**Supplementary Figure 1**  Synthesis of 3′-O-methyl-5(6)-carboxyfluorescein-monophosphate

**Supplementary Figure 2**  Synthesis of dG4P-TM-3′-O-methyl-5(6)-carboxyfluorescein

**Supplementary Figure 3**  Vacuum-based sealing of PDMS microreactor array

**Supplementary Figure 4**  Sample sequencing data processing

**Supplementary Figure 5**  Demonstration of fluorogenic pyrosequencing in high density microreactor arrays.

**Supplementary Figure 6**  Super-Poissonian loading of PDMS microreactors

**Supplementary Table 1**  DNA Sequences used in fluorogenic pyrosequencing experiments

**Supplementary Note 1**  Synthesis of terminal phosphate-labeled fluorogenic nucleotides

**Supplementary Note 2**  The scalability of fluorogenic pyrosequencing

**Supplementary Note 3**  Potential for microfluidic integration of fluorogenic pyrosequencing
Synthesis of 3'-O-methyl-5(6)-carboxyfluorescein-monophosphate (VI) from 5(6)-carboxyfluorescein (see Supplementary Note 1 for details).
Synthesis of dG4P-δ-3′-O-methyl-5(6)-carboxyfluorescein from 3′-O-methyl-5(6)-carboxyfluorescein-monophosphate (VI). This same synthetic scheme is applied to all four nucleotides to generate fluorogenic nucleotide tetraphosphates (see Supplementary Note 1 for details).
Supplementary Figure 3

Vacuum-based sealing of PDMS microreactor array (not to scale). After loading the microreactors with the appropriate reaction mixture (top left), a pulse of nitrogen gas is injected into the flow cell, dewetting the two sealing surfaces of the flow cell and leaving the liquid inside of the microreactors unaffected (top right). When vacuum is applied to the flow cell, the PDMS slab containing the microreactor array deforms and the microreactor array is brought into contact with the PDMS-coated coverslip (bottom right), causing the microreactors to seal (bottom left). Releasing the vacuum causes the microreactor array to rapidly unseal.
Supplementary Figure 4

Sample application of the data processing algorithm to actual sequencing data (see Supplementary Note 6 for details). A) A single exponential is fit to the set of initial single base calls from a normalized, background-subtracted intensity trace. B) Result of first exponential correction obtained by multiplying the trace in A by the inverse of the exponential fit in A. C) A single exponential fit to the second set of single base calls. The second set of single base calls is computed by thresholding the
trace in B. D) Result of second exponential correction obtained by multiplying the trace in A by the inverse of the exponential fit in C. E) Normalized intensity trace obtained by dividing the trace in D by the mean signal for a single base call. F) Final base calling trace obtained by applying thresholds to the trace in E.
Demonstration of fluorogenic pyrosequencing in high density microreactor arrays. A) Fluorescence image of a microreactor array containing ~5 μm diameter microreactors taken after a cycle of fluorogenic pyrosequencing. The data presented in Figs. 3 and 4 of the main text were obtained using this array. B) Fluorescence image of an array containing ~2.5 μm diameter microreactors taken after a cycle of fluorogenic pyrosequencing under similar conditions. The microreactor density for this array is >2.3x higher than in the array shown in A). C) Representative homopolymer ladder sequencing trace obtained from the high density array in B). Only ~2,000 DNA template copies per microreactor were required for the data shown in B) and C). These data show that fluorogenic pyrosequencing can be scaled by increasing the microreactor array density.
Bright field transmission image of a bead-containing PDMS microreactor array demonstrating super-Poissonian loading. Streptavidin-coated superparamagnetic beads (Compel Beads with a mean diameter of 2.8 μm, BangsLabs) were loaded and captured in biotinylated PDMS microreactors (diameter of ~5 μm) that were prepared using the surface chemistry described in the Online Methods section. Because the bead diameter is greater than the microreactor radius, a single microreactor contains either one or zero beads(s), and a single bead loading efficiency of ~80% is achieved. This is significantly greater than that expected from the Poisson distribution.
**Supplementary Table 1: DNA Sequences used in Fluorogenic Pyrosequencing Experiments**

