APPENDIX (Theory of instruments used in the present work)

1. **Sonicator: Modern industrial corporation (Mumbai, India)**

Sonication is the act of applying sound energy to agitate particles in a sample and the technique is used for various purposes. Usually, the ultrasonic frequencies are used and hence the process is also known as ultrasonication. Bath sonicator was used to ensure the complete solubility of the polymer samples.

2. Particle size and zeta analyzer: NanoBrook 90 Plus (Brookhaven, USA)

Particle size can be calculated by measuring any one of the following features of the scattered light:

(i) the average intensity changes as a function of angle or

(ii) the change in polarization,or

(iii) the change in the wavelength, or

(iv) the change in the average intensity of light.

Dynamic light scattering (DLS) OR quasi –elastic light scattering (QELS) is based on the change in the average intensity of the light. In this method, a vehicle containing the nanoparticles (or nanoplexes or complexes) is placed in the path of a monochromatic beam of light and the temporal fluctuations of the scattered light due to the Brownian motion of the particles are determined. In the present investigation, NanoBrook 90Plus Particle Size Analzyer (Brookhaven Instruments, USA) was used to determine the size of the nanoplexes.

Zeta potential measures the surfaces charge of the particles. It is usually of the same sign as the potential actually at the particle surface. Since it reflects the effective charge on the particles, it is, therefore, related to the electrostatic repulsion them. Moreover, surface charge is an important phenomenon to determine various characteristics such as the interaction of DNA with the polymer conjugates and also the complex of pDNA /polymer conjugates (nanoplexes/ nanoparticles) with the cell surface proteoglycans. In the present study NanoBrook 90Plus Particle Size Analzyer (Brookhaven Instruments, USA) was used to carry out zeta potential measurements.

Briefly, plasmid DNA (pDNA) complexes were prepared by adding the various concentrations of polymer conjugates and pDNA followed by quick vortexing and

incubation for 30min at room temperature. References measurements using malvern size and zeta potential standards were run routinely to verify correct instrument operation.

3. Fluorescence Microscope: FSX100 (Olympus, USA)

Fluorescence is the well-established physical phenomena of the absorption and subsequent re-radiation of light by organic and inorganic specimens.The emission of light through the fluorescence process is nearly simultaneous with the absorption of the excitation light due to a relatively short time delay between photon absorption and emission (usually less than a microsecond in duration). Schematic of functioning of fluorescence microscopy is shwon in Fig 16 as below.

The basic function of a fluorescence microscope is to irradiate the specimen with a desired and specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light. In a properly configured microscope, only the emission light should reach the eye or detector so that the resulting fluorescent structures are superimposed with high contrast against a very dark (or black) background. The limits of detection are generally governed by the darkness of the background, and the excitation light is typically several hundred thousand to a million times brighter than the emitted fluorescence.

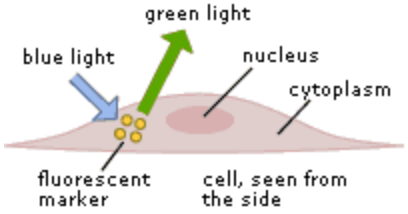

Fig 16: Schematic of functioning of fluorescence microscopy.

The samples were fixed in formaldehyde prior to visualization. Formaldehyde was used as its small molecular weight allows it to penetrate cells and tissues rapidly; making it a choice fixative for thicker samples and autofluorescence of unreacted aldehyde groups is not usually a problem. As the commercial grade formaldehyde solution contains additives such as methanol to formalin, which may be undesirable, the formaldehyde (37%) was freshly preparedeach time before use. The samples were fixed by adding 500 µl

formaldehyde (37%) and then fixed samples were observed under the fluorescence microscope (FSX100, Olympus, USA) by adjusting the optical, condenser and filter adjustments and pictures were captured using the software provided.

4. Flow Cytometer: QUANTA SC (Beckman Coulter, USA)

The flow cytometry is a laser-based, biophysical technology employed in cell counting and cell sorting by suspending cells in a stream of fluid (sheath fluid) and passing them by an electronic detection apparatus. It is a powerful technique that allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. A flow cytometer is similar to a microscope but instead of producing an image of the cell, flow cytometry offers automated quantification of set parameters (such as transfected cells, viable cells etc.).

There are two important terms used in the flow cytometry and those are data acquisition and data analysis. The term 'acquisition' is the process of collecting data from samples using the flow cytometer. Acquisition is mediated by a computer physically connected to the flow cytometer, and the software which handles the digital interface with the cytometer. The software is capable of adjusting parameters (i.e. voltage, compensation, etc.) for the sample being tested, and also assists in displaying initial sample information while acquiring sample data to ensure that parameters are set correctly. The analysis of acquired data is done with the help of software provided with the instrument.

