Chapter 3

Quantitating the Concentration of Py-Im Polyamide-Fluorescein Conjugates in Live Cells

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Abstract

Quantitative fluorescence-based methods have been developed to determine the nuclear concentration of polyamide-fluorescein conjugates in cell culture. Confocal laser scanning microscopy and flow cytometry techniques are utilized to plot calibration curves, from which the nuclear concentration can be interpolated. Upon treatment with polyamide, the concentration in the nucleus of live HeLa cells is calculated to be between 0.1-0.5 μM, which is significantly lower than the 2 μM dosage concentration. In contrast, the observed nuclear concentration in U251 cells is closer to the dosage concentration, indicating a cell line-specific increase in uptake for this class of compounds. Although confocal microscopy and flow cytometry generate disparate values, taken together these experiments suggest that the polyamide concentration inside the cell nucleus is lower than it is outside the cell.

3.1. Introduction

Hairpin pyrrole-imidazole polyamides are synthetic ligands that target predetermined DNA sequences with affinities comparable to those of naturally occurring DNA-binding proteins.[1,2] These cell-permeable small molecules have been shown to localize to the nucleus of living cells[3-7] and regulate endogenous gene expression.[8-12] Polyamides selectively bind in the minor groove of DNA according to a set of "pairing rules," where each heterocyclic ring pair targets a specific Watson-Crick base pair.[1,2] The antiparallel pairing of *N*-methylpyrrole (Py) and *N*-methylimidazole (Im) aromatic rings targets the C•G base pair, while Im/Py discriminates G•C.[13-16] The Py/Py pair recognizes A•T and T•A, while chlorothiophene can be paired with Py to distinguish T•A at the N-terminus.[17]

Polyamide-small molecule conjugates have been utilized in a number of applications.[18-21] In particular, with laser microscopy it is possible to visualize the uptake of polyamide-fluorophore conjugates as they traffic unaided to the nucleus of live cells.[3-6] This advance has helped to usher in an era of gene regulation.[8-11] Traditionally, we have used a qualitative scoring system to rate the extent of nuclear localization in cell culture.[5,6] Upon incubation with polyamide and direct imaging of cells, the fluorescence intensity in the nucleus is compared to that in the medium. Positive scores are assigned when the nuclear staining exceeds that of the medium. The degree of nuclear localization varies across polyamide design, fluorophore selection, and cell type.[3-6] Quantitating the polyamide concentration within the cell nucleus would be an improvement over the current "yes/no" rating, thus creating a digital readout of cellular uptake.

Our quantitative approach utilizes two different fluorescent imaging technologies. First, with confocal laser scanning microscopy, we can compare images of cells to images of calibration standards containing known polyamide concentrations, then interpolate to determine the nuclear concentration in the cell. Next, we can corroborate this value with additional evidence from flow cytometry. Here, the fluorescence of live cells is compared to a calibration curve built using beads functionalized with known amounts of fluorophore,

yielding the interpolated number of fluorophores per cell. Taken together, these values provide a numerical range that is not apparent from previous qualitative experiments. In this study, we investigate the nuclear concentration of two polyamide-fluorescein conjugates in two cell lines using confocal laser scanning microscopy and flow cytometry.

3.2. Polyamide synthesis

For these experiments, we wanted to examine polyamide-fluorescein conjugates that traffic strongly to the nucleus of live cells (Figure 3.1). Polyamide **1** specifically targets the DNA sequence 5'-WTWCGW-3' with an equilibrium association constant of 3.8×10^{9} M^{-1}, while polyamide **2** binds 5'-WGGWCW-3' with an affinity of 6.3×10^{9} M^{-1} (Figure 3.2). For these molecules synthesized with Boc-β-Ala-PAM resin, the 1,3-diaminopropane (C_3) linker is used to connect the polyamide and fluorescein moiety.

Polyamides **1** and **2** show positive nuclear localization in HeLa and U251 cell culture by confocal laser scanning microscopy (Figure 3.1). In each of these images, the nuclear staining exceeds that of the medium, and all are qualitatively rated as + +.[5,6] Furthermore, conjugate **1** binds to the hypoxia response element (HRE) and inhibits vascular endothelial growth factor (VEGF) expression in HeLa cells by quantitative RT-PCR, while the mismatch control compound **2** does not downregulate gene expression (Figure 3.2).[8-10]

3.3. Steady-state fluorimetry

Fluorescence enhancement is observed when polyamide-fluorophore conjugates bind DNA.[22-25] When in solution, the fluorescence of the conjugate is largely quenched, but, upon DNA binding, sequestration of the polyamide in the minor groove restores fluorescence. The fluorescence enhancements of polyamides **1** and **2** were 14- and 36-fold, respectively, as determined by steady-state fluorimetry (Figure 3.3). The fluorescent nuclei observed in confocal microscopy images appear much brighter than the background medium. Does this mean that the nuclear polyamide concentration is very high, or does the fluorescence enhancement dominate?