<table>
<thead>
<tr>
<th>SEQUENCE NAME</th>
<th>SEQUENCE (5’ to 3’)</th>
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<tbody>
<tr>
<td>Dual-biotinylated primer</td>
<td>CCTATCCCTGTGTGCCTGCCTATCCCGTTGCGTGTCTCAG</td>
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<tr>
<td>Homopolymer Ladder template</td>
<td>CCCCCCCCCCCTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTGAGACACGCAACGGGATAGG</td>
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<tr>
<td>R1 quasi-random template</td>
<td>ATGTGTATTAATGAGTACCCCTGAGACACCTCCATCTATTCTTTCGGGCTTAGCT</td>
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<td>R2 quasi-random template</td>
<td>ACTATGAGAGTGTTCCACACACCGCGTTGCCCTACACTGCTGCCGACTCAATGTCT</td>
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<tr>
<td>R3 quasi-random template</td>
<td>GAGAATACACTTCTTAATGTATATCACTATGACGCGCCTGACTCTCTGACTGAGACACGCAACGGGATAGG</td>
</tr>
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</table>

**Supplementary Note 1: Synthesis of Terminal Phosphate-Labeled Fluorogenic Nucleotides**

*Preparation of 3’-O-methyl-5(6)-carboxyfluorescein-monophosphate VI*

Supplementary Fig. 1 shows a synthetic scheme for the preparation of 3’-O-Methyl-5(6)-carboxyfluorescein monophosphate, which was conjugated to all four dNTPs to generate a full set of fluorogenic nucleotide tetraphosphate substrates.

5(6)-carboxyfluorescein (5/6-FAM) I (1.1 g, 2.90 mmol) was dissolved in methanol (60 ml), then H₂SO₄ (conc. 2 ml) was added dropwise under stirring. The mixture was heated under reflux for 10 h. After the reaction completed, the resulting solution was concentrated, diluted with dichloromethane, and neutralized with saturated sodium bicarbonate to pH~6. The aqueous phase was extracted with dichloromethane and the combined organic phase was washed with water and brine and dried over sodium sulfate. After evaporation of the dichloromethane, the residue was purified by silica gel chromatography to afford compound II (703 mg, 60%, 5/6 mixture). H1 NMR (300 MHz, CDCl₃): δ 3.61 (s, 3H-5FAM), 3.64 (s, 3H-6FAM), 3.94 (s, 3H-6FAM), 4.01 (s, 3H-5FAM). 6.90-7.05 (m, 6H), 7.21 (s, 4H), 7.45 (d, J = 7.8 Hz, 2H-5FAM), 7.97 (s, 1H-6FAM), 8.33 (s, 2H-6FAM), 8.49 (dd, J = 1.5Hz, 1.5Hz, 1H-5FAM).

The obtained diester compound II was dissolved in DMF (25 ml), then MeI (604 mg, 4.25 mmol) and cesium carbonate (831 mg, 2.55 mmol) were added sequentially. The reaction mixture was stirred at room temperature and monitored by TLC. After 2h the reaction completed and DMF was removed by vacuum pump. The residue was diluted with dichloromethane (50 ml), washed with 2N HCl and brine, and dried over magnesium sulfate. The organic layer was concentrated *in vacuo* to afford compound III, which was dissolved in methanol (60 ml) for subsequent usage without further purification.

To hydrolyze compound III, 2N NaOH (5.8 ml) was added to a methanol solution of III from the above step. The mixture was then stirred for 8 h at room temperature. Upon completion of the reaction, the methanol was evaporated, and the aqueous residue was acidified with 2N HCl. The resulting precipitate was collected by filtration and further purified by silica gel chromatography to afford 3’-O-methyl-5(6)-carboxyfluorescein IV (488 mg, 72%, 5/6 mixture). 1H 1NMR (300 MHz, CD₂OD) : δ 3.83 (s, 3H), 3.84(s, 3H), 6.53-6.62 (m, 3H), 6.65-6.72 (m, 5H), 6.84-6.86 (m, 2H), 7.28 (s, 1H), 7.31

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(s, 1H), 7.73 (s, 1H), 8.09 (s, 1H), 8.31 (dd, \( J = 1.5 \text{Hz}, 7.8 \text{ Hz}, 1\text{H-6FAM} \)), 8.37 (dd, \( J = 2.0 \text{Hz}, 7.8 \text{ Hz}, 1\text{H-5FAM} \)), 8.58 (s, 1H-5FAM).

