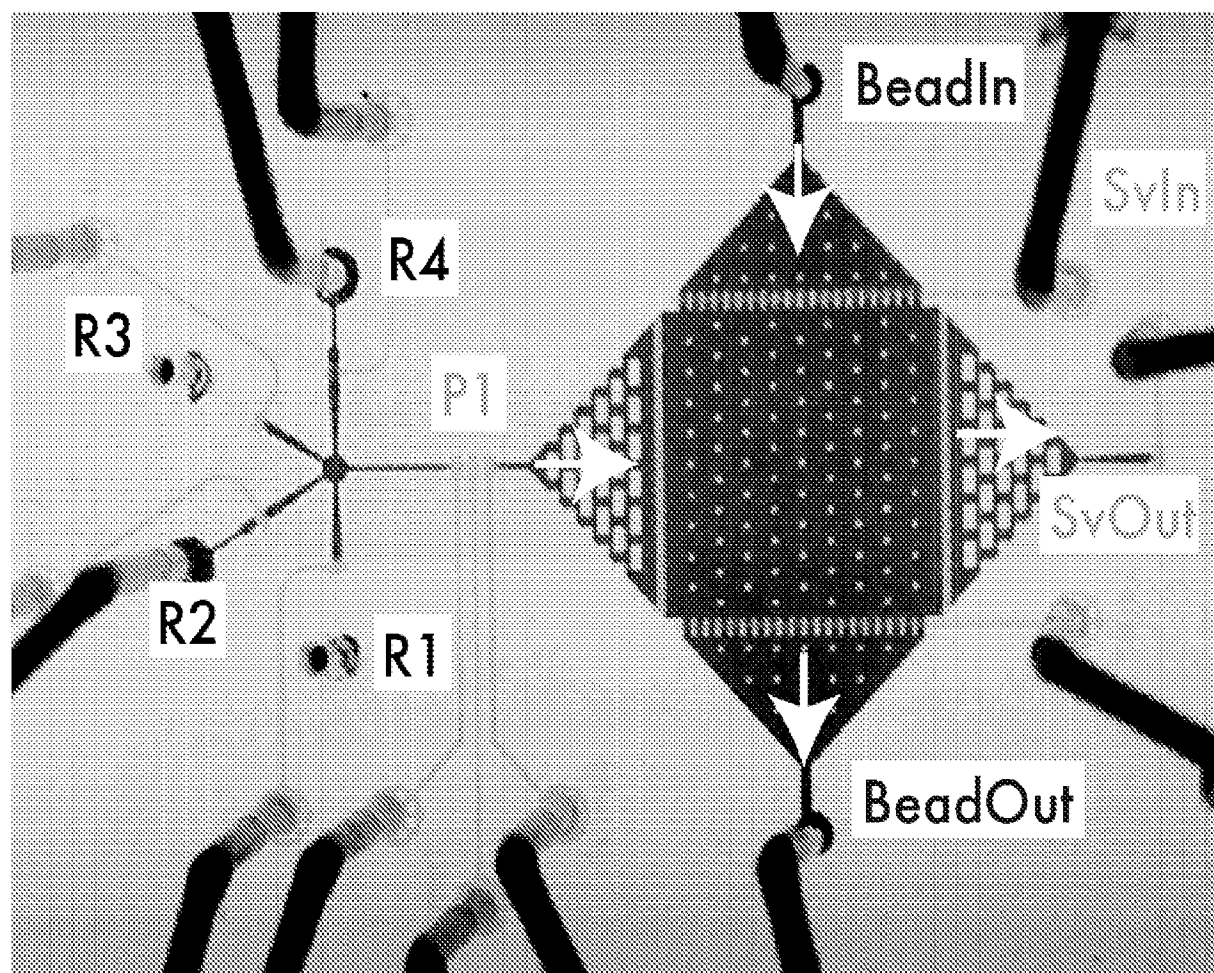
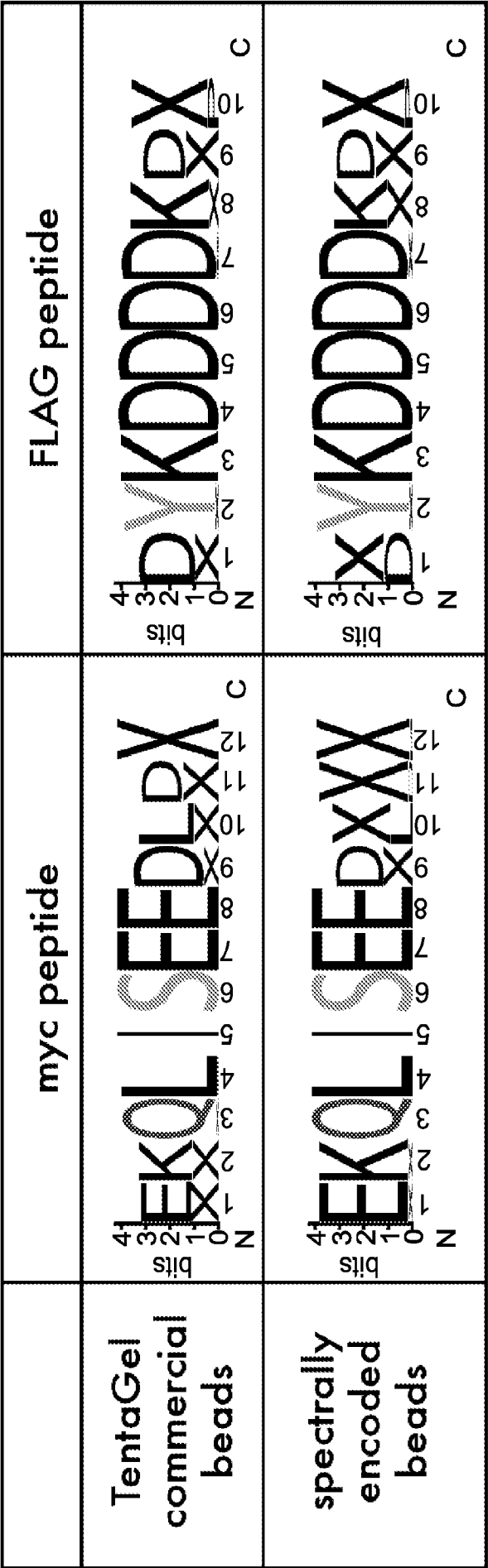