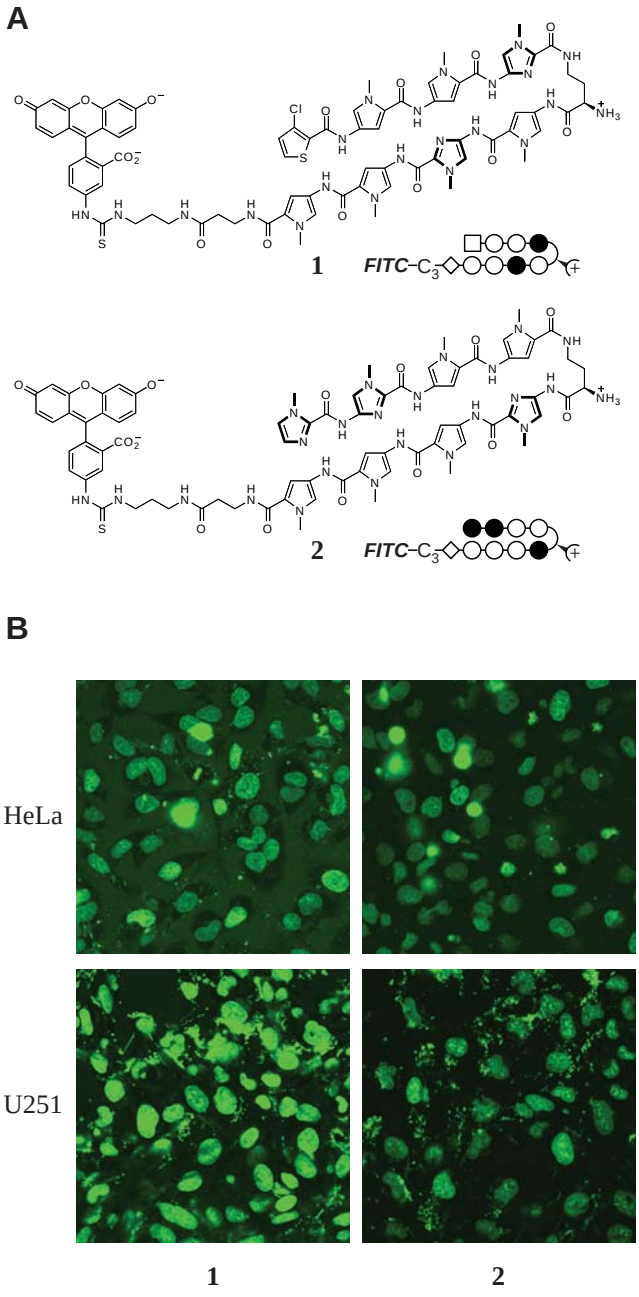

Figure 3.1. (A) Structures of polyamide-fluorescein conjugates **1** and **2**. Imidazole and pyrrole are shown as filled and non-filled circles, respectively; chlorothiophene is shown as a square; β-alanine is shown as a diamond; the 1,3-diaminopropane linker is shown as "C_3"; and the chiral diaminobutyric acid turn residue is shown as a semicircle connecting the two subunits linked to a half-circle with a plus. (B) Confocal laser scanning microscopy images of polyamide-fluorescein conjugates **1** and **2** in HeLa and U251 cell lines. Cells were incubated with 2 μM polyamide for 12-14 hours at 37°C in a 5% CO_2 atmosphere.

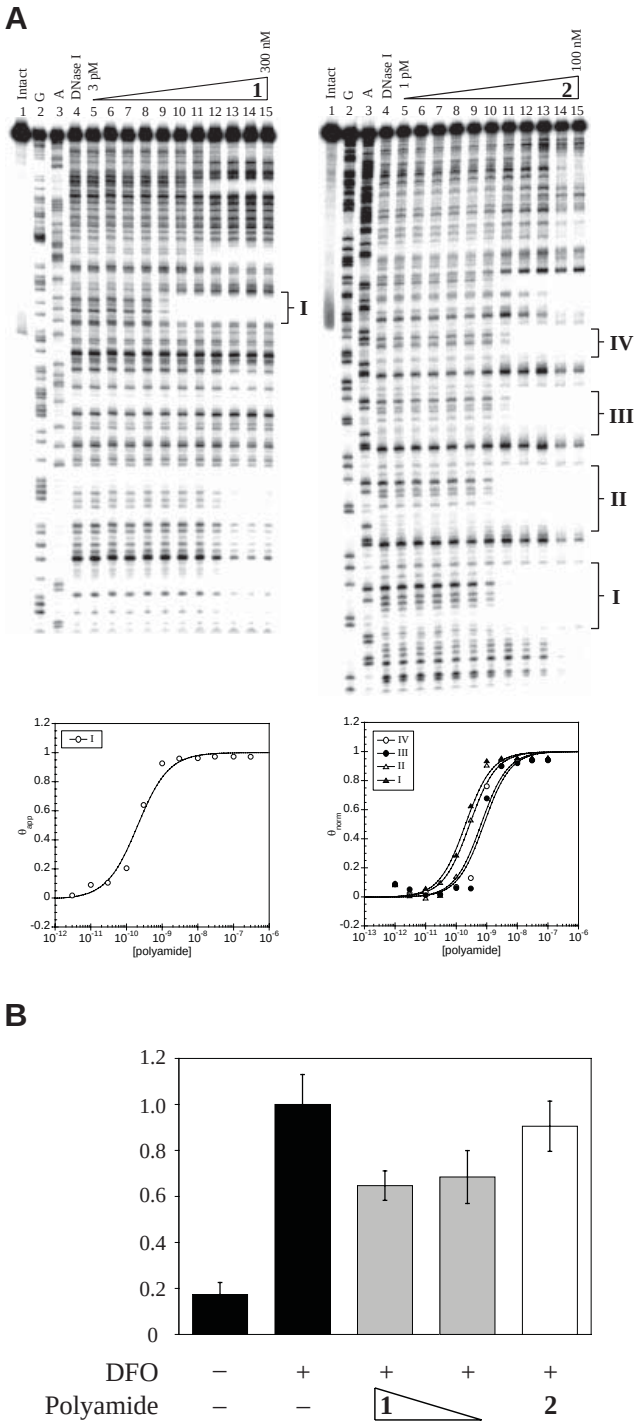

Figure 3.2. (A) Quantitative DNase I footprint titration experiments for polyamides **1** and **2**; lane 1, intact DNA; lane 2, G reaction; lane 3, A reaction; lane 4, DNase standard; lanes 5-15, polyamide concentrations. Binding isotherms are shown below each footprinting gel; θ_{norm} values were calculated according to published methods.[28] (B) Quantitative RT-PCR data for **1** and **2** in HeLa cells induced with deferoxamine (DFO).[8-10] The final concentration of compound **1** was 0.2 μM or 1 μM. The final concentration of compound **2** was 1 μM.

