Cationic Vector Intercalation into the Lipid Membrane Enables Intact Polyplex DNA Escape from Endosomes for Gene Delivery

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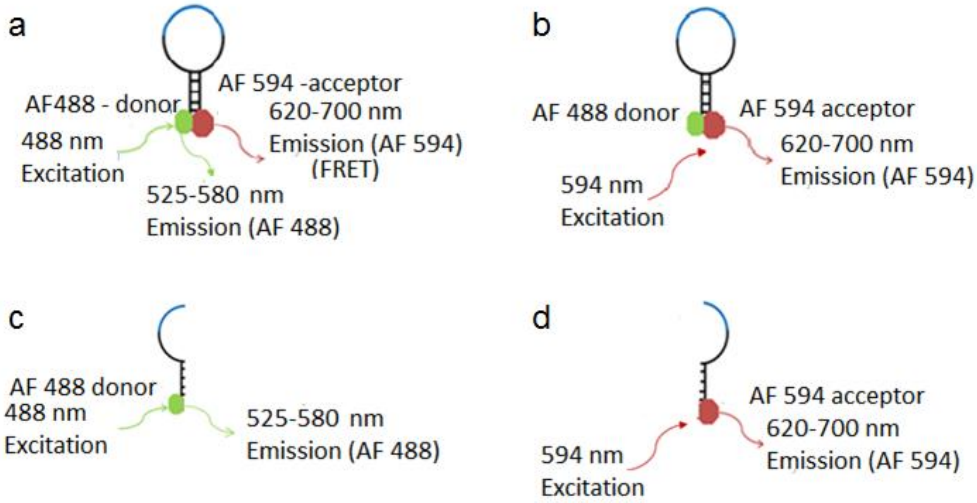


Figure S1. Summary of oligonucleotide molecular beacon (OMB) probe response as a function of structural state and excitation wavelength. (a) Intact beacon shows both FRET red emission (620-700 nm) and green emission (525-580 nm) when excited by 488 nm laser.(b) Excitation of intact beacon by 594 nm laser allows quantification of cleaved beacon by providing a non-FRET level of emission from the AF594 dye. (c) Cleaved beacon exhibits green emission (525-580 nm) upon 488 nm laser excitation. (d) Cleaved beacon exhibits red emission (620-700 nm) upon 594 nm laser excitation.

Quantifying FRET: Several indices to quantify FRET (Intactness, I) using intensities of the FRET donor and acceptor have been described in the past.[9] These include equations A and 1:

$$I = \frac{I_{D\,ex,Aem}}{I_{Dex,Aem}+I_{Dex,Dem}} * 100 \quad \text{(A)}$$

$$I = \frac{I_{D\,ex,Aem}-I_{noise}}{I_{A\,ex,Aem}} * 100 \qquad (1)$$

In the subscripts A denotes FRET acceptor (AF594), D denotes FRET donor (AF488), ex denotes excitation and em denotes emission. For example, $I_{D\ ex,\ A\ em}$ means donor (AF488) is excited and emission from acceptor (AF594) is measured. Preliminary studies indicated that the Intactness measured using the equation A was dependent on the polymer used to make polyplexes. This difference is due to the fact that AF594 but not AF 488 was quenched significantly by jetPEI (Figure S2a, S2b). However, the Intactness measured using equation 1 was insensitive to quenching by the

polymers (Figure S2b) since equation 1 uses only the intensities of the acceptor emission. Hence, all subsequent work uses equation 1 to quantify FRET.

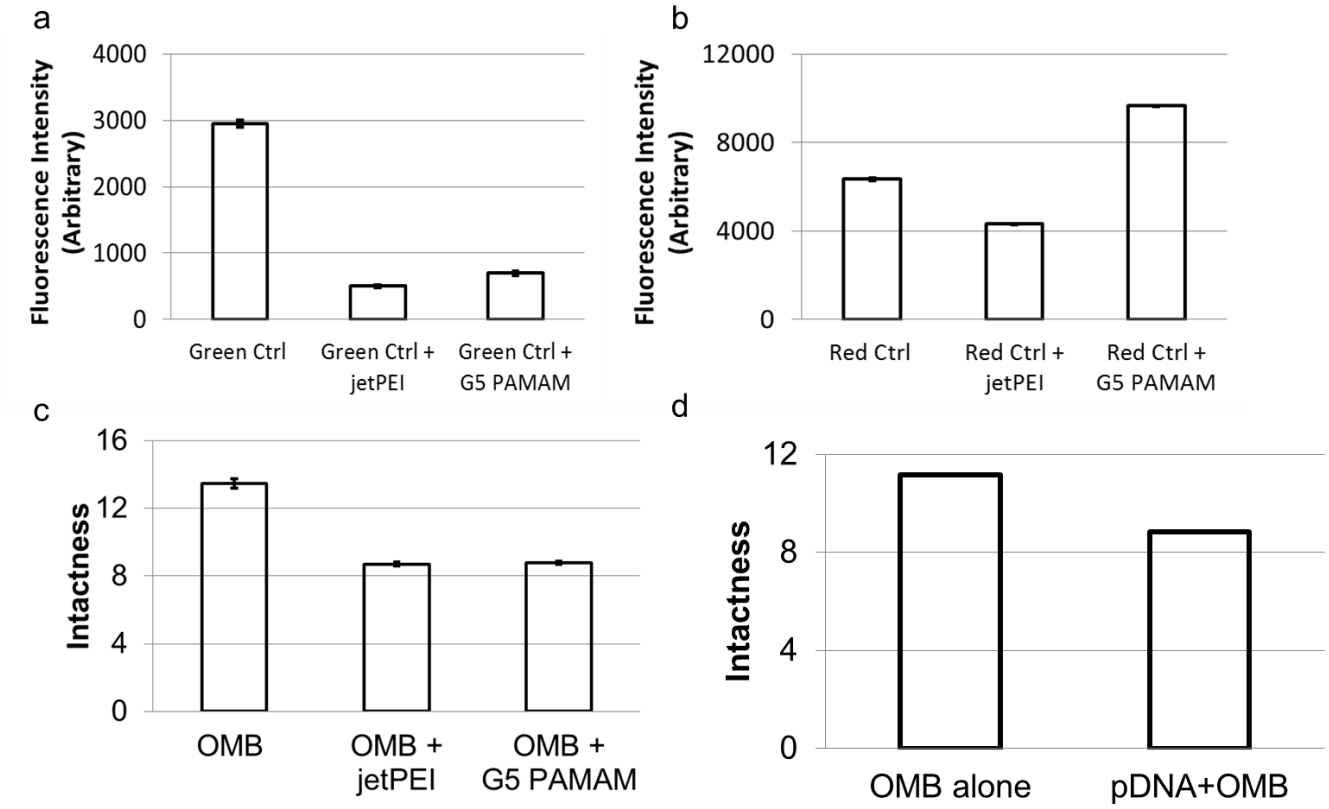
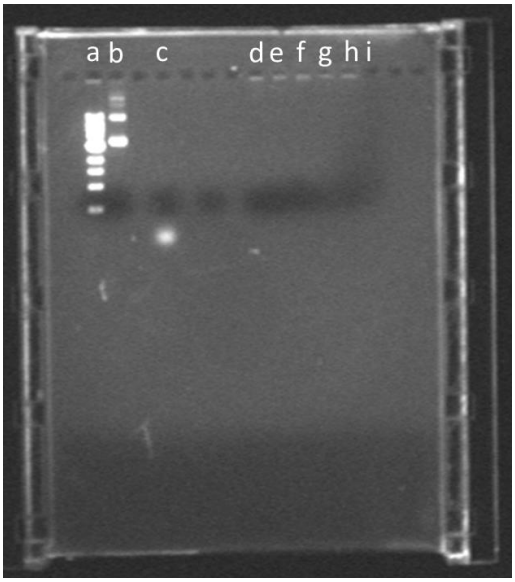