FIGURE 13



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FIGURE 14

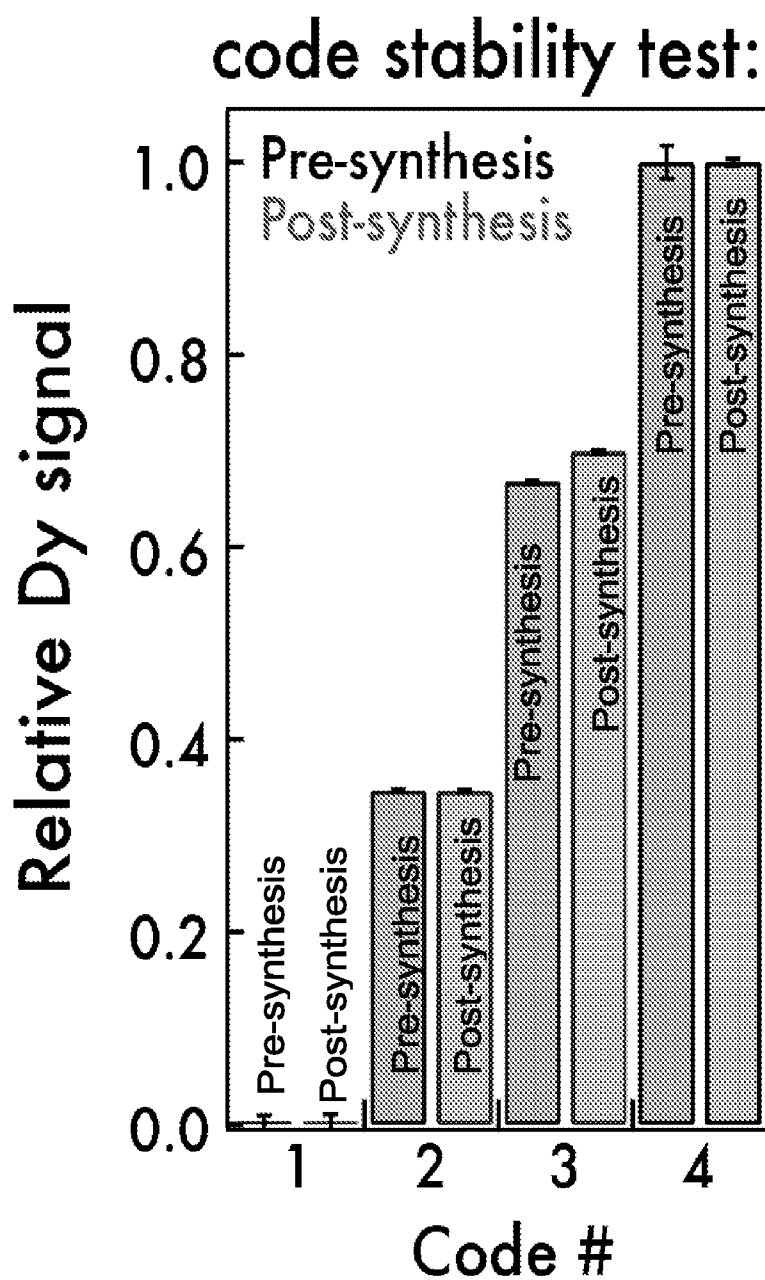


FIGURE 15