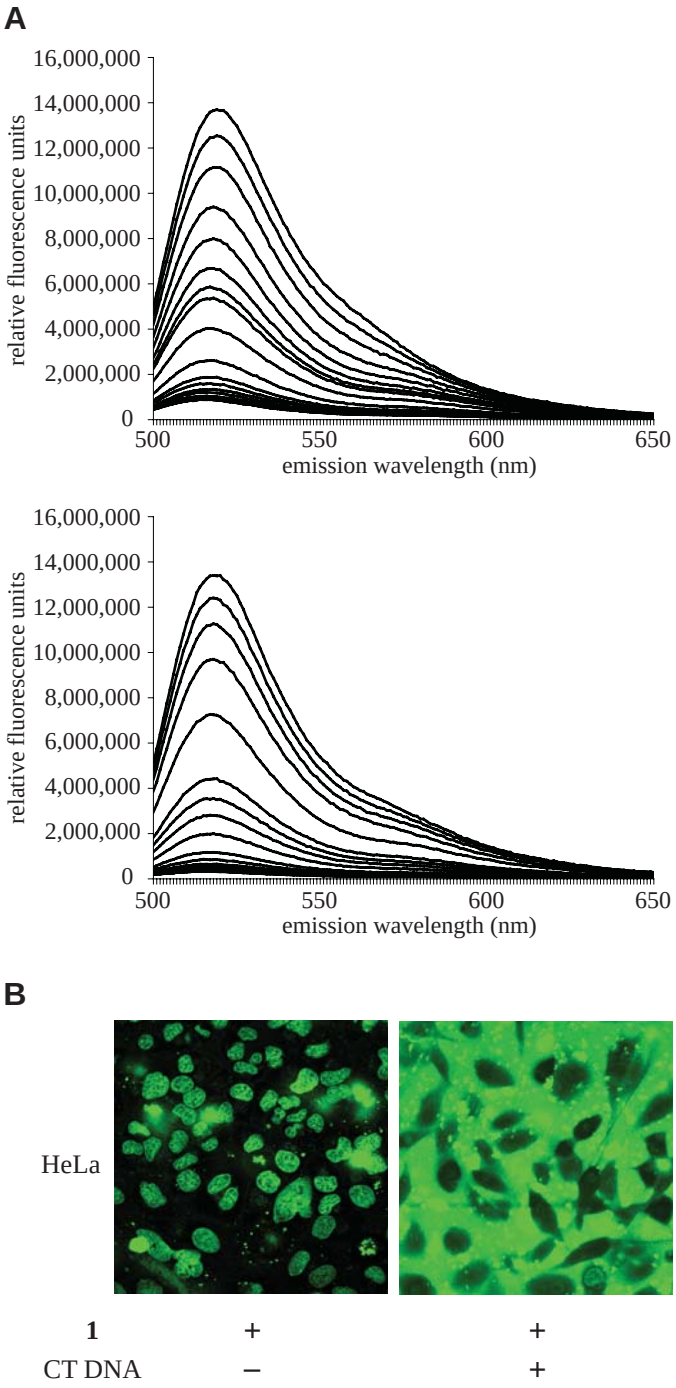

Figure 3.3. (A) Overlaid fluorescence emission spectra for polyamides **1** (top) and **2** (bottom). The polyamide concentration is 2 μM. Calf thymus DNA concentrations are 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100, 200, 400, 600, 800, and 1000 μM bp. (B) Confocal laser scanning microscopy images of polyamide-fluorescein conjugate **1** in HeLa cells. Cells were incubated with 2 μM polyamide for 12-14 hours at 37°C in a 5% CO_2 atmosphere. 500 μM bp calf thymus DNA was added 1 hour prior to imaging.

In order to control for this effect, calf thymus DNA was added to the medium prior to imaging. This allows the direct comparison of polyamide concentration inside the cell nucleus and outside the cell by increasing the fluorescence of both simultaneously. This experiment generates a picture of dark cells in a background of fluorescently enhanced polyamide solution (Figure 3.3). Overlay of the fluorescence and bright-field images indicates that the fluorescence in the cell nucleus is significantly lower than that of the medium. The image of the "dark" nucleus implies that the nuclear concentration is lower than the dosage concentration.