3’-O-methyl-5(6)-carboxyfluorescein IV (128 mg, 0.33 mmol) was dissolved in 10 mL of chloroform, then triethyl amine (333 mg, 3.3 mmol) and 1-H tetrazole (231 mg, 3.3 mmol) were added sequentially. After stirring for 10 min, dibenzyl diisopropylphosphoramidite (252 mg, 0.67 mmol) was added, and the reaction mixture was stirred for 1h at room temperature. TLC analysis (hexane / ethyl acetate / methanol = 10/10/1) revealed that the starting material was consumed. The reaction was cooled to 0 °C, and MCPBA (200 mg) was added. The reaction was then stirred for 30 min to oxidize the phosphite intermediate. Then, the mixture was diluted with dichloromethane (50 ml) and washed with sodium sulfite solution (10 ml, 1.5 M in water). After separation, the organic phase was washed with 2N HCl, water and brine, and dried over sodium sulfate. After evaporation of the solvent, the residue was purified by silica gel chromatography to afford compound V (150 mg, 70%, 5/6 mixture). 1H NMR (300 MHz, CDCl₃): \( \delta 3.78 (s, 3H), 3.84 (s, 3H), 5.11 (d, J = 2.8 \text{ Hz}, 2H), 5.15 (d, J = 2.8 \text{ Hz}, 2H), 6.64-6.70 (m, 3H), 6.77-6.82 (m, 3H), 7.05-7.08 (m, 2H), 7.21 (s, 1H), 7.24 (s, 1H), 7.83 (s, 1H), 8.10 (d, \( J = 4.8 \text{ Hz}, 1H \)), 8.31-8.37 (m, 2H), 8.72 (s, 1H).

Compound V (150 mg, 0.23 mmol) was dissolved in 10 mL of anhydrous methanol at -5°C. Then, 10% Pd/C (40 mg) was added to the solution, and the suspension was stirred for 30 min under 1 atm H₂. TLC showed no remaining starting material (eluting with Hexane/Ethyl acetate/MeOH = 10/10/2). The mixture was filtered through a short celite plug (prewashed with MeOH) to remove palladium. After removal of the solvent, the 3′-O-methyl-5(6)-carboxyfluorescein monophosphate VI was obtained as slightly yellowish oil (75 mg, 70%), which can be further purified by reverse phase HPLC (Agilent). MS (ES): M+H = 471.23 (calc 470.04).

The purified 3′-O-methyl-5(6)-carboxyfluorescein monophosphate VI was coevaporated with anhydrous DMF (2 mL) twice and then dissolved in 1.7 mL anhydrous DMF. Tributylamine (60 mg, 0.32 mmol) was added and the resulting 3′-O-methyl-5(6)-carboxyfluorescein monophosphate tributylammonium salt DMF solution was kept in a -20°C freezer for further usage.

Synthesis of dG4P-δ-3′-O-methyl-5(6)-carboxyfluorescein

3′-O-methyl-5(6)-carboxyfluorescein monophosphate VI was conjugated to dGTP using the synthetic scheme depicted in Supplementary Fig. 2.

