Identifying the underlying cause of limitations in Salmonella mediated gene transfer for the development of a successful cancer gene therapy

Honors Thesis

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Abstract

With cancer being still among the top prevalent diseases, many novel treatment options have been invented to improve on serious side effects experienced by the patients commonly administered with chemotherapy drugs. In the recent decade, salmonella-mediated cancer therapy has gained prominence in its ability to efficiently target and penetrate tumor tissue. Many therapeutics agents including proteins and chemotherapy drugs have been successfully delivered to the tumor microenvironment, however, relatively limited success has been observed in salmonella-mediated gene transfer therapy. Despite a successful bacterial invasion into the mammalian cancer cell, poor efficiency in regards to the number of cells expressing the plasmid is observed. The purpose of this project is to examine how a nuclear localization signal on a plasmid impacts DNA transport from salmonella to the nucleus. Attenuated salmonella strain VNP20009 with overexpressing flhDC increases invasion efficiency. In order to ensure the intracellular occupancy of the vector, optimizing the invasion of salmonella carrying the plasmid in the tumor cell is crucial. A plasmid containing lysis cassette and EGFP gene driven by the CMV promoter with absence or presence of SV40 origin is bactofected into mammalian cancer cells to observe the effect of SV40 origin on nuclear localization of the plasmid. However, no EGFP expression was observed in mammalian cells, which led to the hypothesis that the entrapment of plasmid in salmonella containing vacuoles (SCV) inhibits the plasmid expression in mammalian cells. Understanding the mobility of the delivered plasmid in the cancer cell through in situ hybridization could provide insights critical for the successful development of salmonella-mediated gene transfer therapy for treating cancer.

Introduction

The current era has seen a boom in new, unique, and effective cancer treatments. Research in cancer gene therapy has received notable acknowledgment in offering many promising treatments. Gene therapy has been extensively researched to produce possible future treatments for gliomas, lung, pancreatic, liver, and many other cancer types [1]. A biological understanding of the genetic mechanisms in cancer cells has shone light on many potential genes that can be targeted or developed as part of the gene therapy. Many approaches such as gene silencing of an oncogene, overexpression of tumor suppressor genes, the introduction of genes causing cell apoptosis, immunogenic therapy have been thoroughly researched in the development of many preclinical gene therapies [2].

However, in order to develop effective gene therapy, many challenges on a systemic and molecular level need to be addressed. The clearance time of the therapeutic system should be long enough for the therapeutic to reach the target before the immune system clears it yet short enough that its presence begins negatively affecting the body. The therapeutic system selectively targets the tumor tissue sparing the normal living cells unlike the generic target of chemotherapeutic drugs to dividing cells that infamously develop dangerous side effects aiding in patient's trauma. Even at the tumor site, there are many boundaries that the therapeutic system needs to cross before reaching the gene target. Many chemotherapeutic drugs are struck with diffusion limitation such that they can efficiently clear out the outer layers of tumor tissue, but fail to penetrate deep into the necrotic and quiescent regions of the tissue. In terms of direct gene

transfer, direct injection of the therapeutic to the tumor site has resulted in low uptake of gene transfer vectors by the cells [3]. Moreover, the therapeutic has to penetrate both the cell membrane and nucleolar membrane to reach the nucleus of the cell and get expressed by the mammalian cells.

Considering these limitations, using salmonella typhimurium overcomes many of these hurdles because they preferentially target and are cytotoxic to cancer cells, sense the cell microenvironment and respond to the surrounding stimuli, have the ability to transfect cells, and can be easily detected externally [4], and therefore it is perfect for delivering plasmid to the cancer cells. Administration of attenuated salmonella also minimizes the immune stress due dampened expression of the virulence gene.

Salmonella consists of type III secretion systems 1 and 2 (T3SS1 and T3SS2) whose interplay allows it to inject effector protein in host cells' cytoplasm. In a T3SS dependent *Salmonella* invasion, T3SS1 effector proteins are secreted into the host cells' cytoplasm through a needle apparatus. Some of these effectors drive actin mediated ruffling of cytoskeleton and endocytosis of *salmonella* into salmonella containing vacuoles (SCV). T3SS2 is induced for further development of SCV biogenesis. Within 1-2 hours, the majority of SCVs become enriched in proteins like Lamp1, Rab7, and vacuolar ATPase. Some SCVs do not undergo biogenesis and lyse leading the bacteria to escape from vacuole to the cytosol or are targeted by the cell's autosome system. The mature SCV begins extending long tubular structures (Sifs), enriched in Lamp1, stemming from the surface of SCV post 4-6 hour invasion [5].