3.4. Confocal microscopy

For the first quantitative approach, the fluorescence of cells was compared to the fluorescence of calibration standards containing known polyamide concentrations, using images taken with a confocal laser scanning microscope. After building a linear calibration curve with the known concentration values, the nuclear concentration in the cell was determined by interpolation (Figure 3.4 and Figure 3.5). Cells were incubated with 2 μM polyamide for 12-14 hours in a 5% CO_2 atmosphere at 37°C and imaged directly. Calibration standards contained both polyamide and 500 μM bp calf thymus DNA. To investigate nuclear concentration, calf thymus DNA was included in the calibration standards to ensure that the fluorescence was turned "on." For these experiments, the concentration of calf thymus DNA (500 μM bp) is lower than the estimated DNA concentration in the nucleus (5 mM bp). Attempts to increase the concentration to the low mM bp range were limited by DNA solubility in medium. Similar experiments in TKMC buffer resulted in fluorescence quenching at the highest DNA concentrations. The actual DNA bp concentration in the nucleus could produce increased fluorescence enhancement and might bring the calculated polyamide concentration closer to the values observed in flow cytometry experiments. In addition, these experiments were performed on cells with mixed cell cycles, i.e., these cells were not grown in synchronized culture. A minimum of three images were taken per condition, with at least ten cells from each image used for analysis.

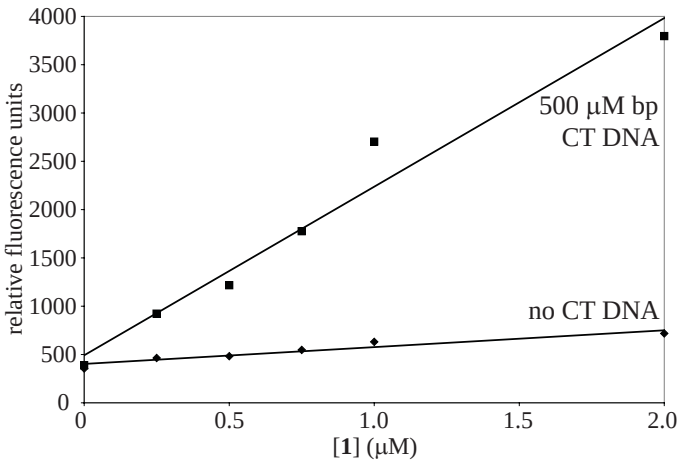

Figure 3.4. Sample calibration curve for confocal microscopy. Squares indicate equilibration with 500 μM bp calf thymus DNA, while diamonds indicate no calf thymus DNA control. R-squared values are 0.9639 (500 μM bp calf thymus DNA) and 0.9171 (no calf thymus DNA).

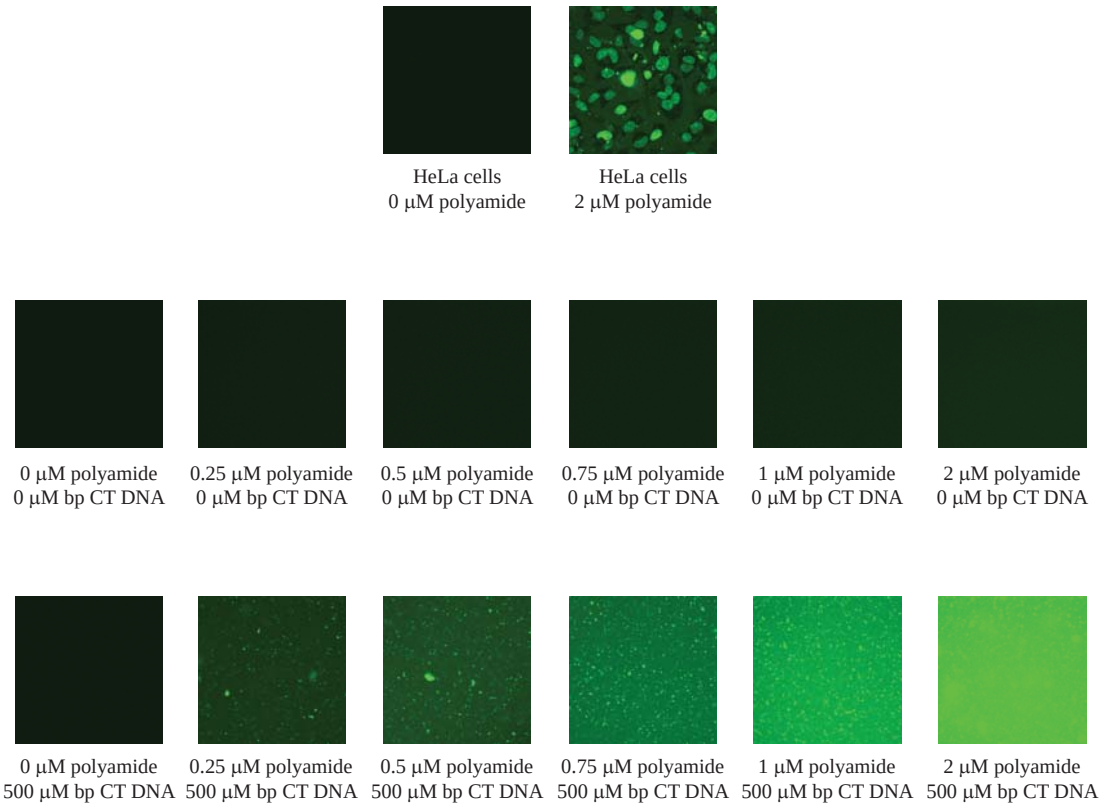

Figure 3.5. Confocal laser scanning microscopy images of calibration standards.

The experiments in HeLa cells indicated that the concentration in the cell nucleus was 0.5 μM and 0.3 μM for polyamides **1** and **2**, respectively (Table 3.1). These values were significantly lower than the 2 μM dosage concentration, which was surprising given that the fluorescence images show the cell nucleus lighting up brightly against a dark background of cytoplasm and medium. The fluorescence signal from the nucleus exceeds the external signal even though the nuclear concentration is lower than the external concentration, as a result of fluorescence enhancement upon binding DNA.