2′-deoxyguanosine-5′-triphosphate (dGTP) disodium salt (6.8 mg, 14.0 µmol) was converted to the tributylammonium salt by treatment with ion-exchange resin (BioRad AG-50W-XB) and tributylamine. After removal of water, the obtained tributylammonium salt was coevaporated with anhydrous DMF (2 mL) twice and then dissolved in 0.3 mL anhydrous DMF. To the solution, carbonyldiimidazole (CDI, 11.3 mg, 70 µmol, 5 eq) was added, and the mixture was stirred at room temperature for 12 h (monitored by LCMS). After that, MeOH (3.2 µL) was added, and the solution stirred for 0.5 h to destroy the excess CDI. The 3′-O-methyl-5(6)-carboxyfluorescein monophosphate VI tributylammonium salt (28 µmol) DMF solution (0.3 ml) from the previous step was transferred into the reaction by syringe, and MgBr₂ (28 mg, 112 µmol, 8 eq) in DMF was also added at the same time. The mixture was stirred for 3 days at room temperature. Then, the reaction mixture was concentrated, diluted with water, and purified on a Hi-Trap Q-HP 5 mL anion exchange column (GE Healthcare) using a step gradient: first water then 50 mM PIPES/1 M NaCl buffer. Fractions containing the product were collected, and shrimp alkaline phosphatase (USB) was added to destroy the unreacted
monophosphate. After 30 min, the solution was concentrated and repurified by HPLC (Agilent) on an Xterra RP C-18 19-150 mm column (Waters) using 0-30% acetonitrile in 50 mM triethylammonium acetate buffer (PH 7), flow rate 5 ml/min. Fractions containing pure product were concentrated and further purified by a HiTrap Q-HP 1 mL anion exchange column (GE Healthcare) to give a 0.7 mL of a 1.5 mM solution. UV/VIS $\lambda_{\text{max}} = 271$ nm and 422 nm. MS (MALDI-TOF): M+1 = 960.71 (calc 959.03).

**Preparation of dC4P-$\delta$-3\textsuperscript{-}'O-methyl-5(6)-carboxyfluorescein**

dC4P-$\delta$-3\textsuperscript{-}'O-methyl-5(6)-carboxyfluorescein was synthesized from dCTP by following the similar procedure as above. UV/VIS $\lambda_{\text{max}} = 273$ nm and 434 nm. MS (MALDI-TOF): M-1 = 918.36 (calc 919.02)

**Preparation of dA4P-$\delta$-3\textsuperscript{-}'O-methyl-5(6)-carboxyfluorescein**

dA4P-$\delta$-3\textsuperscript{-}'O-methyl-5(6)-carboxyfluorescein was synthesized from dATP by following the similar procedure as above. UV/VIS $\lambda_{\text{max}} = 261$ nm and 439 nm. MS (MALDI-TOF): M+1 = 943.57 (calc 942.04)

**Preparation of 3\textsuperscript{-}' dT4P-$\delta$-3\textsuperscript{-}'O-methyl-5(6)-carboxyfluorescein**

dT4P-$\delta$-3\textsuperscript{-}'O-methyl-5(6)-carboxyfluorescein was synthesized from dTTP by following the similar procedure as above. UV/VIS $\lambda_{\text{max}} = 272$ nm and 432 nm. MS (MALDI-TOF): M+1 = 935.84 (calc 934.02)

**Supplementary Note 2: The Scalability of Fluorogenic Pyrosequencing**

Fluorogenic pyrosequencing has two significant advantages over conventional pyrosequencing that are critical for future high-throughput applications. First, about $10^3$ times fewer copies of DNA per reaction center are required than in pyrosequencing. Hence, in fluorogenic pyrosequencing, each sequencing reaction takes up a much smaller surface area than in conventional pyrosequencing. In all of the sequencing experiments described here, we used 1 $\mu$m diameter beads in either ~5 $\mu$m diameter microreactors (Figs 3 and 4) or ~2.5 $\mu$m diameter microreactors (Supplementary Figs. 5B-C). However, in the pyrosequencing platform commercialized by 454/Roche, bead diameters are in the tens of microns range which sets an upper limit on microreactor packing density. For example, the 454 GS FLX Titanium PicoTiterPlate has a total area of 5,250 mm$^2$ and yields about one million reads. An equivalently-sized hexagonally close-packed array of our ~5 $\mu$m diameter microreactors (with a center-to-center distance of 7.5 $\mu$m) would contain >100 million microreactors. An array made from the higher density pattern shown in Supplementary Fig. 5B would contain >250 million microreactors. Even if such an array were loaded according to the Poisson distribution (without taking advantage of super-Poissonian loading as shown in Supplementary Fig. 6), a ~100x throughput advantage could be expected over the PicoTiterPlate.