Figure S2: OMB FRET in the presence of polymers. (a) A decrease in fluorescence emission in the 525-580 nm range is observed for beacons containing only AF488 in the presence of jet PEI or G5 PAMAM (485 nm excitation).(b) An increase in fluorescence emission in the 620-700 nm range is observed for beacons containing only AF 594 in the presence of G5 PAMAM (590 nm excitation). (c) FRET from free OMB as well as OMB in mixed polyplexes containing a 1:1 mixture of pDNA and OMB were measured. Equation 1 was used to quantify FRET because it is unchanged by the changes in emission of AF594 induced by the vectors. As shown in panel C, this method showed similar FRET in the presence of both jetPEI and G5 PAMAM vectors. OMB in polyplexes presented a 35% reduction in FRET. (d) The FRET from the OMB was also tested in the presence and absence of pDNA. The presence of the pDNA resulted in a 20% reduction in FRET, which may partially account for the reduction in FRET seen in polyplexes.



a. 1 kb ladder
b. pDNA
c. OMB
d. jetPEI (N:P = 2)
e. jetPEI (N:P = 5)
f. jetPEI (N:P = 10)
g. G5 PAMAM (N:P = 2)
h. G5 PAMAM (N:P = 5)
i. G5 PAMAM (N:P = 10)

Figure S3: Gel electrophoresis of pDNA, OMB and polyplexes in 0.9% agarose gel. The gel was run at 60 V for 2 h and 15 minutes in TAE buffer. Polyplexes contained 0.5 µg of pDNA and 0.5 µg of OMB. The aliquot added to well contained 0.2 µg of DNA (pDNA + OMB). Lane a shows 1 kb DNA ladder. pDNA in lane b shows two bands. It is likely that one of the bands represents supercoiled pDNA. OMB in lane c migrated the furthest. Results from lanes d-i show that both jetPEI and G5 PAMAM completely complex pDNA and OMB at N:P = 2 as they do not migrate far in the lanes. Moreover, the results also show that the OMB and pDNA travel together in the polyplexes.

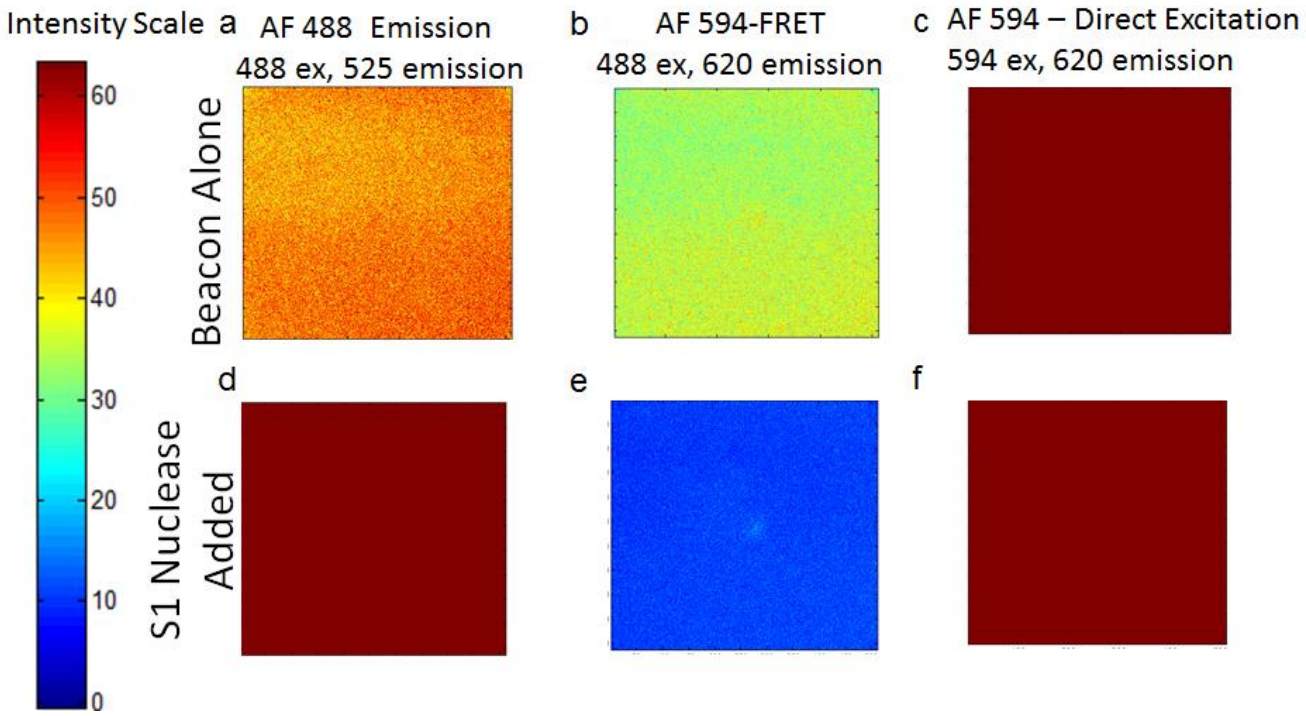


Figure S4: OMB was dissolved in 1X - S1 nuclease buffer and the fluorescence assessed using a microscope. Color bar on the left indicates intensity of emission. (a) The excitation of the solution using a 488nm laser resulted in emission in the 525-580 nm channel. (b) When excited using the 488 nm laser, OMB emission was also observed in the 620-700 nm channel as expected. (c) The emission of AF 594 excited using a 594 nm laser was also measured. (d) When S1 nuclease was added to the same droplet, AF488 emission increased since energy was no longer being transferred by FRET. (e) FRET emission by AF594 also reduced to ~ 0 after the addition of S1 nuclease. (f) AF594 emission due to excitation by 594 nm laser did not change.

FRET from Cleaved Beacons

A factor that could confound the experiments is the possibility that cleaved beacons are held together by the polymer resulting in a FRET signal. To test for this possibility, cells were incubated with polyplexes containing both single labeled AF488 and AF594. Figures S6 and S6 show that polyplexes made using a mixture of singly labeled beacons containing AF488 and AF594 can exhibit some FRET signal. However, the Intactness for these samples was 3 compared with 7-10 for jetPEI and G5 PAMAM beacons at 4h. Thus our controls confirm that the results obtained from the flow cytometry experiments and confocal experiments were representative of beacon cleavage within the cell.