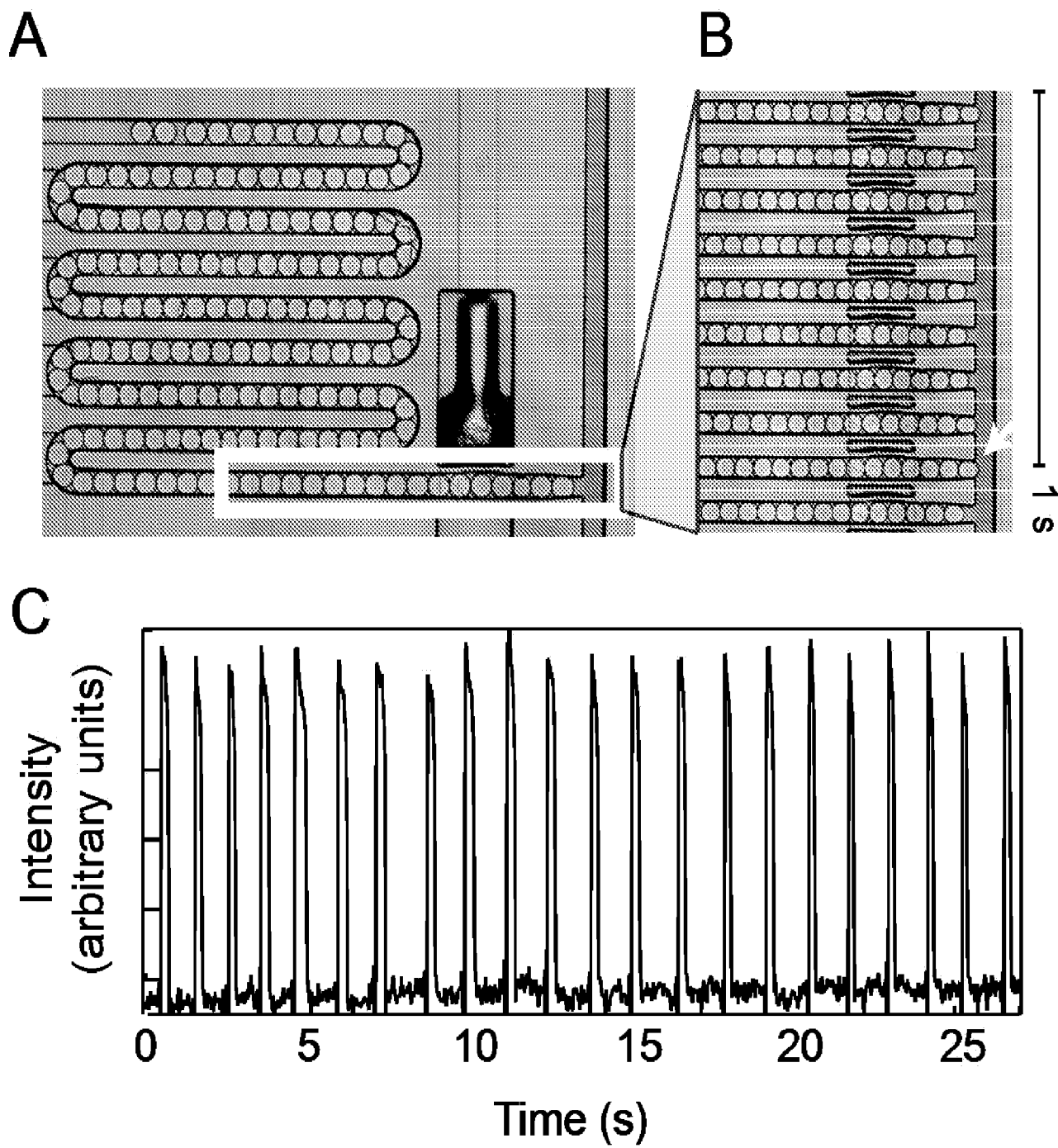


FIGURE 16

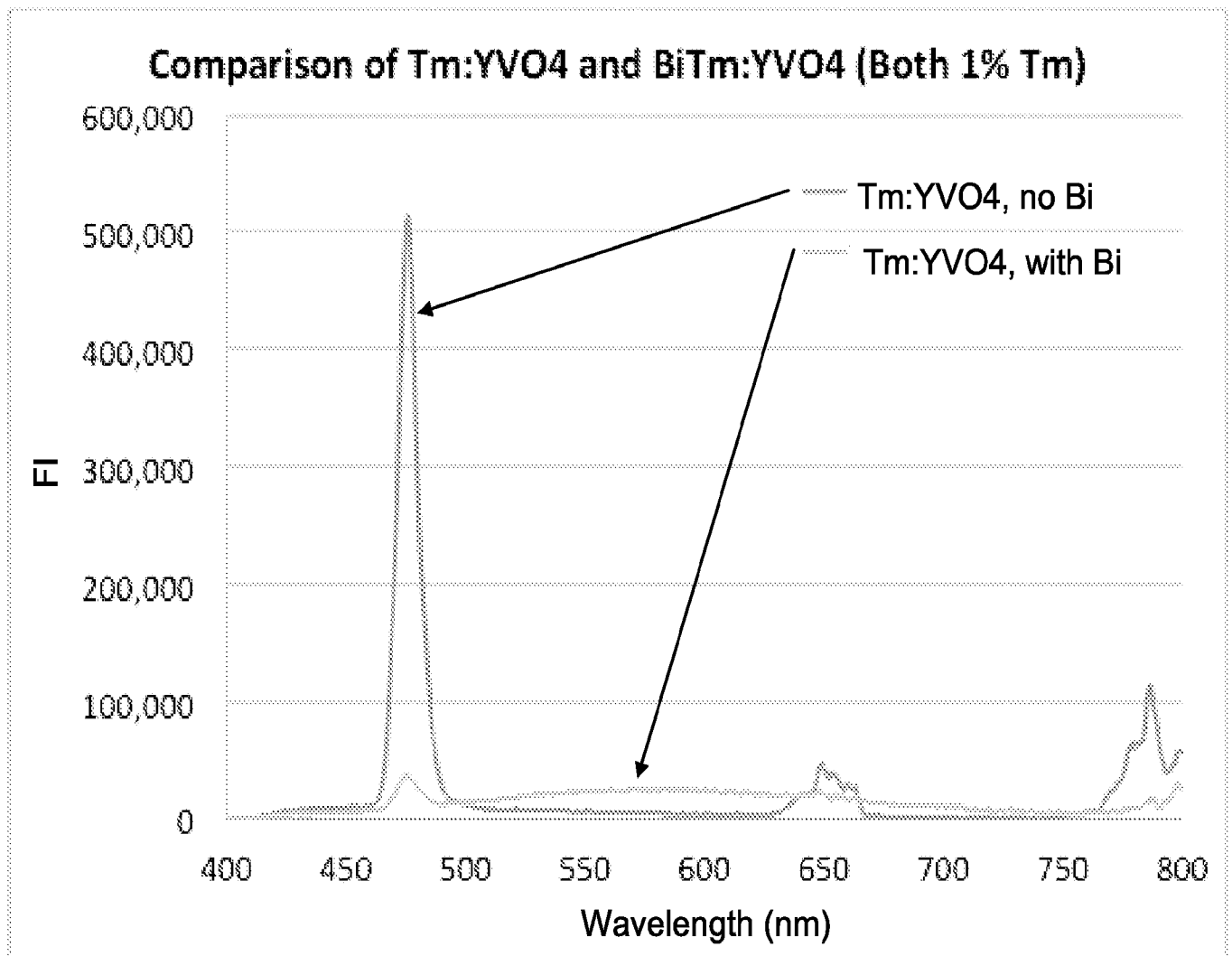
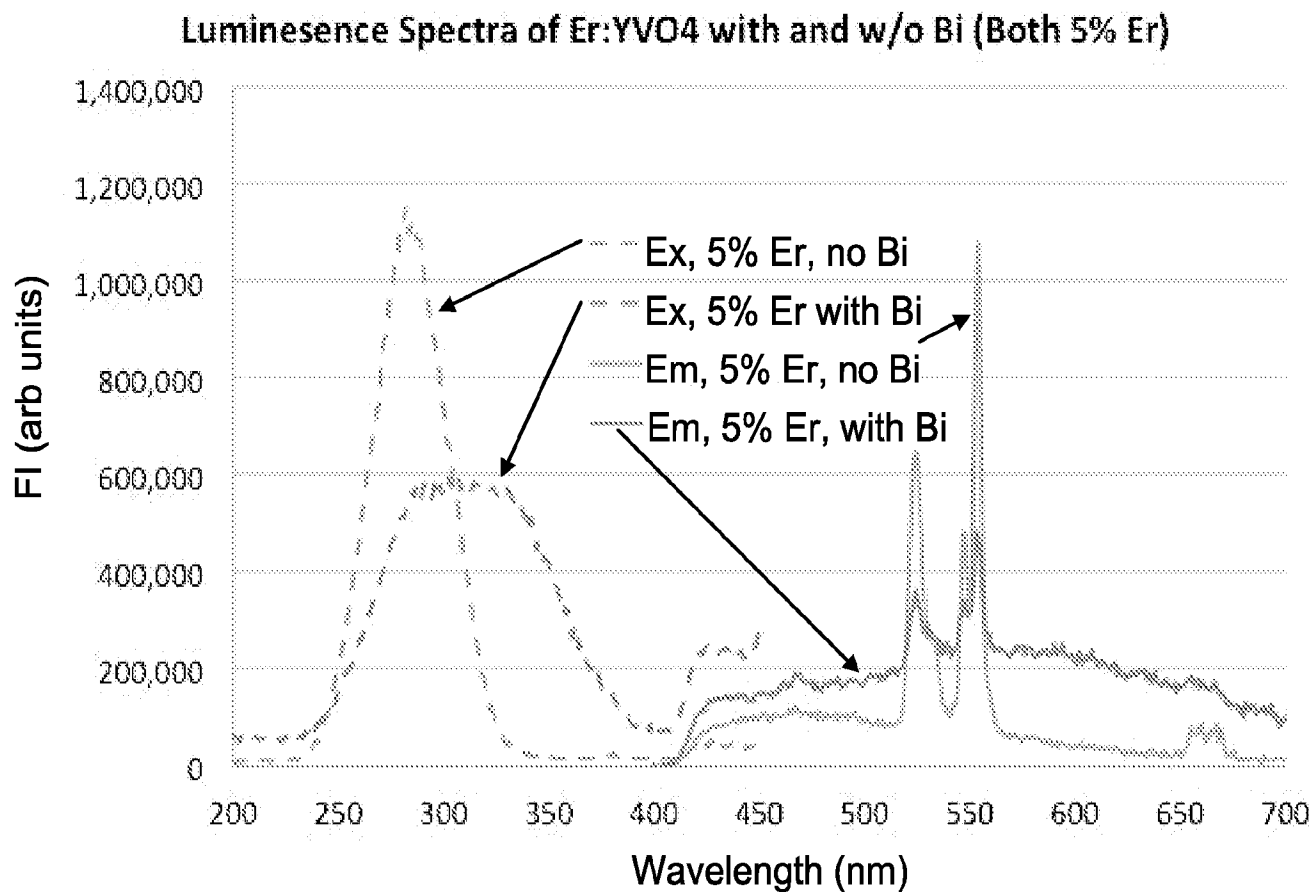