The second advantage is that simultaneous, real-time monitoring of all reaction centers is not required in fluorogenic pyrosequencing. This aspect has two potential benefits with respect to scalability. First, the detector does not have to be the same size as the sample. A very large, dense array, such as that described above, could be mechanically scanned relative to a detector. Second, sequencing reads could be acquired from multiple flow cells in a single run. Each sequencing cycle has multiple stages (e.g.
polymerase incubation, washing, and imaging), and different flow cells could undergo different stages of a sequencing cycle simultaneously. In the simplest case, a sequencing system could have two flow cells and one flow cell could be washed and incubated with the TPLFN reaction mixture while the other is imaged.

Supplementary Note 3: Potential for Microfluidic Integration of Fluorogenic Pyrosequencing

Fluorogenic pyrosequencing is demonstrated here using PDMS, a material that has been widely used for the construction of microfluidic devices (McDonald JC, Whitesides GM. Poly(dimethylsiloxane) as a material for fabricating microfluidic devices. (2002) Acc. Chem. Res. 35 (7) 491-499). The use of PDMS as the “native” material for fluorogenic pyrosequencing opens the door to the integration of sequencing with microfluidic devices for sample processing. A device capable of taking raw biomaterials, extracting nucleic acids, generating and quantifying a library of nucleic acid fragments suitable for clonal amplification followed by sequencing would require a number of demonstrated capabilities of microfluidic devices to be integrated in a single instrument. The outlines of such an instrument have also been described elsewhere (Coupland P. Microfluidics for the upstream pipeline of DNA sequencing – a worthy application? (2010) Lab Chip 10, 544–547). First, one of the many demonstrated devices capable of extracting and purifying nucleic acids would be used to generate DNA fragments (Wen et al. Purification of Nucleic Acids in Microfluidic Devices (2008) Anal. Chem 80, 6472-6479; Kim et al. Microfluidic sample preparation: cell lysis and nucleic acid purification.(2009) Integr. Biol., 1, 574–586). Next, these nucleic acid fragments would be ligated to adapters, generating a library for sequencing. Microfluidic library preparation for targeted enrichment application of massively parallel sequencing has already been demonstrated. The Fluidigm Access Array system allows simultaneous adapter integration and amplification of specific target sequences using microfluidic PCR (Anderson M et al. High-throughput microfluidic preparation of targeted resequencing libraries using the Access Array system. (2010) J. Biomol. Tech. 21, S26).

One of many available on-chip clonal amplification technologies such as PCR (Zhang C. et al PCR microfluidic devices for DNA amplification (2006) Biotechnology Advances 24, 243-284), bridge PCR, solid-state rolling circle amplification, or droplet-based PCR (Tewhey et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. (2010) Nat. Biotech. 27(11) 1025-1031) can be used to generate clonal amplicons from single molecules of DNA in this DNA library. This amplification could occur on a bead which is then transferred to the microreactors for flurogenic pyrosequencing, or the amplicons might be generated directly on the microreactor surface, primed, and sequenced directly.

Microfluidic sample processing has already been shown to impart a number of advantages upstream of massively parallel sequencing workflows. For example, the primary reason why sequencing libraries require large amounts of input DNA is the need for accurate quantification. A microfluidic platform for digital PCR has been shown to dramatically reduce the amount of sample required for Illumina and 454 sequencing by providing accurate quantification of small DNA samples (White et al. Digital PCR provides sensitive and absolute quantification for high throughput sequencing. (2009) BMC Genomics, 10, 116). In addition, microfluidic devices have recently been applied to single cell sequencing, and capabilities ranging from on-chip whole genome amplification to single chromosome sequestration and amplification have been demonstrated (Fan H.C. et al. Whole genome molecular haplotyping of single cells. (2011) Nat. Biotech., 29, 51-57; Blainey et al. Genome of a low-salinity ammonia-oxidizing archaeon determined by single-cell and metagenomic analysis. (2011) PloS One, 6, e16626).