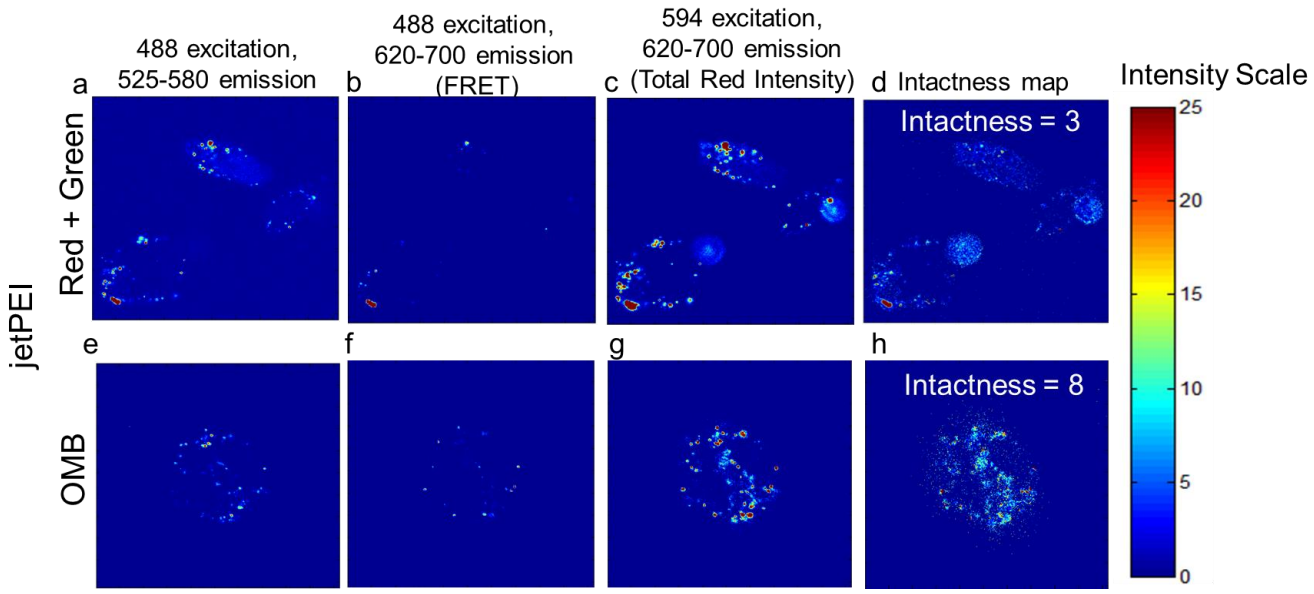


Figure S5: FRET from mixed jetPEI polyplexes containing AF488 and AF594. FRET may occur between AF 488 and AF 594 from neighboring beacon molecules when present in the polyplex and can result in an elevated measure of intactness of the beacon. To test for this possibility, controls experiments were performed in which cells were treated with polyplexes made with a mixture of singly labeled beacons containing only AF 488 dyes and AF 594 dyes (1 ug each) for 3 h. (a) AF488 emission from polyplexes. (b) AF594 emission by excitation with 488 nm. These polyplexes with a mixture of singly labeled beacons still show some FRET signal inside cells. (c) AF594 emission by excitation with 594 nm. (d) Intactness map of mixed polyplexes shows an average value of 3. (e-g) As a comparison, fluorescence from the same channels are shown for a regular OMB. (f) Average intactness for a regular OMB is 8.

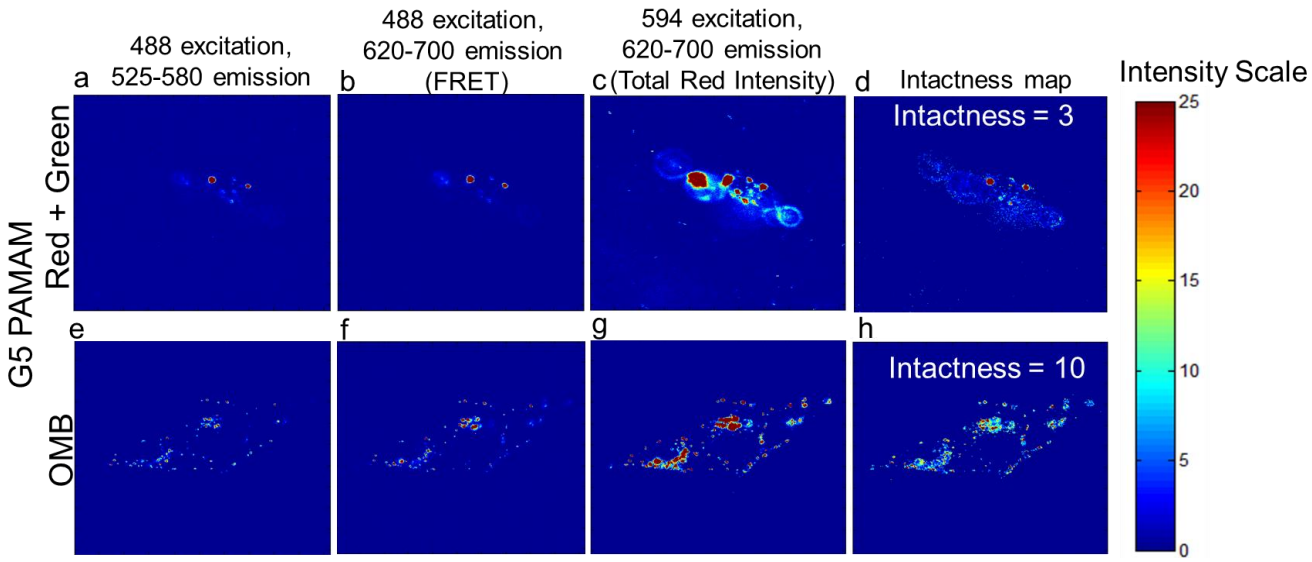


Figure S6: FRET from mixed G5 PAMAM polyplexes containing AF488 and AF594. FRET may occur between AF 488 and AF 594 from neighboring beacon molecules when present in the polyplex and can result in an elevated measure of intactness of the beacon. To test for this possibility, controls experiments were performed in which cells were treated with polyplexes made with a mixture of singly labeled beacons containing only AF 488 dyes and AF 594 dyes (1 ug each) for 3 h. (a) AF488 emission from polyplexes. (b)AF594 emission by excitation with 488 nm. These polyplexes with a mixture of singly labeled beacons still show some FRET signal inside cells. (c) AF594 emission by excitation with 594 nm. (d) Intactness map of mixed polyplexes shows an average value of 3. (e-g) As a comparison, fluorescence from the same channels are shown for a regular OMB. (f) Average intactness for a regular OMB is 10.

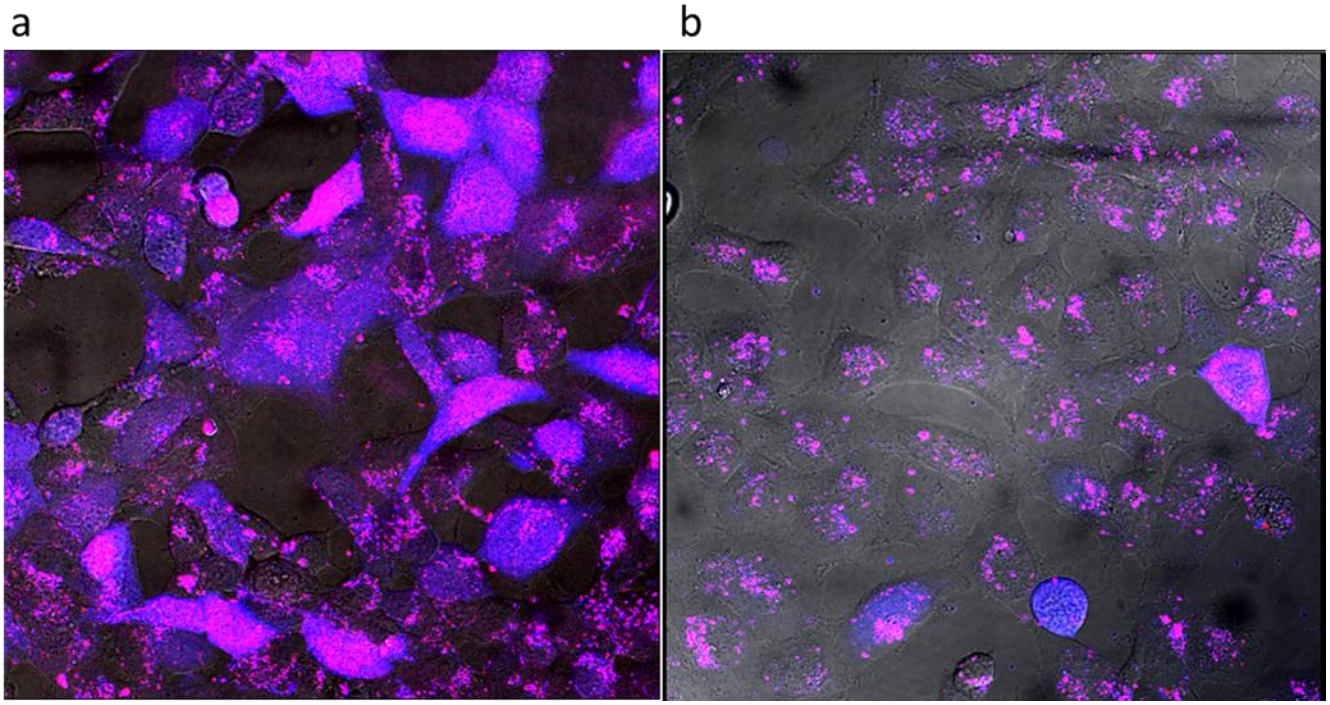


Figure S7. Fluorescence images from Figure 1a and 1b overlaid with transmitted light images to show cell boundaries. For both cases, majority of the fluorescence was confined within cells.

