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Electronic Supplementary Information

Image-based feedback control for real-time sorting of microspheres in microfluidic devices

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Control mold fabrication

The control layer mask was printed as a 20 000 dpi transparency from the design drawing (CAD/Art Services, Bandon, OR) with all features scaled by a factor of 1.015, and the photolithography for the control mold was performed at the NIST Center for Nanoscale Science and Technology. For control mold fabrication, a virgin 100 mm silicon wafer (University Wafer, Boston, MA) was baked at 200 °C for 30 min and allowed to cool in air to room temperature. Approximately 5 mL of SU8-2010 was dispensed onto the wafer and processed following the manufacturers recommendation for 20 µm features. The wafer was then prebaked at 95 °C for 3 min on a hotplate and allowed to cool. Exposure was done using a Karl-Suss MA8 mask aligner at approximately 15 $mW \cdot cm^{-2}$ at 365 nm. The exposed wafer was postbaked and developed according the manufacturer's protocol. After visual inspection to ensure complete development, the wafer was heated to 200 °C for approximately 30 min to anneal any cracks in the photoresist and promote permanent adhesion to the wafer.

Fluorescent Bead Imaging

A custom filter cube (Semrock, Rochester, NY) was used for all fluorescence imaging, consisting of an excitation filter (FF01-615/24-21.8-D, 615/24 Brightline Bandpass Filter), a dichroic beamsplitter (FF648-Di01-22.0x29.0, 648 Brightline Dichroic Beansplitter), and an emission filter (FF01-660/13-23.3-D, 660 Brightline Bandpass Filter).

Device fabrication

PDMS devices were fabricated using GE RTV-615 (R.S. Hughes, Sunnyvale, CA) according to standard multilayer soft lithography fabrication protocols.[29] Briefly, the control layer was cast by adding RTV-615

mixed at a ratio of 5:1 base:catalyst over the control mold to a depth of approximately 3 mm, and the control layer PDMS was degassed under active vacuum for approximately 60 min. The flow layer was fabricated by spin-coating the flow mold with RTV-615 mixed at a ratio of 20:1 base:catalyst to a target height of 25 µm to 30 µm. The control and flow layers were then partially cured at 80 °C for 75 min. The control layer was then peeled from the control mold, the 635 µm diameter input holes for the control lines were punched using a manual press (Technical Innovations, Brazoria, TX), and the control layer was aligned to the flow layer. After baking overnight at 80 °C, the bonded device was excised from the flow mold, the 635 µm diameter input holes for all flow layer channels were punched as above, the devices were diced to size, and then plasma bonded to 50 mm x 75 mm glass slides using a Basic Plasma Cleaner (Harrick Plasma, Ithaca, NY).

Upon testing the device, we observed that PDMS shards created during the punching process would sporadically enter the device and become lodged within the sorter sieve valves, limiting future functionality. To mitigate this, four preventative measures were taken. First, a square post array with a 15 µm channel spacing was added within the flow input channels of the flow mold to block large pieces of debris from entering the sorter region. Second, new or minimally used punches were used during device fabrication. Third, the number of times that the connector pin was removed and inserted into the device was minimized. Fourth, the flow pressure used for the wash line was elevated relative to the bead input line, providing both additional force to drive any PDMS obstruction from the sorter region and increasing the speed at which beads could be delivered.

The complete channel layout is presented in Figure S1. In this figure, red lines represent channels on control layer, green lines represent flow channel features made of SU-8 at a height of approximately 15 µm, light blue lines represent SU-8 features at a target height of 3 µm, and dark blue lines represent features made of re-flowed positive photo resist