When U251 cells were treated with 2 μM of polyamides **1** and **2**, the interpolated nuclear concentration values were 1.6 μM and 1.5 μM, respectively. This observation suggests that the nuclear concentration is dependent on cell line, just as it varies for each polyamide. The quantitative approach indicates that U251 cells are more permissive than HeLa cells with respect to uptake of this type of molecule.

Table 3.1. Calculated nuclear concentration from confocal microscopy[a]

Polyamide	Cell line	2 μM
1	HeLa	0.5 (±0.1)
2	HeLa	0.3 (±0.1)
1	U251	1.6 (±0.1)
2	U251	1.5 (±0.3)

[a] Calculated nuclear concentration values reported in μM; column headings indicate dosage concentration

3.5. Flow cytometry

The fluorescence of polyamide-containing cells was compared to fluorescent beads in the flow cytometer, and interpolation of the linear calibration curve produces the number of fluorophores per cell (Figure 3.6). Dividing this value by the volume of the nucleus yields the calculated nuclear concentration. Based on confocal microscopy images of HeLa and U251 cells, the cell nucleus was modeled as a cylinder with radius 10 μm and height 5 μm to give a calculated nuclear volume of 1×10^{-12} L. As above, cells were incubated with polyamide for 12-14 hours in a 5% CO_2 atmosphere at 37°C. After washing, fluorescence was measured by analyzing 10,000 cells in the flow cytometer. Under identical instrument settings, SPHERO Rainbow Calibration Particles were analyzed in the fluorescein filter set. The functionalized particles contain eight different amounts of fluorophore per bead and are designed to produce eight separate peaks. Due to the high fluorescence levels of the cells, only the five highest fluorophore peaks were resolved within the fluorescence range and used for constructing the linear calibration curve. The fluorescence intensity of each peak is correlated with a known amount of fluorophore in solution, although the quantum yield of a fluorophore can change upon binding.[26] For analysis, 10,000 events were used in flow cytometry experiments and calibrations.

For flow cytometry experiments, cells were dosed with three different polyamide concentrations of 0.4 μM, 2 μM, and 10 μM. In HeLa cells, the calculated nuclear concentrations for compound **1** were 0.02 μM, 0.09 μM, and 0.20 μM (Table 3.2). While the concentration values increase with dosage, each 5-fold increase in dosage does not produce a proportional increase in nuclear concentration, and this plateau effect is more pronounced at higher dosage concentrations. In addition, these values were lower than those observed in confocal microscopy experiments. For the same dosage concentration of 2 μM, the calculated concentration from flow cytometry was 0.09 μM, which is 5-fold lower than the interpolated concentration from confocal microscopy (0.5 μM). The results from experiments with compound **2** in HeLa cells are calculated nuclear concentrations of