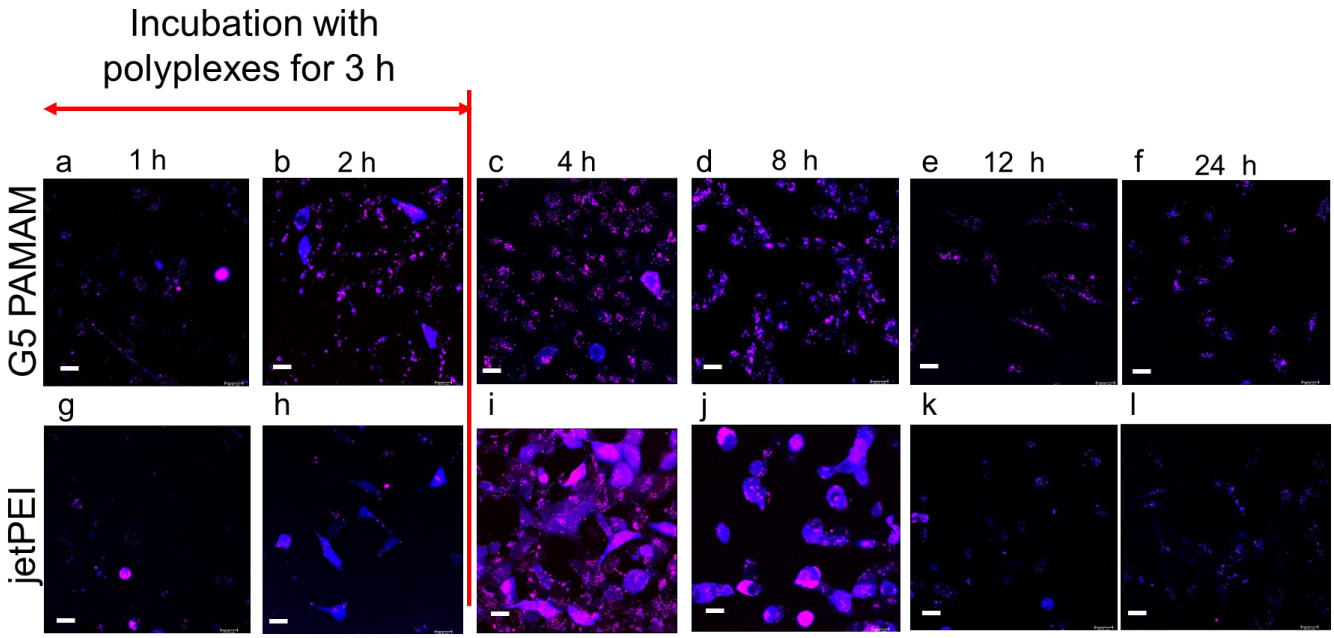


Figure S8. Release and degradation of OMB over time in multiple cells. (a-f) HEK 293A cells exposed for 4 h to either G5 PAMAM polyplexes (N:P 10:1) formed using 0.5 μg OMB and 0.5 μg blank pDNA. OMB fluorescence was imaged using a confocal microscope. OMBs delivered using G5 PAMAM were mostly confined to vesicles and were intact in the punctate regions for 24 h. (g-l) OMB delivered using jetPEI polyplexes exhibited a punctate distribution at 1 h. At 2h, 4h and 8 h, a diffuse distribution of intact OMB in the cytosol was observed which dissipated by 12 h.The OMB left in punctate spots at 12 and 24 h had a substantial fraction of intact OMBs. Scale bar represents 20 μm for all images.

A quantitative analysis of cytosolic DNA cleavage in a cell population

Degradation of the OMB in a cell population was quantifed using flow cytometry. HEK 293A cells were incubated with G5 PAMAM and jetPEI polyplexes (N:P 10:1) formed using a 1:1 mixture (0.5 μg each) of OMB and blank pDNA for 3 h and analyzed using flow cytometry (Figure S9) at 4 h. In order to quantify the difference in FRET intensity as function of polymer vector, the experiment was performed using a flow cytometer that contained both 488 nm and 594 nm lasers. In this experiment, cells were first exposed to the 488 nm laser and emission from 525-580 nm was collected. The same cells were then exposed to a 594 nm laser and emission from 620-700 nm was then collected. This allowed us to quantify FRET emission using equation 1. Results in Figure S9 show that the FRET signal from cells treated using G5 PAMAM beacons was higher than the FRET signal from cells treated using jetPEI polyplexes. The Intactness (eq 1) for the OMBs formed using G5 PAMAM and jetPET at 4 h were 20% and 7%, respectively (Figure S9). Figure S10 also shows results from cells at 30 min, 4 h and 16 h after incubation with polyplexes using a different flow cytometer with only a 488 nm laser. In cells exposed to polyplexes for 16 h, the level of FRET decreased. Nevertheless, G5 PAMAM polyplexes exhibited higher FRET that jetPEI polyplexes.