FIGURE 17



Ex (excitation wavelength)

Em (emission wavelength)

FIGURE 18

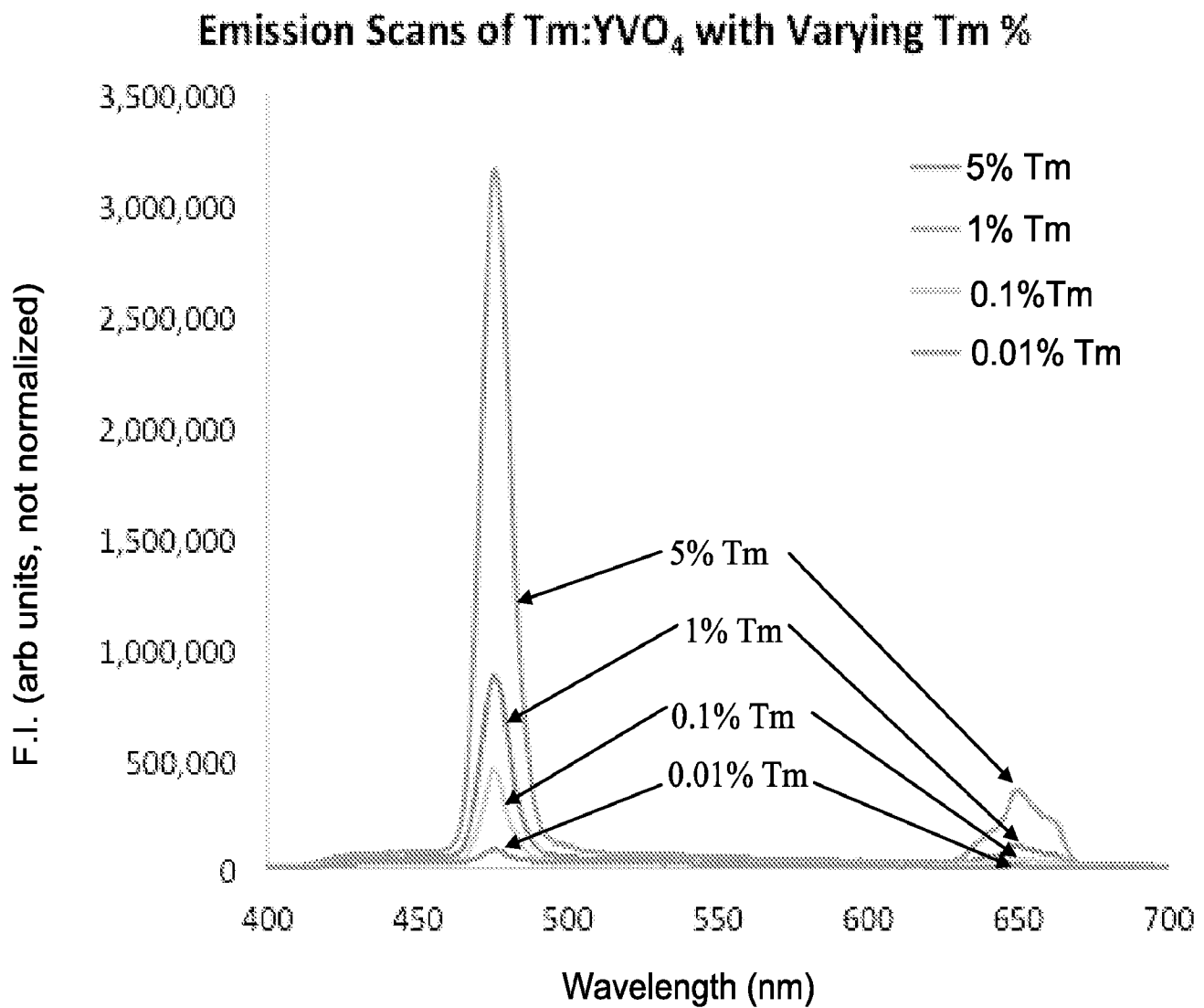