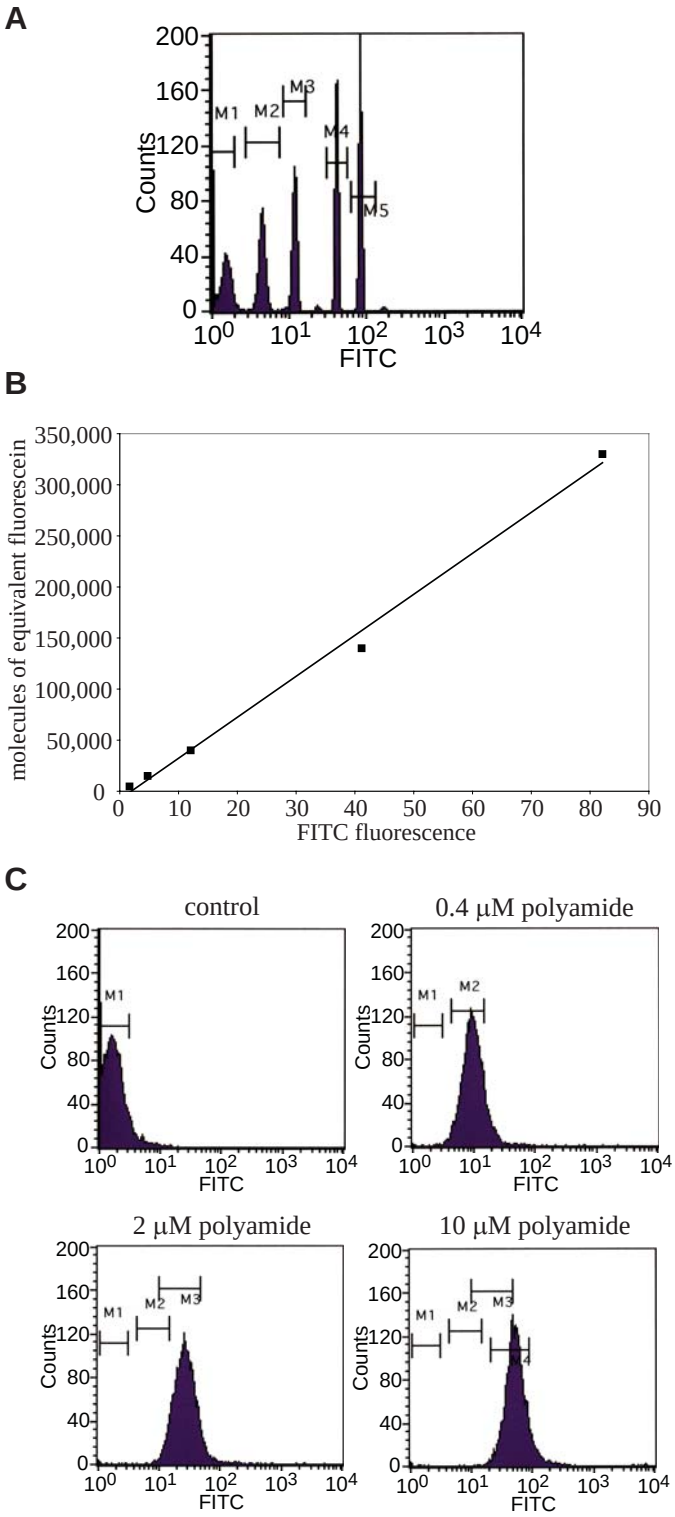

Figure 3.6. (A) Flow cytometry experiment with calibration particles. (B) Sample calibration curve for flow cytometry. R-squared value is 0.9944. (C) Flow cytometry experiments with HeLa cells incubated with 0 μM (top left), 0.4 μM (top right), 2 μM (bottom left), and 10 μM (bottom right) polyamide.

Table 3.2. Calculated cellular concentration from flow cytometry[a]

Polyamide	Cell line	0.4 μM	2 μM	10 μM
1	HeLa	0.02 (±0.01)	0.09 (±0.03)	0.20 (±0.06)
2	HeLa	0.04 (±0.02)	0.14 (±0.05)	0.35 (±0.08)
1	U251	0.09 (±0.04)	0.34 (±0.23)	0.84 (±0.41)
2	U251	0.04 (±0.02)	0.14 (±0.06)	0.34 (±0.17)

[a] Calculated cellular concentration values reported in μM; column headings indicate dosage concentration

0.04 μM, 0.14 μM, and 0.35 μM, indicating that uptake is polyamide core-dependent.

When U251 cells were treated with compound **1**, the calculated nuclear concentration values were 0.09 μM, 0.34 μM, and 0.84 μM. The relatively high nuclear concentrations support the observation that U251 cells are more permissive than HeLa cells, as seen in the above confocal microscopy experiments.

3.6. Comparison of methods

For each polyamide and cell line explored here, the confocal microscopy method produces a higher calculated nuclear concentration value than the flow cytometry method. For instance, at the 2 μM dosage level in HeLa cells, the polyamides exhibit 0.3-0.5 μM nuclear concentration by confocal microscopy, as compared to 0.09-0.14 μM by flow cytometry. However, both methods support the observation that U251 cells allow increased uptake of polyamide-fluorescein conjugates relative to HeLa cells. Overall, the calculations suggest that the concentration in the nucleus is much lower than that in the external medium. This is the opposite of what one might expect upon seeing brightly fluorescent cell nuclei with a dark background.

Both of the techniques explored here have strengths and limitations. Confocal microscopy allows data analysis from direct images of cell culture, requiring no further cell processing steps. This method also uses the same compound for calibration and imaging, as opposed to a generic fluorophore. On the other hand, flow cytometry lends itself well to the quantitative nature of this study and benefits from large sample sizes. However, the nuclear volume is not known with certainty, and the flow cytometer measures the fluorescence of the entire cell, not just the nucleus. Also, the calibration does not take into account the fluorescence enhancement effect; therefore, the calculated nuclear concentration from flow cytometry represents an upper bound for this value.

In this study, we elaborate methods for quantitating the concentration of polyamide-fluorescein conjugates in live cells, incorporating data gathered from confocal microscopy and flow cytometry techniques. When cultured HeLa cells are treated with 2 μM polyamide, the calculated nuclear concentration is between 0.09-0.5 μM, suggesting that the intracellular concentration is 4- to 25-fold lower than the external concentration. As observed in previous studies, cellular uptake is cell-line dependent.[3,5,6] Although qualitative images in HeLa and U251 cell lines both receive the same positive rating, it appears that the nuclear concentration in U251 cells is actually several-fold higher than in HeLa cells.

3.7. Experimental

3.7.1 Polyamide synthesis. Polyamides were synthesized with Boc-β-Ala-PAM resin (Peptides International) according to published manual solid-phase synthesis protocols.[27] The protected ^{FmocHN}γ-turn amine was deprotected with 20% piperidine in DMF and reprotected as the Boc derivative with a solution of Boc_2O (Fluka) and DIEA in DMF. The Boc-protected resin was cleaved with 1 mL of 1,3-diaminopropane at 37°C with agitation for 16 h. Products were purified by preparatory reverse-phase HPLC on a Beckman Gold system using either a Waters Delta-Pak 25×100 mm, 15 μm 300 Å C_{18} PrepPak Cartridge reverse-phase column or a Varian Dynamax 21.4×250 mm Microsorb 8 μm 300 Å C_8 reverse-phase column in 0.1% (w/v) TFA with acetonitrile as the eluent. The appropriate fractions were lyophilized after characterization by analytical HPLC, UV-visible spectroscopy, and MALDI-TOF or ESI mass spectrometry. Conjugates were formed by reacting fluorescein-5-isothiocyanate (FITC, Invitrogen) with the polyamide in a solution of DIEA (20 equiv) and DMF for 1 h at room temperature. Conjugates were deprotected with neat TFA (Halocarbon) and triethylsilane for 30 min at room temperature before purification by preparatory reverse-phase HPLC. Lyophilization of the appropriate fractions yielded the polyamide conjugates **1** and **2**, which were characterized as described above. Extinction coefficients were calculated according to standard protocols.[28] Chemicals not otherwise specified were from Aldrich.