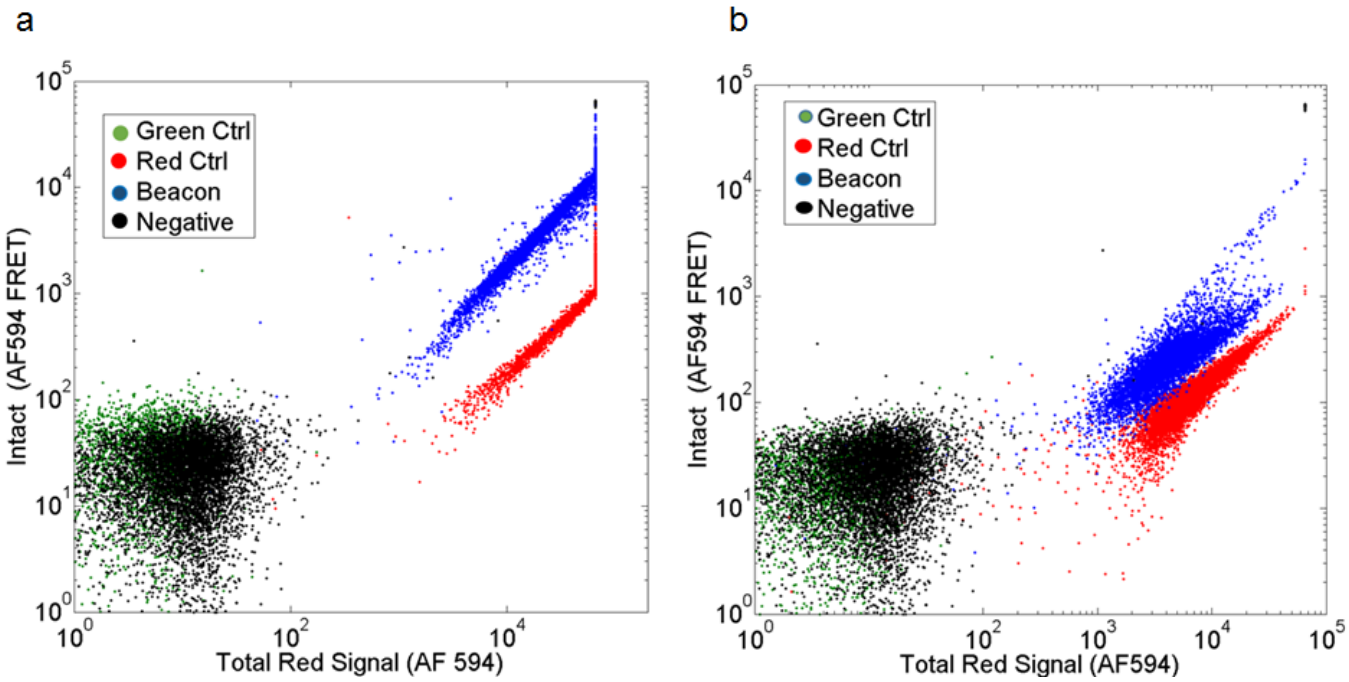


Figure S9. OMB degradation in a population of cells. (a) HEK 293A cells treated for 4 hours with G5 PAMAM polyplexes (N:P = 10:1) formed using a 1:1 ratio of OMB and blank pDNA (0.5 μg each). The x-axis shows fluorescence emission (620-700 nm) upon 594 nm excitation. The y-axis shows the FRET fluorescence emission (620-700 nm) signal upon 488 nm excitation. (b) HEK 293A cells treated for 4 hours with jetPEI polyplexes (N:P = 10:1) formed using a 1:1 ratio of OMB and blank pDNA (0.5 μg each). jetPEI polyplexes (N:P 10:1) Polyplexes formed using G5 PAMAM exhibit 10x greater FRET signal than those formed with jet PEI as judged by the relative y-shift of the blue and red cell distributions.

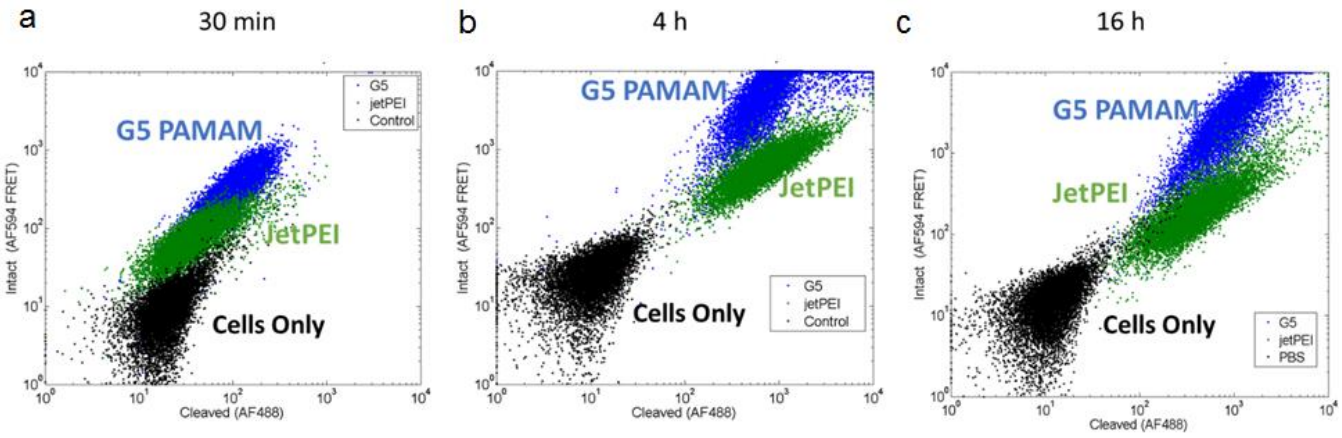
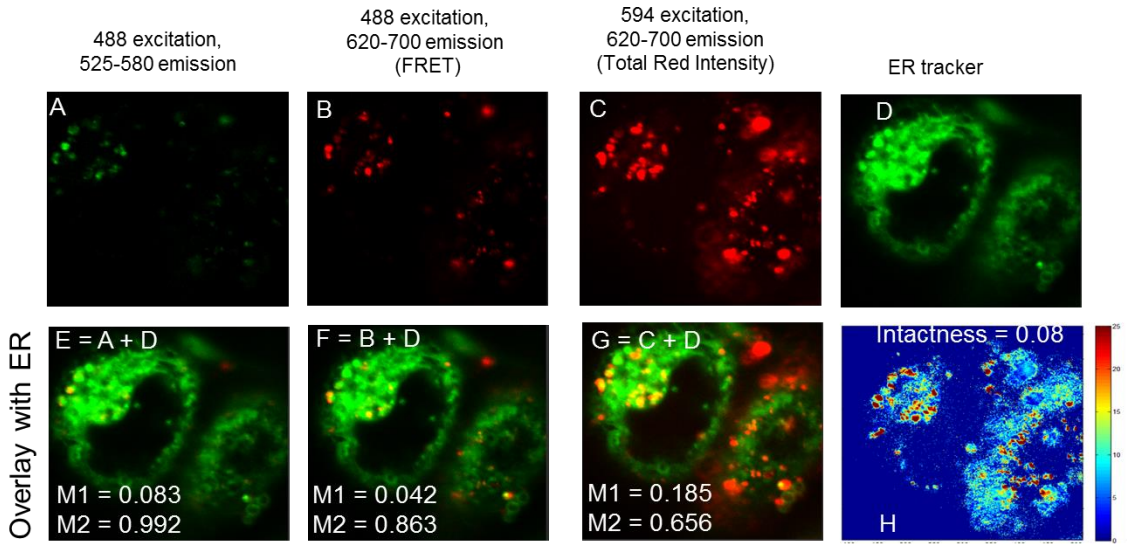


Figure S10. OMB Degradation Over Time Studied Using Flow Cytometry. (a) HEK 293A cells exposed for 30 minutes to either G5 PAMAM or jet PEI polyplexes (N:P 10:1) formed using 0.5 μg of oligonucleotide molecular beacon and 0.5 μg blank pDNA. The x-axis shows fluorescence emission (525-580 nm) upon 488 nm excitation. The y-axis shows the FRET fluorescence emission (620-700 nm) signal upon 488 nm excitation. OMBs delivered using jetPEI show less AF594 FRET emission than beacons delivered using G5 PAMAM. (b) HEK293A cells exposed to G5 PAMAM or jetPEI polyplexes for 3 h in serum free media followed by 1 h incubation in complete media. OMBs delivered using jetPEI show less AF594 FRET emission than beacons delivered using G5 PAMAM. (c) HEK293A cells exposed to G5 PAMAM or jetPEI polyplexes for 3 h in serum free media followed by 13 h incubation in complete media. OMBs delivered using jetPEI show less AF594 FRET emission than beacons delivered using G5 PAMAM.