FIGURE 19

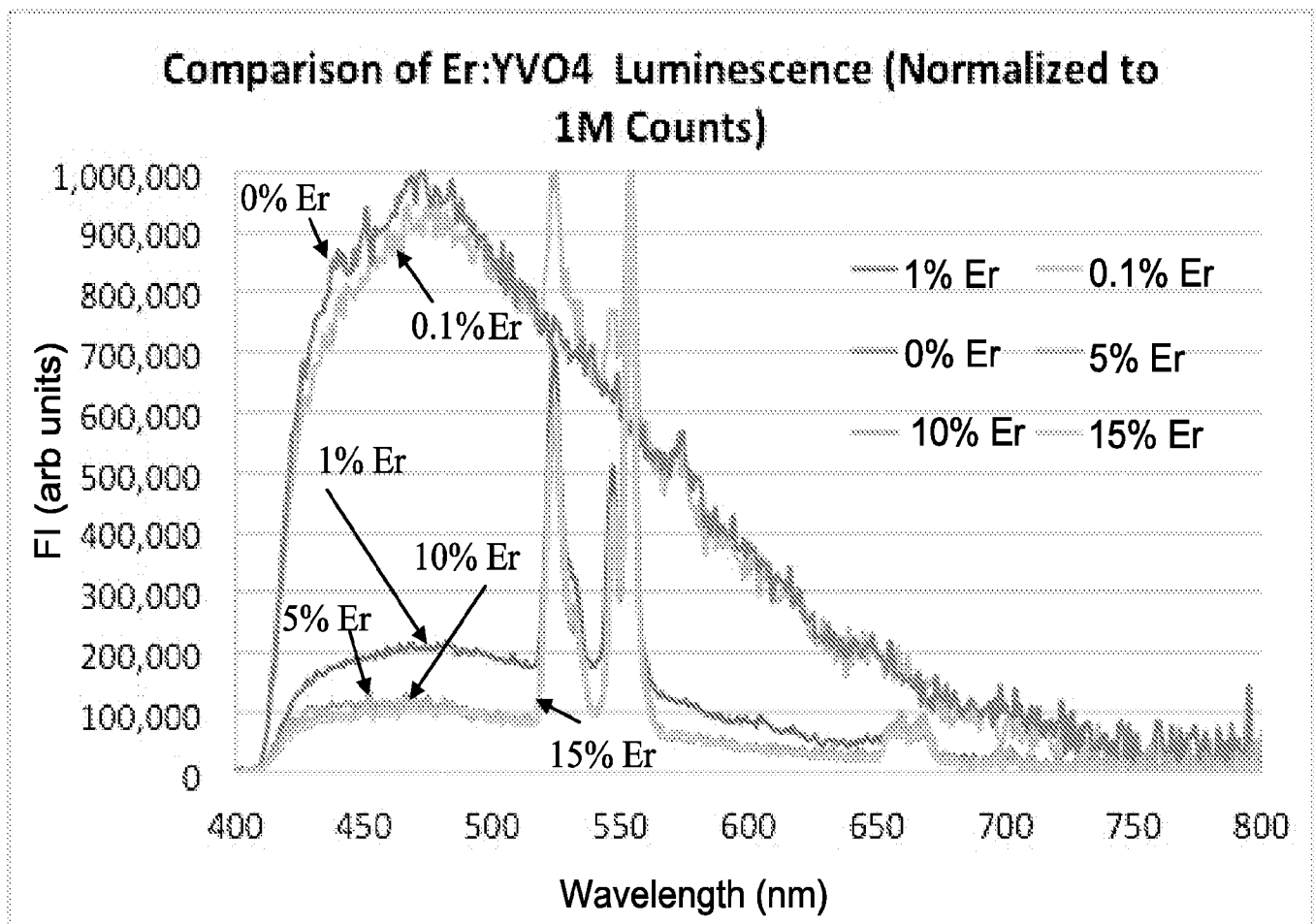


FIGURE 20

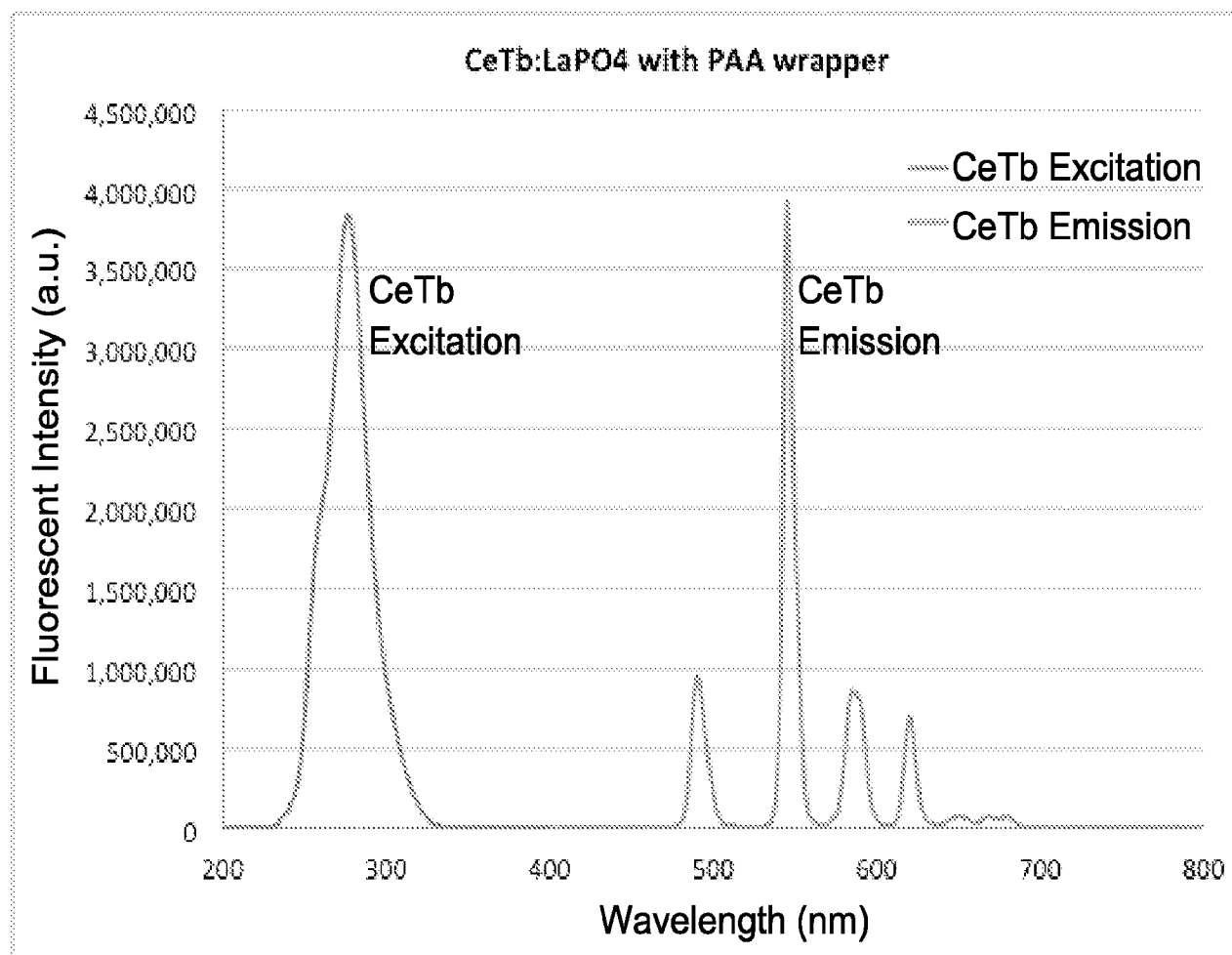