Research has shown that S. typhimurium accumulates in tumor tissue in relatively large numbers compared to the normal body cells [6]. The engineered bacterial system has enhanced features that optimized the mobility of the cell and its invasion into the mammalian cell. Vishnu et al. have demonstrated that *salmonella* overexpressing *flhDC* increases intracellular accumulation in tumor cells and drive tumor colonization. Therefore, the developed recombinant *Salmonella* strain is characterized by improved colonization, invasion, and delivery of therapeutics [7].

The goal of the project is to understand the limitations associated with mammalian expression of therapeutic to further engineer the S. typhimurium system that optimizes the nuclear localization of therapeutic (a vector encoding EGFP under mammalian promoter). Mechanism underlying plasmid delivery after *salmonella* invasion and lysis are unknown. To better understand the delivery process fluorescence in-situ hybridization technique is used for plasmid localization in tumor intracellular environments. Understanding the underlying mechanisms preventing the expression of the vector delivered by S.typhimurium would aid in the development of a novel gene therapy that addresses the boundaries mentioned earlier.

Experimental plan

A recombinant *salmonella* strain can be produced by integrating a vector consisting of lysis cassette, tdTomato cassette, and EGFP cassette. The expression of tdTomato is controlled by lacUV5 bacterial promoter, so the gene is constantly expressed in the bacteria. While EGFP is

controlled by CMV mammalian promoter, and therefore the expression is not activated until the plasmid reaches the nucleus of the tumor cell.

The 4T1 breast cancer cell line will be infected with *salmonella* carrying the designed vector, and it is observed that the tumor cells do not express the EGFP cassette. It is hypothesized that after *salmonella* lysis upon invasion into the mammalian cell, the vector remains trapped in salmonella containing vacuoles and the vector does not localize to the nucleus. To validate the vector, it will be transfected to the cells using lipofectamine 3000 reagent, which is known to transport nucleic acid to the nucleus using vesicles. In-situ hybridization is used to locate the plasmid within the intracellular environment. As a negative control, the technique is also performed on cells infected with scrambled plasmid to ensure probe specificity to just plasmid.

Methods

Plasmid Design and Cloning

SSEJ-lysE plasmid constructed by cloning lysis cassette (lysinE under control of SSEJ promoter) into PBAD-his-myc plasmid (*Invitrogen*, Carlsbad, CA). tdTomato coding sequence is isolated from tdTomato-N1 plasmid (*addgene*) through PCR amplification. The tdTomato cassette (tdTomato under lacUV5 bacterial promoter) is cloned into SSEJ-lysE plasmid through 2pc-gibson assembly. The forward primer of the cassette was designed such that the lacUV5 promoter and shine Dalgarno sequence was inserted between the overhangs. An EGFP cassette

consisting of EGFP sequence, amplified from PEGFP-N1 plasmid (addgene), under CMV mammalian promoter was cloned into SSEJ-lysE-tdTomato plasmid.