CtPyPyIm-(*R*)^{H_2N}γ-PyImPyPy-β-C_3-FITC (1). UV-vis (H_2O) λ_{max} 310, 444 nm; ESI-MS *m/z* 1633.6 ($C_{76}H_{74}ClN_{22}O_{15}S_2^-$ calculated $[M-H]^-$ 1633.48)

ImImPyPy-(*R*)^{H_2N}γ-ImPyPyPy-β-C_3-FITC (2). UV-vis (H_2O) λ_{max} 312, 444 nm; ESI-MS *m/z* 1597.5 ($C_{76}H_{77}N_{24}O_{15}S^-$ calculated $[M-H]^-$ 1597.57)

3.7.2. Steady-state fluorimetry. For steady-state fluorimetry experiments, titration samples were prepared with sonicated calf thymus DNA (GE Healthcare) as serial dilutions with supplemented medium. Addition of 3 μL of the 100 μM polyamide solution

to each dilution yielded a final polyamide concentration of 2 μM. Fluorescence spectra were measured with a Jobin Yvon/SPEX Fluorolog spectrofluorimeter (Model FL3-11) equipped with a Hamamatsu R928 PMT. Samples were excited at 490 nm using 2 nm emission and excitation slits. Fluorescence was measured from 500 nm to 650 nm at room temperature.

3.7.3. Cell culture. For cell culture experiments, human cancer cell lines HeLa and U251 were cultured in a 5% CO_2 atmosphere at 37°C in supplemented DMEM (HeLa, GIBCO) or RPMI medium 1640 (U251, GIBCO).[5,6] All media were supplemented with 10% fetal bovine serum (HeLa, Omega Scientific) or 5% fetal bovine serum (U251, Omega Scientific) and 1% penicillin/streptomycin solution (Mediatech). HeLa cells were purchased from ATCC. U251 cells were received as a gift from Dr. Giovanni Melillo of the National Cancer Institute.[29] Quantitative RT-PCR experiments were performed using HeLa cells according to published protocols.[8,9]

3.7.4. Confocal microscopy. For confocal microscopy experiments, cell lines were trypsinized (Mediatech) for 5 min at 37°C, centrifuged for 10 min at 4°C at 100×*g*, and resuspended in fresh medium to a concentration of 1.33×10^5 cells/mL.[5,6] Incubations were performed by adding 150 μL of cells into culture dishes equipped with glass bottoms for direct imaging (MatTek). Cells were grown in the glass-bottom dishes for 24 h. The medium was then removed and replaced with 147 μL of fresh medium, followed by addition of 3 μL of the 100 μM polyamide solution for a final polyamide concentration of 2 μM. Cells were incubated in a 5% CO_2 atmosphere at 37°C for 12-14 h. Imaging was performed with a 40× oil-immersion objective lens on a Zeiss LSM 5 Pascal inverted laser scanning microscope. Polyamide-fluorescein conjugate fluorescence and visible-light images were obtained using standard filter sets for fluorescein.[5,6] 12-bit images were analyzed using Zeiss LSM and ImageJ software. Calibration standards were prepared from the 100 μM

polyamide solution as serial dilutions with fresh medium and were directly imaged in glass-bottom dishes in a volume of 150 μL. Sonicated calf thymus DNA solution (GE Healthcare) was added to appropriate calibration standards for a final DNA concentration of 500 μM bp.

3.7.5. Flow cytometry. For flow cytometry experiments, cell lines were trypsinized (Mediatech) for 5 min at 37°C, centrifuged for 10 min at 4°C at 100×*g*, and resuspended in fresh medium to a concentration of 1.33×10^5 cells/mL. Incubations were performed by adding 3 mL of cells into 6-well culture plates (BD Falcon). Cells were grown in the culture plates for 24 h. The medium was then removed and replaced with fresh medium, followed by addition of the appropriate volume of the 100 μM polyamide solution for a final polyamide concentration of 0, 0.4, 2, or 10 μM in a final volume of 3 mL. Cells were incubated in a 5% CO_2 atmosphere at 37°C for 12-14 h. Following incubation, cells were trypsinized for 5 min at 37°C, centrifuged for 10 min at 4°C at 100×*g*, and resuspended in HBSS solution (no Mg^{2+}, no Ca^{2+}, no phenol red, 2.5 mg/mL BSA fraction V, 10 mM HEPES, pH 7.0-7.2) (Sigma). Cell viability was checked with trypan blue stain, and >95% appeared to be viable. Cells were centrifuged again for 10 min at 4°C at 100×*g* and resuspended in the above HBSS solution to a concentration of 5×10^5 cells/mL. The cell suspension was pipetted through a cell strainer (BD Falcon) into a 5 mL polystyrene round-bottom tube (BD Falcon). Samples were analyzed using a BD FACSCalibur System flow cytometer. SPHERO Rainbow Calibration Particles (Spherotech) were used as calibration standards.

3.7.6. Quantitative DNase I footprinting. For quantitative DNase I footprint titration experiments, plasmid pCFH6 was constructed according to standard protocols for DNA manipulation.[30] The two 87-mer oligonucleotides 5'-GATCGTGTAATCAAATGGTCATAGCTGTGTAATCATATGGTCATAGCTGT