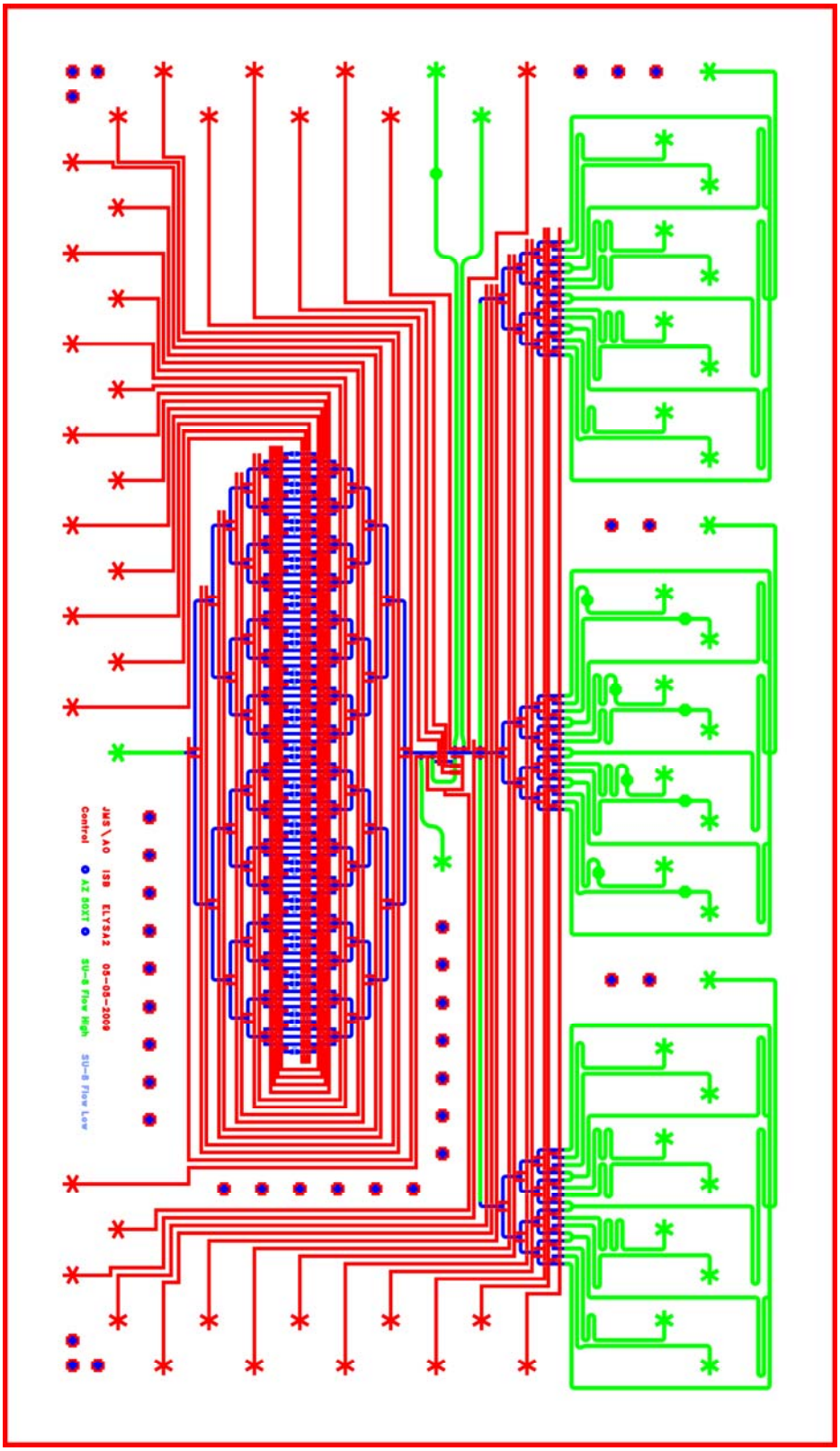

Figure S1: Complete device layout.