FIGURE 21

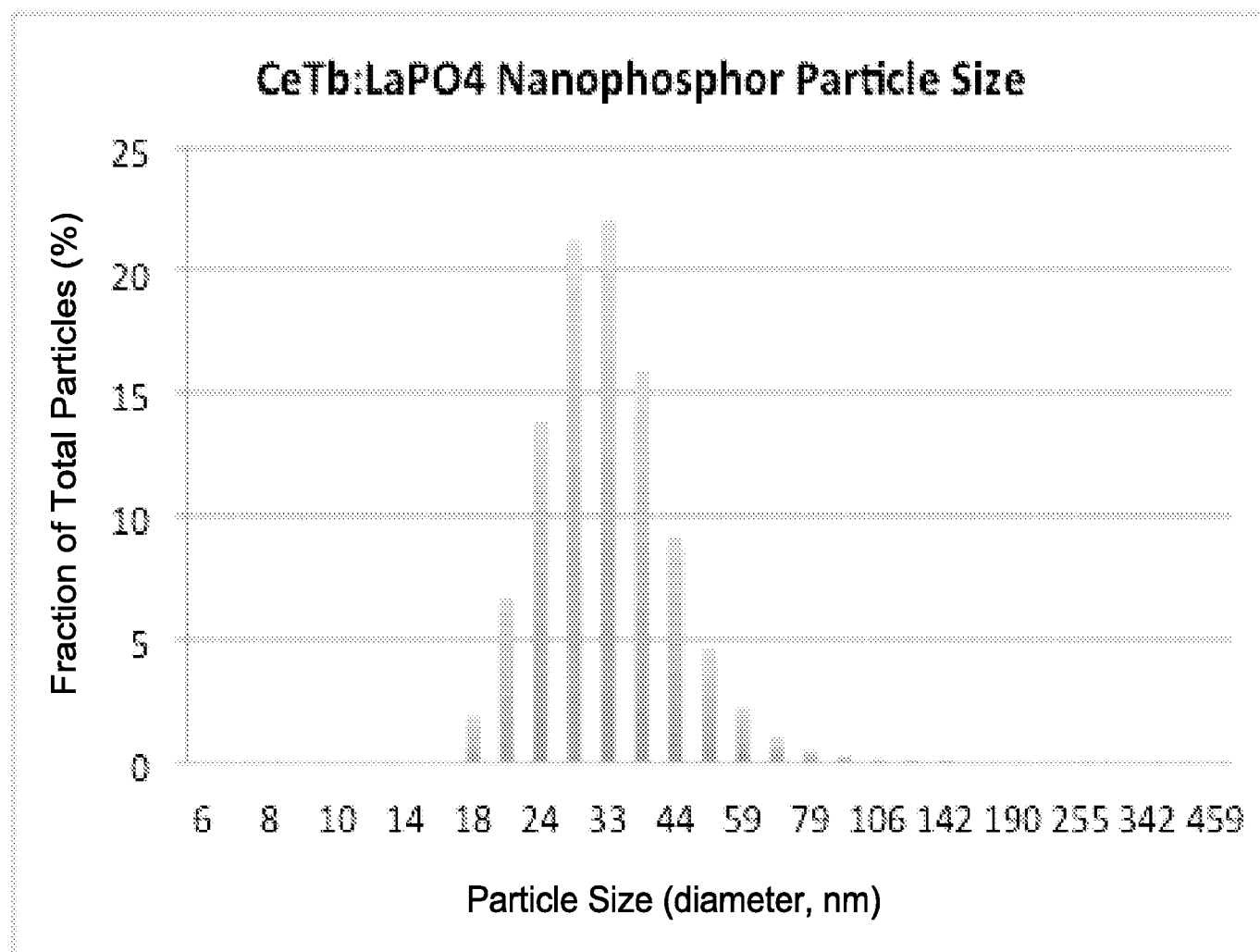


FIGURE 22