GTAATCACATGGTCATAGCTGTGTAATCAGATGGTCATAGC-3' and 5'-AGCTGCTATGACCATCTGATTACACAGCTATGACCATGTGATTACACAGC

TATGACCATATGATTACACAGCTATGACCATTTGATTACAC-3' (Integrated DNA Technologies) were annealed and ligated into the BamHI/HindIII (Roche) restriction fragment of pUC19 (Sigma). DNA sequencing of the constructed plasmid was performed at the Sequence Analysis Facility at the California Institute of Technology. The forward primer 5'-AATTCGAGCTCGGTACCCGGG-3' was ^{32}P-labeled at the 5'-end. The reverse primer 5'-CTGGCACGACAGGTTTCCCGAC-3' was used to amplify plasmid pCFH6 as previously described.[28] pGL-VEGF-*Luc* was 5'-end-labeled and amplified as previously described.[8,9] PCR products (5'-end-labeled, 291 bp for pCFH6, 197 bp for pGL-VEGF-*Luc*) were isolated according to standard protocols.[28] Quantitative DNase I footprint titration experiments were performed on the 5'-^{32}P-end-labeled PCR products of plasmids pGL-VEGF-*Luc* and pCFH6 with polyamides **1** and **2**, respectively, according to standard protocols.[28] Radiolabeled DNA was equilibrated with polyamide solutions for 14-16 h at 22°C in a buffer of 10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$ at pH 7.0 prior to DNase I cleavage. Chemical sequencing reactions were performed according to published methods.[31,32]

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References

1. Dervan, P. B. *Bioorg. Med. Chem.* **2001,** *9,* 2215.

2. Dervan, P. B.; Edelson, B. S. *Curr. Opin. Struct. Biol.* **2003,** *13,* 284.

3. Belitsky, J. M.; Leslie, S. J.; Arora, P. S.; Beerman, T. A.; Dervan, P. B. *Bioorg. Med. Chem.* **2002,** *10,* 3313.

4. Crowley, K. S.; Phillion, D. P.; Woodard, S. S.; Schweitzer, B. A.; Singh, M.; Shabany, H.; Burnette, B.; Hippenmeyer, P.; Heitmeier, M.; Bashkin, J. K. *Bioorg. Med. Chem. Lett.* **2003,** *13,* 1565.

5. Best, T. P.; Edelson, B. S.; Nickols, N. G.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2003,** *100,* 12063.

6. Edelson, B. S.; Best, T. P.; Olenyuk, B.; Nickols, N. G.; Doss, R. M.; Foister, S.; Heckel, A.; Dervan, P. B. *Nucleic Acids Res.* **2004,** *32,* 2802.

7. Xiao, X. S.; Yu, P.; Lim, H. S.; Sikder, D.; Kodadek, T. *Angew. Chem. Int. Ed.* **2007,** *46,* 2865.

8. Olenyuk, B. Z.; Zhang, G. J.; Klco, J. M.; Nickols, N. G.; Kaelin, W. G.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2004,** *101,* 16768.

9. Nickols, N. G.; Jacobs, C. S.; Farkas, M. E.; Dervan, P. B. *Nucleic Acids Res.* **2007,** *35,* 363.

10. Nickols, N. G.; Jacobs, C. S.; Farkas, M. E.; Dervan, P. B. *ACS Chem. Biol.* **2007,** *2,* 561.

11. Nickols, N. G.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2007,** *104,* 10418.

12. Fukuda, N.; Ueno, T.; Tahira, Y.; Ayame, H.; Zhang, W.; Bando, T.; Sugiyama, H.; Saito, S.; Matsumoto, K.; Mugishima, H.; Serie, K. *J. Am. Soc. Nephrol.* **2006,** *17,* 422.

13. Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Nature* **1996,** *382,* 559.

14. White, S.; Szewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B. *Nature* **1998,** *391,* 468.

15. Kielkopf, C. L.; Baird, E. E.; Dervan, P. D.; Rees, D. C. *Nat. Struct. Biol.* **1998,** *5,* 104.

16. Kielkopf, C. L.; White, S.; Szewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B.; Rees, D. C. *Science* **1998,** *282,* 111.

17. Foister, S.; Marques, M. A.; Doss, R. M.; Dervan, P. B. *Bioorg. Med. Chem.* **2003,** *11,* 4333.

18. Wurtz, N. R.; Dervan, P. B. *Chem. Biol.* **2000,** *7,* 153.

19. Kwonj, Y.; Arndt, H. D.; Qian, M.; Choi, Y.; Kawazoe, Y.; Dervan, P. B.; Uesugi, M. *J. Am. Chem. Soc.* **2004,** *126,* 15940.

20. Stafford, R. L.; Arndt, H. D.; Brezinski, M. L.; Ansari, A. Z.; Dervan, P. B. *J. Am. Chem. Soc.* **2007,** *129,* 2660.

21. Cohen, J. D.; Sadowski, J. P.; Dervan, P. B. *Angew. Chem. Int. Ed.* **2007,** *46,* 7956.

22. Rucker, V. C.; Foister, S.; Melander, C.; Dervan, P. B. *J. Am. Chem. Soc.* **2003,** *125,* 1195.

23. Rucker, V. C. Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 2003.

24. Rucker, V. C.; Dunn, A. R.; Sharma, S.; Dervan, P. B.; Gray, H. B. *J. Phys. Chem. B* **2004,** *108,* 7490.

25. Chenoweth, D. M.; Viger, A.; Dervan, P. B. *J. Am. Chem. Soc.* **2007,** *129,* 2216.

26. SPHERO Technical Notes 8 and 9 (http://www.spherotech.com/).

27. Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1996,** *118,* 6141.

28. Trauger, J. W.; Dervan, P. B. *Methods Enzymol.* **2001,** *340,* 450.

29. Kong, D. H.; Park, E. J.; Stephen, A. G.; Calvani, M.; Cardellina, J. H.; Monks, A.; Fisher, R. J.; Shoemaker, R. H.; Melillo, G. *Cancer Res.* **2005,** *65,* 9047.

30. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual.* 2nd ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

31. Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499.

32. Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823.