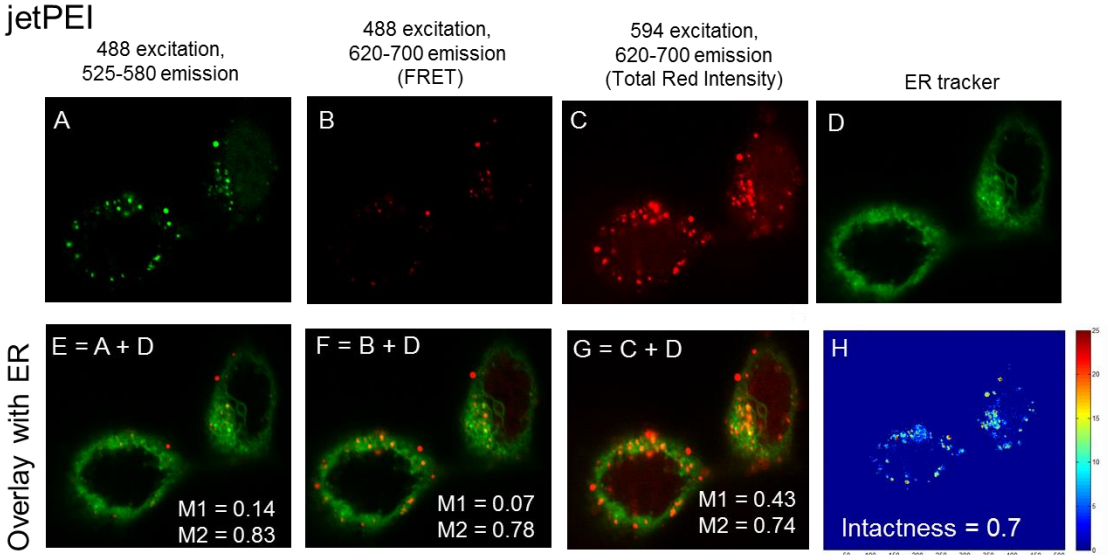
Colocalization of OMB with Organelle Markers

In order to test if there were differences in the amount of intact OMB present in the endoplasmic reticulum (ER) and Golgi apparatus, we stained cells using organelle markers for ER (ER tracker blue) and Golgi apparatus (wheat germ agglutinin-AF 350, WGA). Figures S11 and S12 illustrate the colocalization of beacons with ER tracker blue and WGA. Colocalization was quantified using Mander's coefficients combined with manual thresholds to ensure that background noise did not confound colocalization coefficients.[10] M1 (fraction of organelle markers that overlap with OMB) and M2 (fraction of OMB that overlap with the organelle marker) have been calculated for both FRET signal (intact beacons) and AF488 or AF594 signal by direct excitation (the sum of intact and cleaved beacons). Since the organelle markers are widespread, a higher fraction of beacon signal was colocalized with the organelle markers. Hence, M2 was greater than M1 for all cases. We observed that the colocalization of OMB with ER was higher than the colocalization with the Golgi for both L-PEI and G5 PAMAM polyplexes. Two sample t-test was used to test if the colocalization of the OMB delivered using jetPEI and G5 PAMAM with organelle markers were significantly different. The results are presented in tables I and II. The experiments are designed such that a 2 fold difference in colocalization can be detected with a power greater than 0.8. In cases where the t-test showed a significant difference in colocalization of OMB, the power was ≥ 0.8. For cases where the means were not significantly different, the power was lower.

a. G5 PAMAM

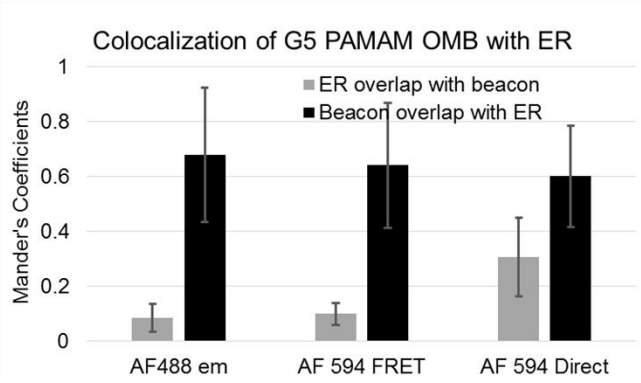


b. jetPEI



c. Average Mander's coefficients from multiple cells

A



B

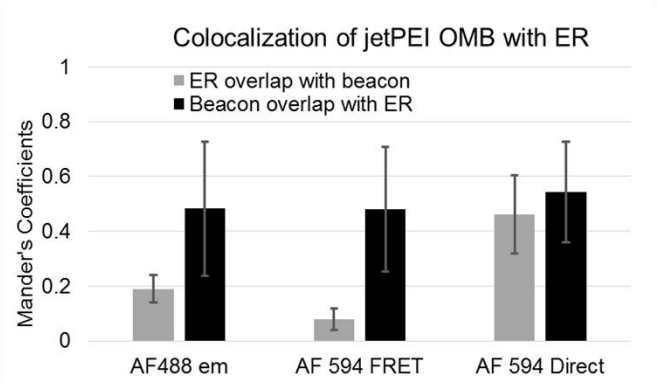
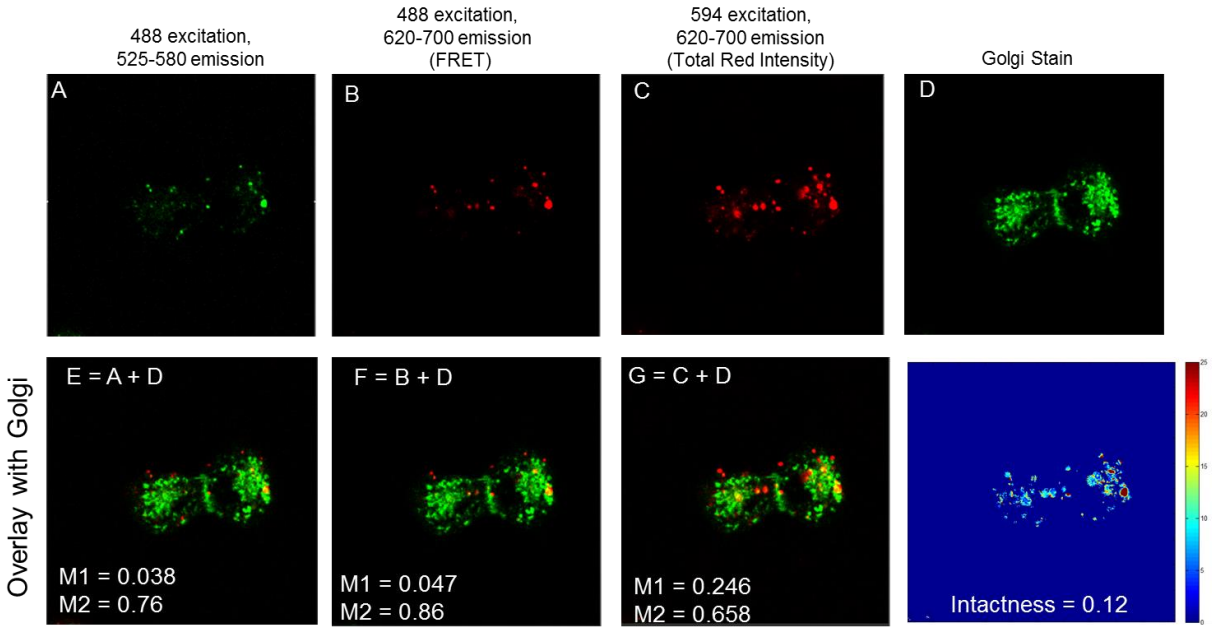


Figure S11: Colocalization of OMB with ER tracker blue (a) Images A-D show the signal from beacons and ER tracker respectively for beacons delivered using G5 PAMAM. Images E-G show the overlay of A, B and C with D. (b) Images show the signal from the beacons and ER for beacons delivered using jetPEI. For beacons delivered using both jetPEI and G5 PAMAM, a large fraction of intact (B) and cleaved beacons (A) are distributed close to the ER (D) as indicated by the Mander's coefficients (M2). JetPEI beacons also show a diffuse distribution throughout the cell. (c) Since the diffuse beacons do not colocalize with ER, the average Mander's coefficients presented in B for jetPEI beacons are less than the average Mander's coefficients presented for G5 PAMAM beacons in A.