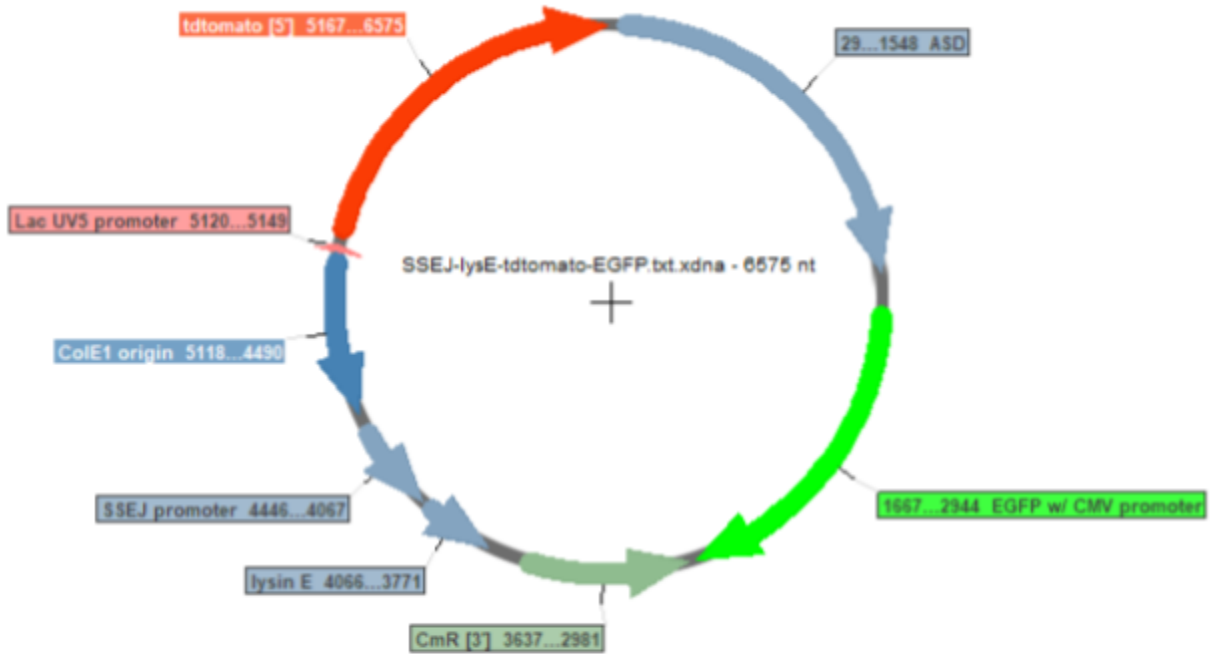

Figure 1 SSEJlysE-tdTom-EGFP vector map that includes lysis cassette (lysinE under SSEJ promoter), tdTomato cassette (tdTomato under Lac UV5 promoter) and EGFP cassette (EGFP under CMV promoter)

Bacterial transformation and invasion assay

The SSEJ-lysE-tdTom-EGFP plasmid is transformed in DH5α before *salmonella* transformation since it is more stable strain, has better transformation efficiency, produce greater yield in terms of purified plasmid. Moreover, the plasmid purification before *salmonella* transformation removes salts and other impurities that may affect electroporation and lower transformation efficiency. Transformed salmonella is grown and the OD of the culture is measured. 4T1 cells

with ~70% confluency grown in a 6 well plate were infected with 1E7 bacterial cells (calculated based on OD) per well. The cells were washed and treated with gentamicin to kill extracellular *salmonella* in the tumor microenvironment.

Immunofluorescence Staining Protocol

The infected 4T1 cells were fixed at 6 ,12 and 18 hours post infection using 10% formaldehyde. Cells are permeabilized by incubating them in a blocking buffer (3% BSA, 0.5% triton in 1xPBS) for 1 hour at room temperature. The coverslips are stained with primary LAMP1 rabbit unconjugated antibody, followed by conjugated anti-rabbit secondary antibody to detect SCV. Conjugated FITC antibody is stained last for salmonella detection. It is important that staining is important in a dark moist chamber, and the coverslips are stored in a blocking buffer, covered in tin foil, and refrigerated overnight.

Fluorescence in-situ hybridization Protocol

Cells are washed with 1x PBS, dehydrated with 80%, 95%, and 100% ethanol for 3 minutes each, and air dried for 20 minutes. The FISH probe along with the cells are next denatured in hybridization (4M Urea, 5x Saline sodium citrate buffer(SSC), 1% dextran sulfate powder) at 80 ℃ for 30 minutes. The probe (1 µg/µL) is added in 1:500 proportion to the cells and allowed to hybridize at 42 ℃ overnight with the well plate wrapped in tin foil. The cells were then washed with 4M Urea/2x SSC (50:50) three times at 42 ℃ and blocked with 4x SSC/0.1% triton/5%

BSA for 15 minutes at room temperature. The coverslips with cells are mounted on slides using mounting media containing DAPI after three 2x SSC washes. The mounting media is allowed to dry and fluorescence microscopy at 100x was performed.

Results

Cells infected with *Salmonella* carrying SSEJlysE-tdTom-EGFP vector did not fluoresce green. Upon reinfection, the intracellular microenvironment of tumor cells was observed 6 hours, 12 hours, and 18 hours post *salmonella* infection by fixing the cells at each time point using paraformaldehyde. The fixed cells are visualized using fluorescence microscopy post FISH and immunoblotting. Figure 2,3, and 4 show three different views of intracellular microenvironment of tumor cells at 18 hours post infection. The figures are arranged such that all the frames display at least DAPI and LAMP1 stain, while the right column and bottom row respectively display FISH and FITC stain in addition to the former two.