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Statistical Model

The fundamental property of a Poisson distribution is the mean value, λ, which physically is a function of bead concentration and flow rate. We sought to simulate the sorter to determine the value of λ that would minimize bead delivery time. Using MATLAB, we generated a 20 000-element array of random integers using a Poisson random number generator for a given value of λ. Each element in the array was a surrogate for the number of beads present within an image of the detection region. Each element of the array was processed to determine the sorting time necessary to complete the appropriate duty cycle based on the number of observed beads. Elements with a value of zero were assigned an elapsed time of 0.6 s since this was the experimentally determined camera frame rate. Non-zero elements were assigned the experimentally observed value of 12.8 s to simulate the total time required for the wash, counting, and delivery phases. The cumulative bead count was determined by summing the elements of the array that were less than or equal to two, and the total time required to deliver 512 beads was computed. The mean of the distribution, λ, was increased in increments of 0.05 from 0.05 to 2.0. The simulation was repeated 100 times at each λ to accommodate the statistically small number of events required in each simulation.

The central assumption of the model was that the number of beads arriving at the trap would be appropriately represented by a Poisson distribution. The validity of this assumption is supported by Q-Q plots (Figure S2) which provide a semi-quantitative method to assess how well the experimental data matched a theoretical Poisson distribution with the same mean. Each point on the graphs represents a single observation (y axis) and the corresponding value from a Poisson distribution with the same mean (x axis). In this plot, data that were Poisson distributed will lie along the line y = x.

We plotted the data for the observed bead counts over 11 experimental conditions and added a small amount of random noise to aid visualization. Qualitatively, the observed agreement supports the assumption of the model to first order. The percentage of the points that lay on the line y = x was calculated to range between 99.2% and 47.3%. We noted an apparent deviation from ideal Poisson behavior for those experimental conditions where higher values of the mean were observed. Most likely, these deviations represent a systematic error arising from a reduction in fluid flow through the sorter over time caused by debris build-up in the particle filter over multiple sorting runs on a single device. The planned analytical work will not suffer this same loss of performance, since each chip will only be used once.

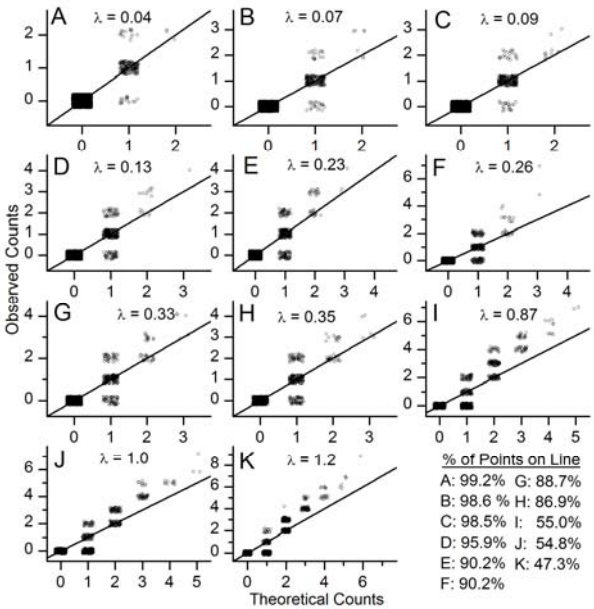

Fig. S2 Validation of model assumptions. Q-Q plots were constructed to assess whether the number of beads detected per image during sorting experiments was Poisson distributed. Due to the discrete nature of the count data small random noise has been added to each point (in both X and Y) to aid in visualization of the number of events displayed. The pressure, bead concentration and fit Poisson mean (λ) are: A) P = 7 kPa, [Beads] = 200 beads μL^{-1}, λ = 0.04 B) P = 21 kPa, [Beads] = 200 beads μL^{-1}, λ = 0.07 C) P = 10 kPa, [Beads] = 200 beads μL^{-1}, λ = 0.09 D) P = 7 kPa, [Beads] = 400 beads μL^{-1}, λ = 0.13 E) P = 10 kPa, [Beads] = 400 beads μL^{-1}, λ = 0.23 F) P = 7 kPa, [Beads] = 700 beads μL^{-1}, λ = 0.26 G) P = 21 kPa, [Beads] = 400 beads μL^{-1}, λ = 0.33 H) P = 10 kPa, [Beads] = 700 beads μL^{-1}, λ = 0.35 I) P = 28 kPa, [Beads] = 400 beads μL^{-1}, λ = 0.87 J) P = 21 kPa, [Beads] = 700 beads μL^{-1}, λ = 1.0 K) P = 28 kPa, [Beads] = 700 beads μL^{-1}, λ = 1.2. The percentage of points lying on the line y = x is taken as a qualitative measure of the "Poisson-ness" of the data.