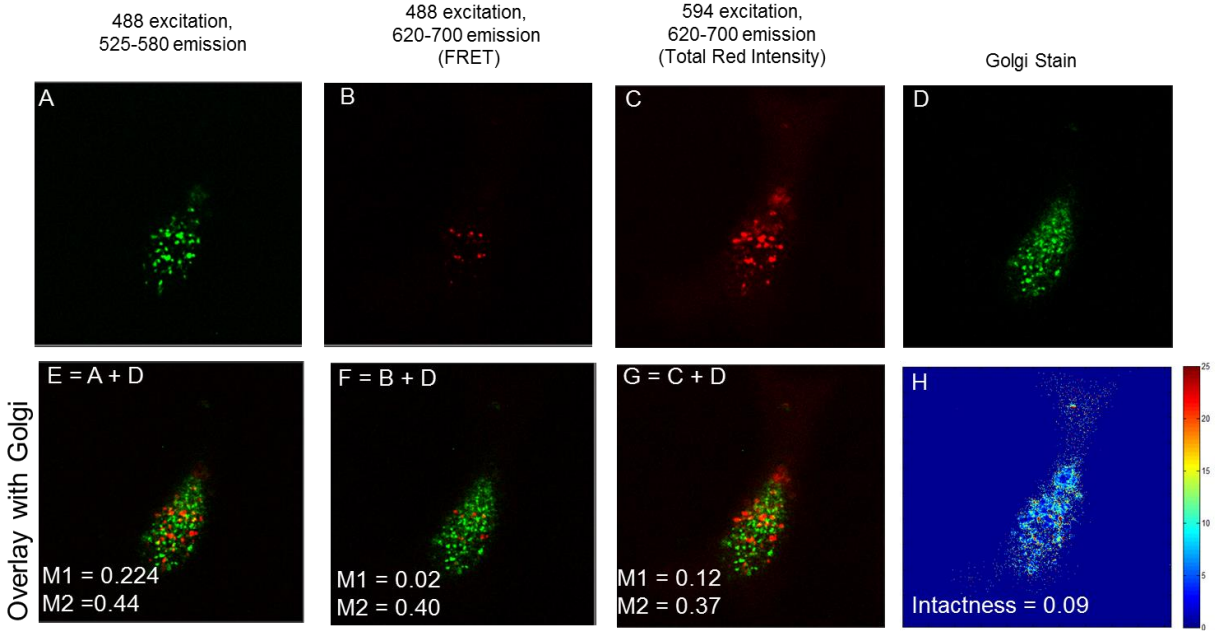
Table I: Colocalization of OMB with ER tracker

	AF 488	**AF594 FRET**	**AF594 direct**	
M1	0.0126	0.1075	0.0324	p value
	1	0.5	0.8	power
M2	0.0531	0.0754	0.4761	pvalue
	0.8	0.8	0.1	power

a. G5 PAMAM



b. jetPEI



c. Average Mander's coefficients from multiple cells

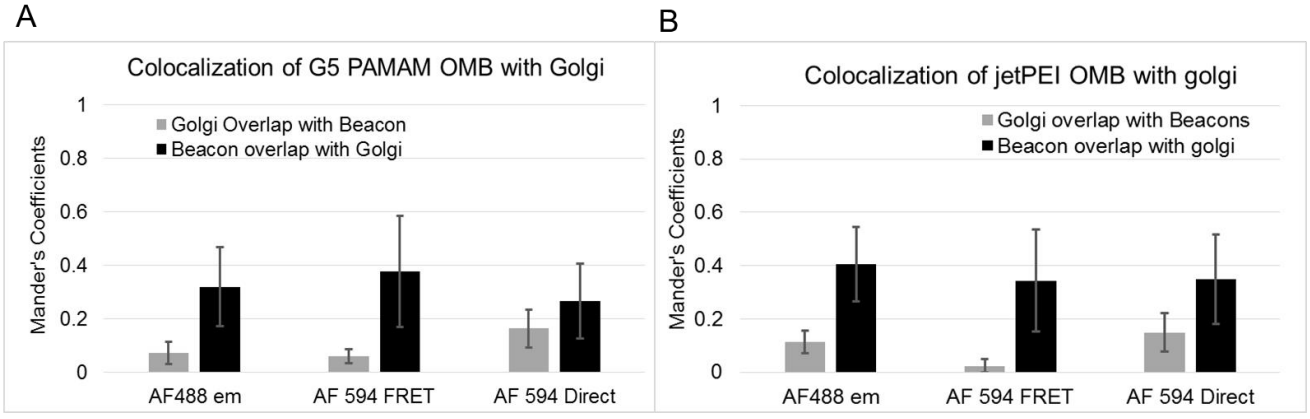


Figure S12: Colocalization of OMB with golgi stain (WGA-AF350)(a) Images A-D show the signal from beacons and wheat germ agglutinin (golgi marker) respectively for beacons delivered using G5 PAMAM. Images E-G show the overlay of A, B and C with D. (b) Images show the signal from the beacons and ER for beacons delivered using jetPEI. More overlap is seen between the beacon and and wheat germ agglutinin (golgi marker) for jetPEI polyplexes. (c) For both G5 PAMAM polyplexes and jetpEI polyplexes, the fraction of beacons overlapping with golgi signal were less than the average fraction of beacons overlapping with ER tracker. Interestingly, the fraction of beacons overlapping with golgi was less for jetPEI polyplexes than G5 PAMAM polyplexes.

Table II: Colocalization of OMB with WGA

	AF 488	**AF594 FRET**	**AF594 direct**	
M1	0.088	<0.0001	0.6879	p value
	0.5	1	0.08	power
M2	0.1716	0.6872	0.2131	pvalue
	0.5	0.09	0.3	power

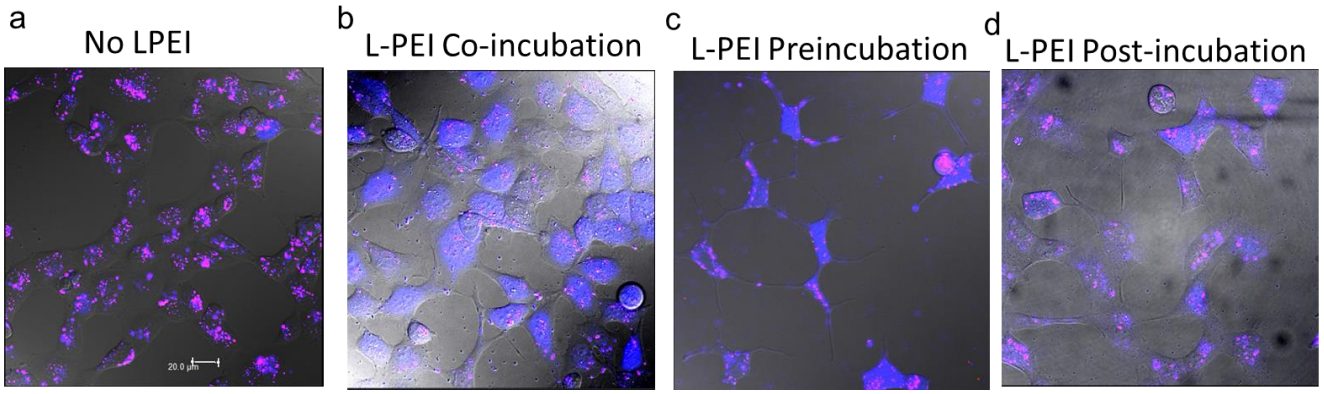


Figure S13. Fluorescence images from Figure 3a-d overlaid with transmitted light images to show cell boundaries. For all cases, majority of the fluorescence was confined within cells.