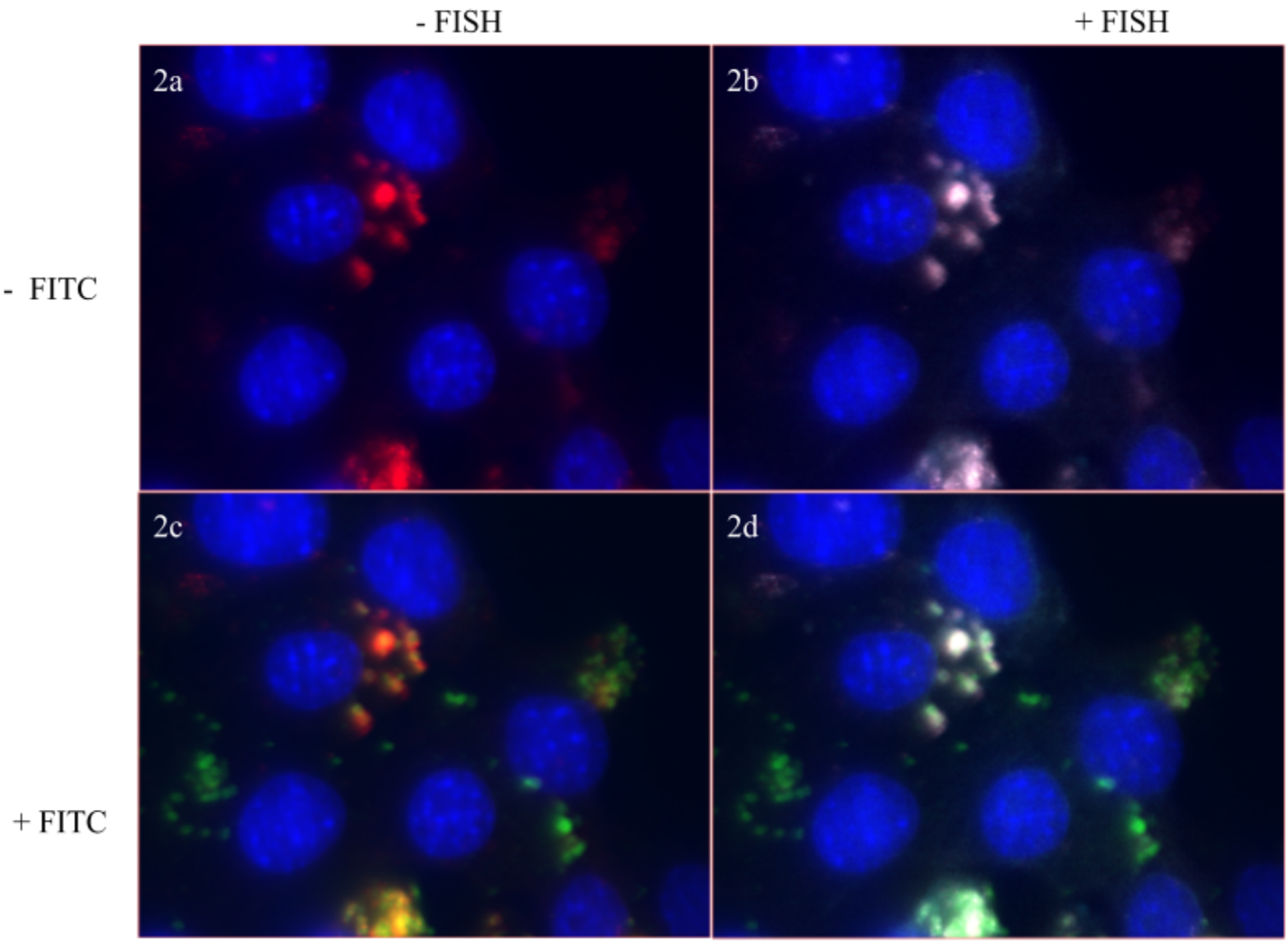

Figure 2. Fluorescence microscopy post FISH and immunohistochemistry of **Bla** cells infected with *salmonella* carrying SSEJlysE-tdtom-EGFP plasmid. All four frames represent cell microenvironment at 18hr post infection in which DNA is stained with DAPI (blue), *salmonella* with FITC (green), SCV with LAMP1 (red), and plasmid using FISH probe (cyan).
2a. Frame including DAPI and LAMP1 staining
2b. Frame including DAPI, LAMP1, and FISH staining
2c. Frame including DAPI, LAMP1, and FITC staining
2d. Frame including DAPI, LAMP1, FITC, and FISH staining