Image Processing Scripts:

The MATLAB scripts used to process the images are included as supplementary information. The function of each script is described fully in the text. Each script is summarized below.

Trigger.m

This script provides the triggering function for microfludic real-time microsphere sorter. Triggering can occur only if the maximum intensity of the 3x3 median-filtered image is more than 500 counts above a reference intensity value. The microsphere sorter uses the mean background intensity from the first image of each sorting sequence as the reference intensity value. The function returns the number of regions detected by Otsu's thresholding method, but the microsphere sorter uses the output as a binary trigger only.

- Input:
  - img 16-bit grayscale image
  - ref reference intensity
- Output:
  - N number of beads in the image

Count.m

This script counts the number of fluorescent beads in an image. Initial detection of bead areas is done by thresholding the image by Otsu's method. Individual beads are detected from clumps of beads by a seeded watershed method. Local maxima of the original bead image are used as seeds.

- Input:
  - img 16-bit grayscale image
- Output:
  - N number of beads in the image

Error modes:

The first error mode was triggered if more than 5% of the pixels in the thresholded binary image were classified as foreground. This error code could be generated, for example, if an image included 30 or more beads within the trapping region (as might occur if beads sedimented in the input pin after a period of non-use). The second error mode was triggered if the largest segmented region was more than twice the area of the smallest. Sources of this error could include light scattering from a non-fluorescent particle in solution, bead size disparities, or unequal sized regions arising from the watershed segmentation algorithm. The third error mode was triggered if more than 5 small objects comprising less than 100 pixels were removed from the image. This error also addressed potential errors in the watershed segmentation. The fourth error was returned if any segmented region was below the roundness threshold as described above.

Flocmaxima.m (called by count.m)

This script finds the local maxima in an image. Only maxima within the area defined by a binary mask are considered.

- Input:
  - x image
  - ws window size for finding the local maxima
  - mask binary mask
- Output:
  - y binary image with the maxima marked as ones

Seededwatershedcut.m (called by count.m)

This script performs the segmentation for each identified bead-containing region using the seeded watershed transformation.

- Input:
  - x binary image
  - seeds seeds
- Output:
  - y binary image with split regions
  - wslines watershed lines

Formfactor.m (called by count.m)

This script calculates the form factor (or roundness), defined as $4*\pi*A/P^2$, where A is the area of an object and P is its perimeter.

- In:
  - x binary image
- Out:
  - y vector of form factors

Improvedcount.m

This script counts the number of fluorescent beads in an image. Initial detection of bead areas is done by thresholding the image by Otsu's method. Individual beads are detected from clumps of beads by a seeded watershed method. Local maxima of the original bead image are used as seeds. This improved version adds dithering to solve the problem of multiple local maxima with the same value within a single bead, see line 42.

- Input:
  - img 16-bit grayscale image
- Output:
  - N number of beads in the image

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Movie File

A movie was created by appending the successive images taken during a sorting experiment. The contrast of the images was adjusted to aid visualization. Each frame is branded with the elapsed time in seconds since the start of the sorting experiment, the accumulated number of beads, and the state of the sorting valves. Images that were processed with the counting script further annotated to show the resulting segmentation and are duplicated in the movie to be displayed as 15 consecutive frames. The flow pressure was 7 kPa and the bead concentration was 400 beads μL^{-1}, corresponding to the condition denoted by the filled black triangles in Figure 8. Only the first 64 accumulated beads are shown.