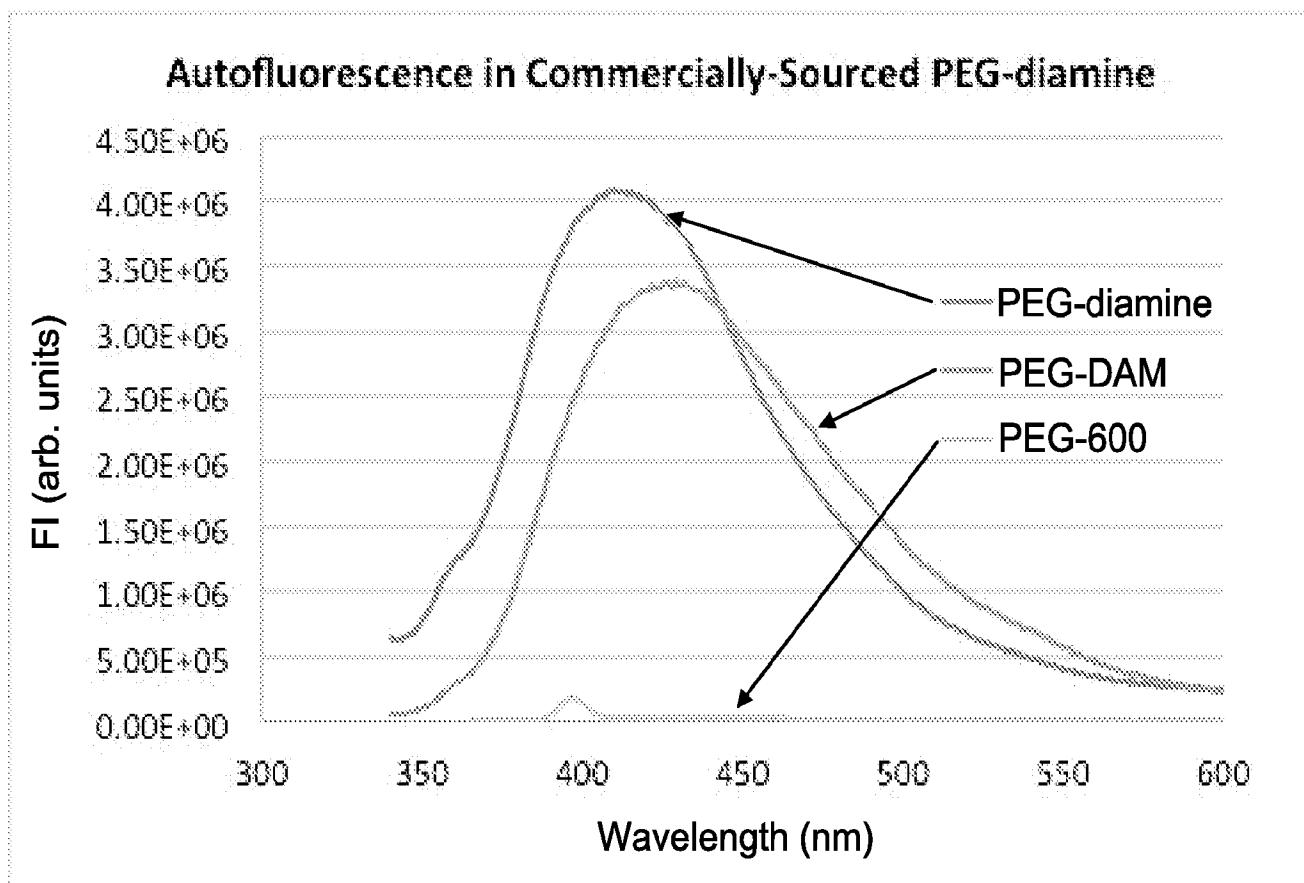
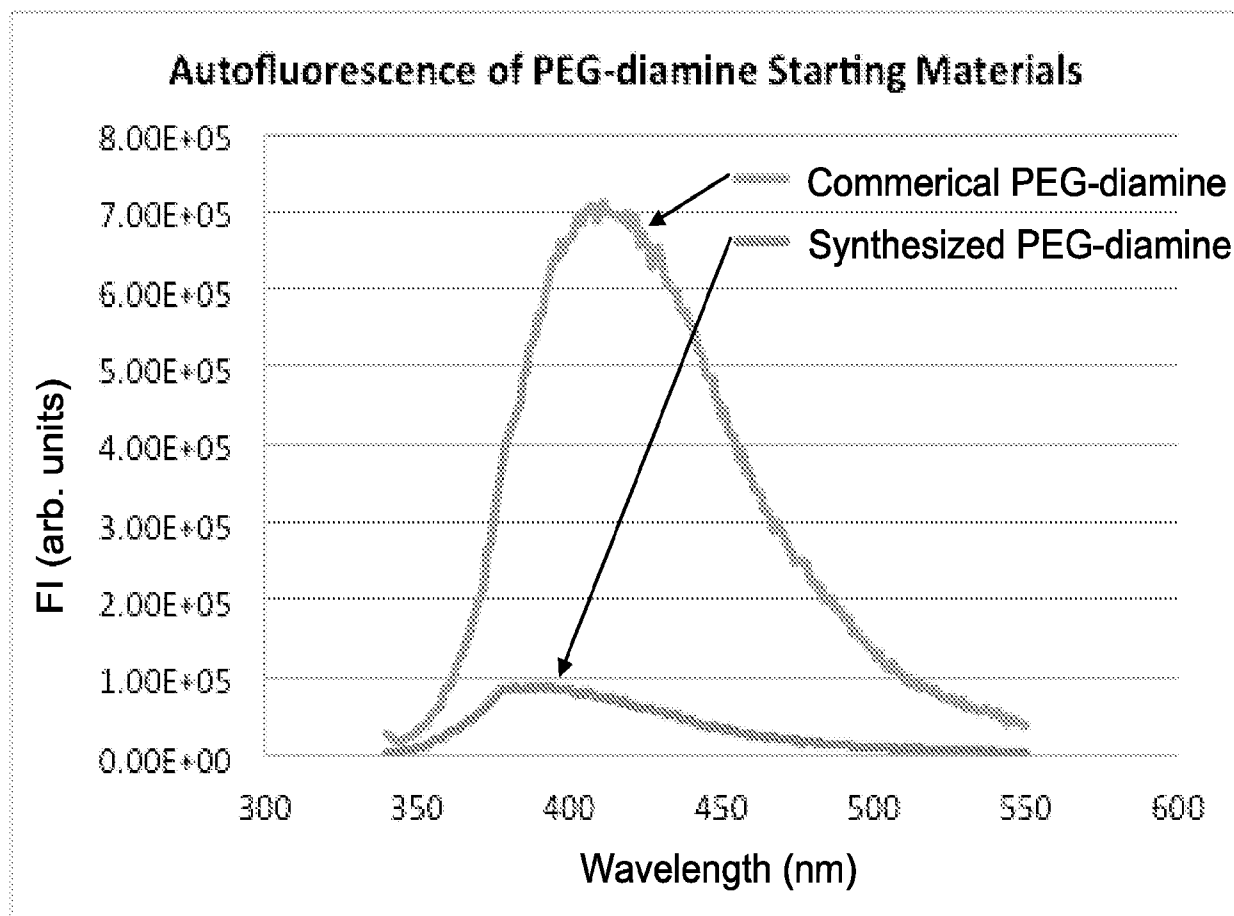
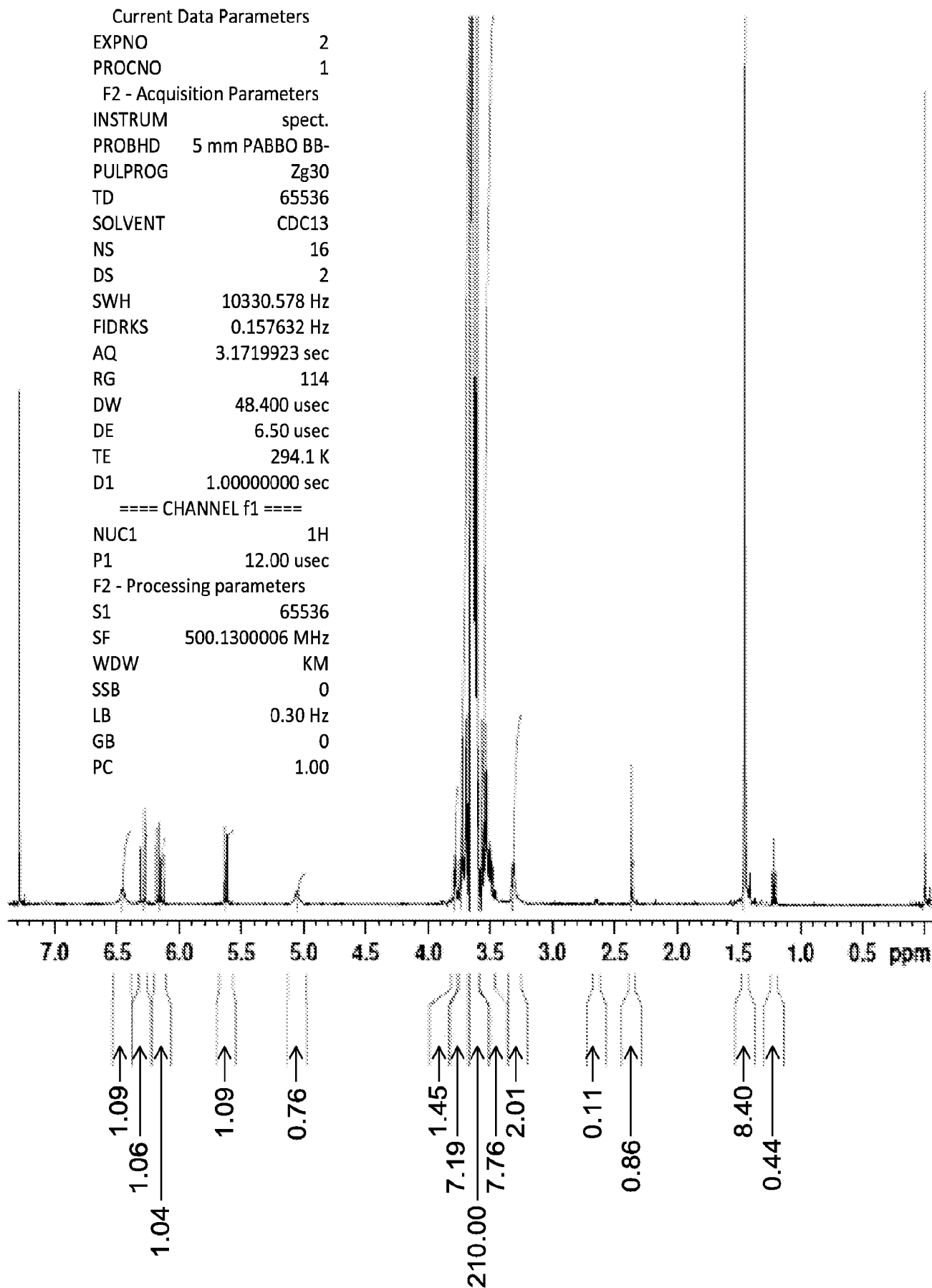


FIGURE 23



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FIGURE 24

SUBSTITUTE SHEET (RULE 26)