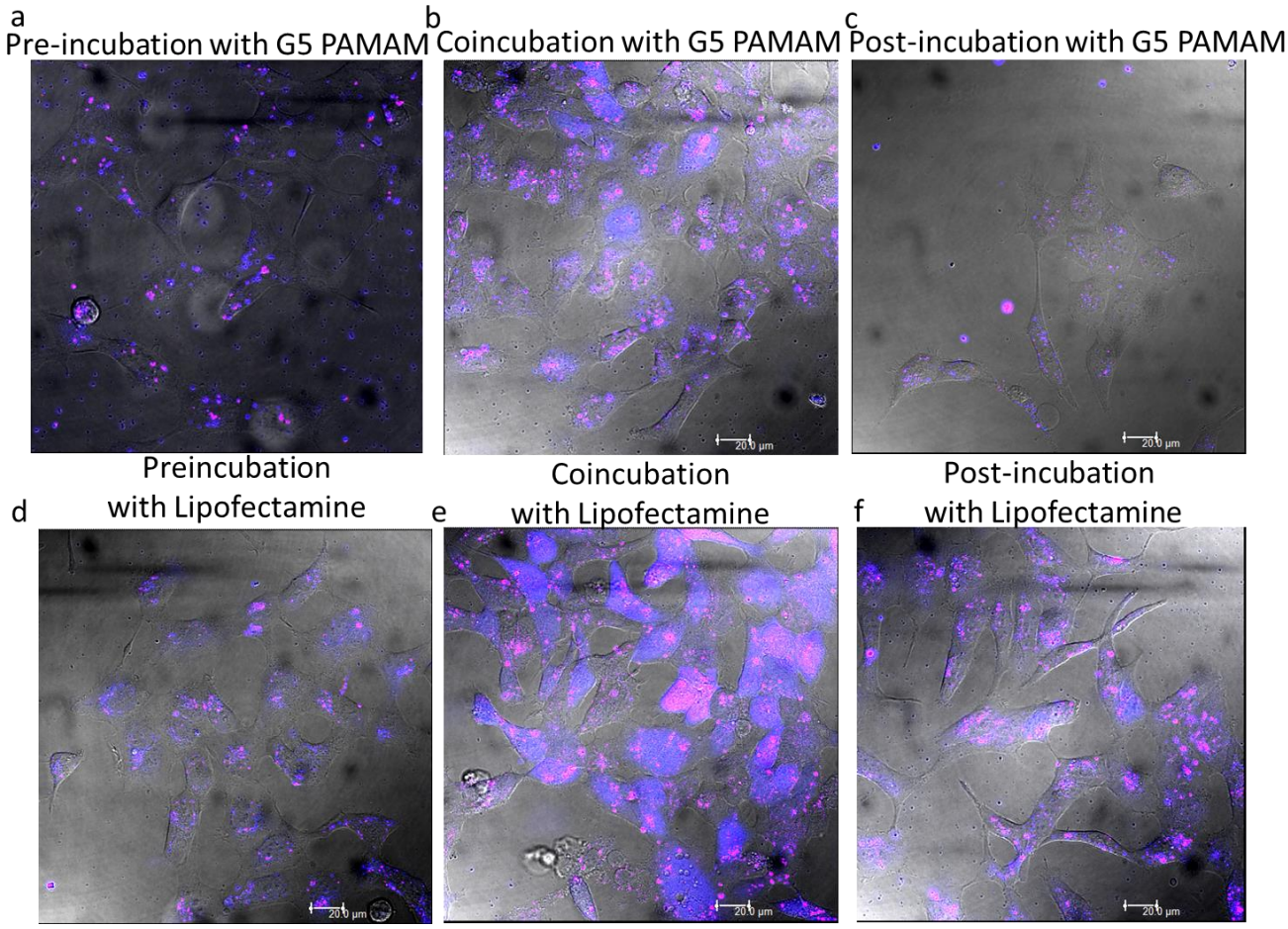


Figure S14: Fluorescence images from Figure 4a-f overlaid with transmitted light images to show cell boundaries. For both cases, majority of the fluorescence was confined within cells.

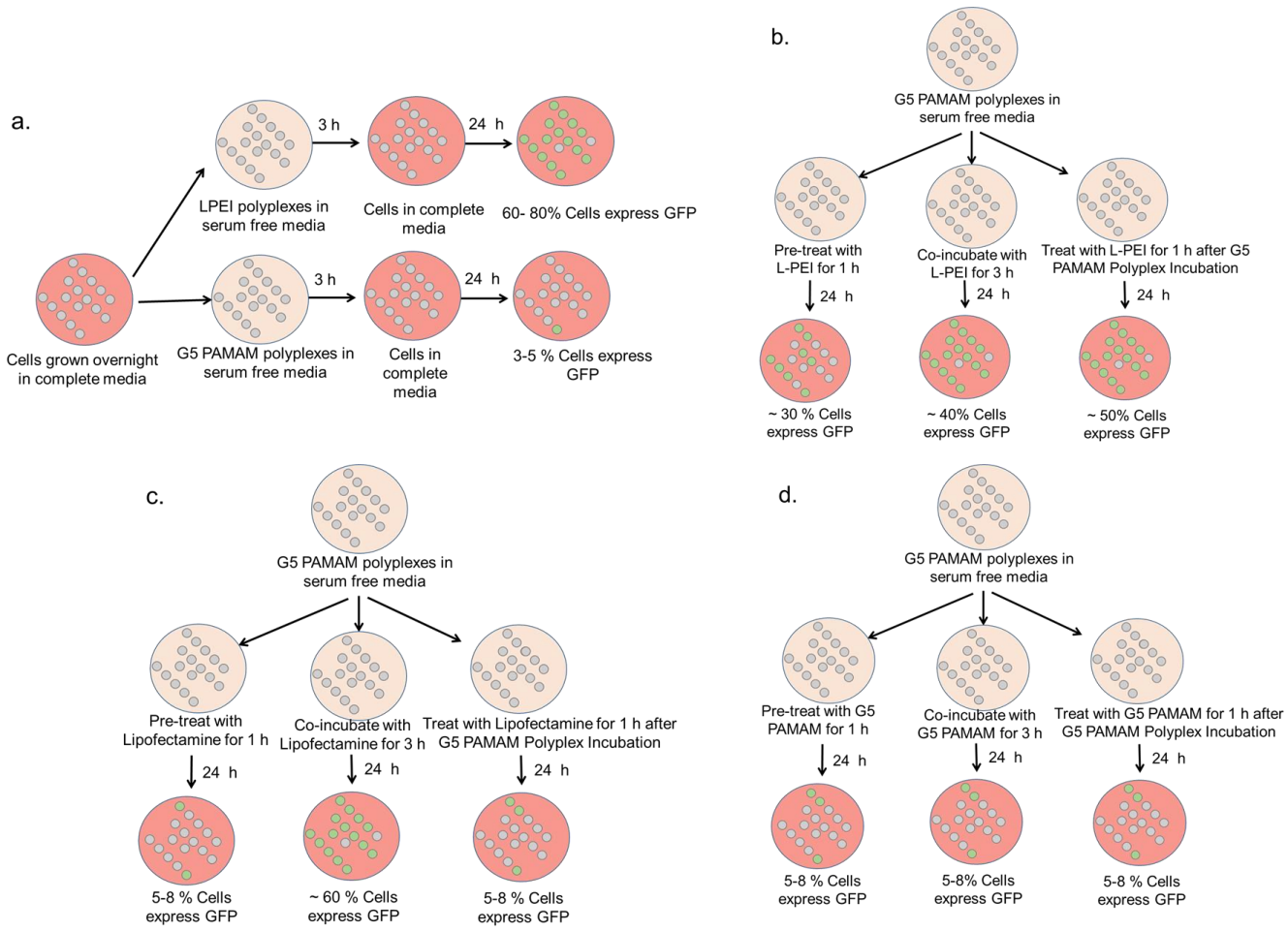


Figure S15: Summary of experiments that tested GFP expression in cells. (a) 60-80% of HEK 293A cells treated with L-PEI polyplexes (N:P = 10) showed GFP expression. Under 5% of cells treated with G5 PAMAM polyplexes (N:P = 10) showed gene expression.(b) Adding L-PEI to cells treated with G5 PAMAM polyplexes resulted in increased genen expression. (c) Lipofectamine only increased expression dramatically if co-incubated with G5 PAMAM polyplexes. (d) Adding extra G5 PAMAM does not increase gene expression.

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