Gating: The data generated by flow-cytometer can be plotted in a single dimension, to produce a histogram, or in two-dimensional dot plots or even in three dimensions. The regions on these plots can be sequentially separated, based on fluorescence intensity, by creating a series of subset extractions, termed "gates." Specific gating protocols exist for desired purposes especially in relation to transfection and hematology. The plots are often made on logarithmic scales and because different fluorescent dyes' emission spectra overlap signals at the detectors have to be compensated electronically.

Sample Preparation: As a general requirement, all samples should be prepared in a single-cell suspension form at a concentration of 6-8 million per ml to 10-20 million/ml (depending on cell type). In case of the experiments conducted in 48 well plate, the cells

were taken in a volume of 500 µl as the cell count would be too low.But in case of cell cycle analysis the DNA analysis is need to be performed and there fewer cells/mL were sufficient. Immediately before it's brought for analysis, samples should be filtered through a 0.45 micron filter to remove any clumps or aggregates prior to analysis. This will ensure the removal of any larger clumps before the sample is introduced into the cell sorter (for avoiding clogging of the flow cell which would cause a considerable delay in the sorting of the sample).

All the experiments were carried out in adherent tissue culture cell lines, and the protocol for adherent cells is as below:

Protocol:

1. The PBS buffer (phosphate buffered saline pH 7.4 and 1% BSA) was prepared and in some experiments readily available commercial HBSS buffer was use.
2. The cells were washed with 500 µl of PBS or HBSS.
3. The cells were trypsinized using 0.25% trypsin solution (aliquot of frozen solutionwere removed just before experiment). Trypsinization was done for 20 sec to 1.5 min depending on cell type.
4. Trypsin was removed carefully by applying gentle suction.
5. The cells were then washed with PBS or HBSS to remove any residual trypsin.
6. One ml of serum containing media (DMEM with 10 % FBS) was added and cells were suspended into it. It should be note that serum containing media was used for suspending the cells so as to counter the activity of trypsin, in case it is not removed completely.
7. The cells were transferred to a 1.5 ml tube containing 1 ml of PBS/HBSS.
8. The tube was centrifuge at 600 g for 5 minutes.
9. Supernatant wasdiscarded and resuspended the pellet in fresh PBS or HBSS.
10. The tube was again centrifuged at 600 g for 5 minutes
11. Supernatant was discarded and resuspended the pellet in fresh PBS or HBSS.

5. Animal Imager: IVIS Lumina II (Caliper Life Sciences, UK)

Fluorescence imaging works on the basis of fluorochromes inside the subject that are excited by an external light source whereupon it emits light of a different wavelength in

response. Traditional fluorochromes include GFP, RFP, YFP and their many mutants. However significant challenges emerge in vivo due to the autofluorescence of tissue at wavelengths below 700 nm. This has led to a transition to near-infrared dyes and infrared fluorescent proteins (700 nm-800 nm) which have demonstrated much more feasibility for in vivo imaging due to the much lower autofluorescence of tissue and deeper tissue penetration at these wavelengths.

Despite the numerous published reports on the efficiency of imaging with fluorescent proteins, there are comments in the literature denying these facts. In spite of their extensive use for in vivo imaging, GFP and fluorescent proteins are associated with many misconceptions about GFP imaging especially compared to luciferase. GFP is not toxic and cancer cells with GFP are as aggressive and malignant as the cells without GFP. Cell lines can be made very bright with fluorescent proteins with no toxicity. The in vivo signal from fluorescent proteins is at least 1,000 times greater than luciferase and is so bright that a single molecule of GFP can be seen in a bacterium. Moreover, GFP can be observed through the skin on deep organs. Skin autofluorescence presents no problem for in vivo GFP imaging with proper filters and even further fur can be rapidly clipped removing this autofluorescence.

The IVIS Lumina II (Caliper Life Sciences, UK) is coupled with charge-coupled device (CCD) camera having 13 x 13 mm square dimensions and has resolution of 1024 x 1024 pixels that yields higher imaging resolution. The CCD is thermoelectrically (Peltier) cooled to -90 ºC ensuring low dark current and low noise. These types of CCDs have found applications in the field of biomedical research in low-light applications including small animal imaging. The instrument is provided with different set of filters out of which few combinations were tried and one set is selected. The filters used for GFP are for emission λ_{max} is 430 nm and for excitation λ_{max} is 500 nm.