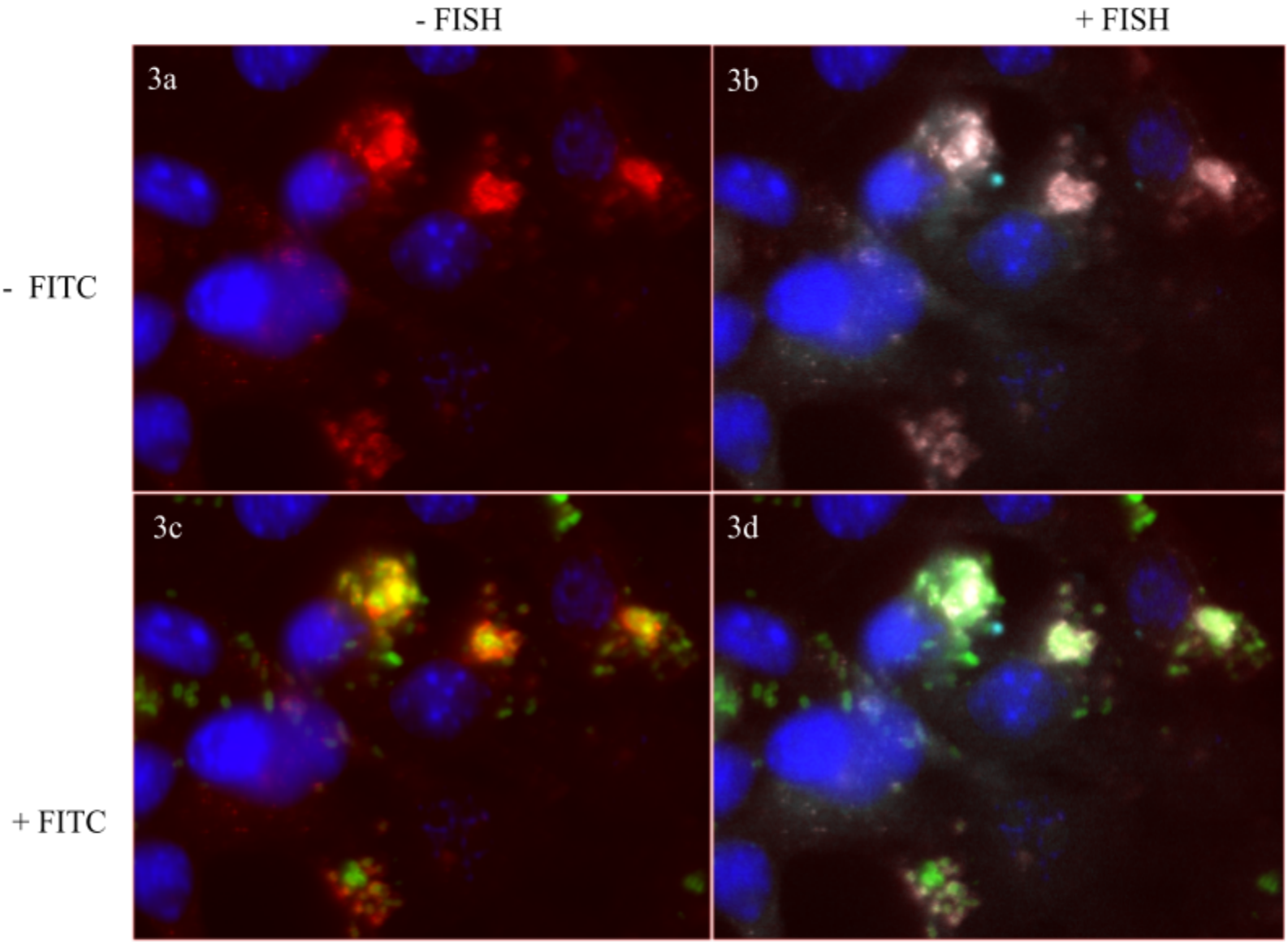

Figure 3. [Location 2] Fluorescence microscopy post FISH and immunohistochemistry of **Bla** cells infected with *salmonella* carrying SSEJlysE-tdtom-EGFP plasmid. All four frames represent cell microenvironment at 18 hour post infection in which DNA is stained with DAPI (blue), *salmonella* with FITC (green), SCV with LAMP1 (red), and plasmid using FISH probe (cyan).
3a. Frame including DAPI and LAMP1 staining
3b. Frame including DAPI, LAMP1, and FISH staining
3c. Frame including DAPI, LAMP1, and FITC staining
3d. Frame including DAPI, LAMP1, FITC, and FISH staining

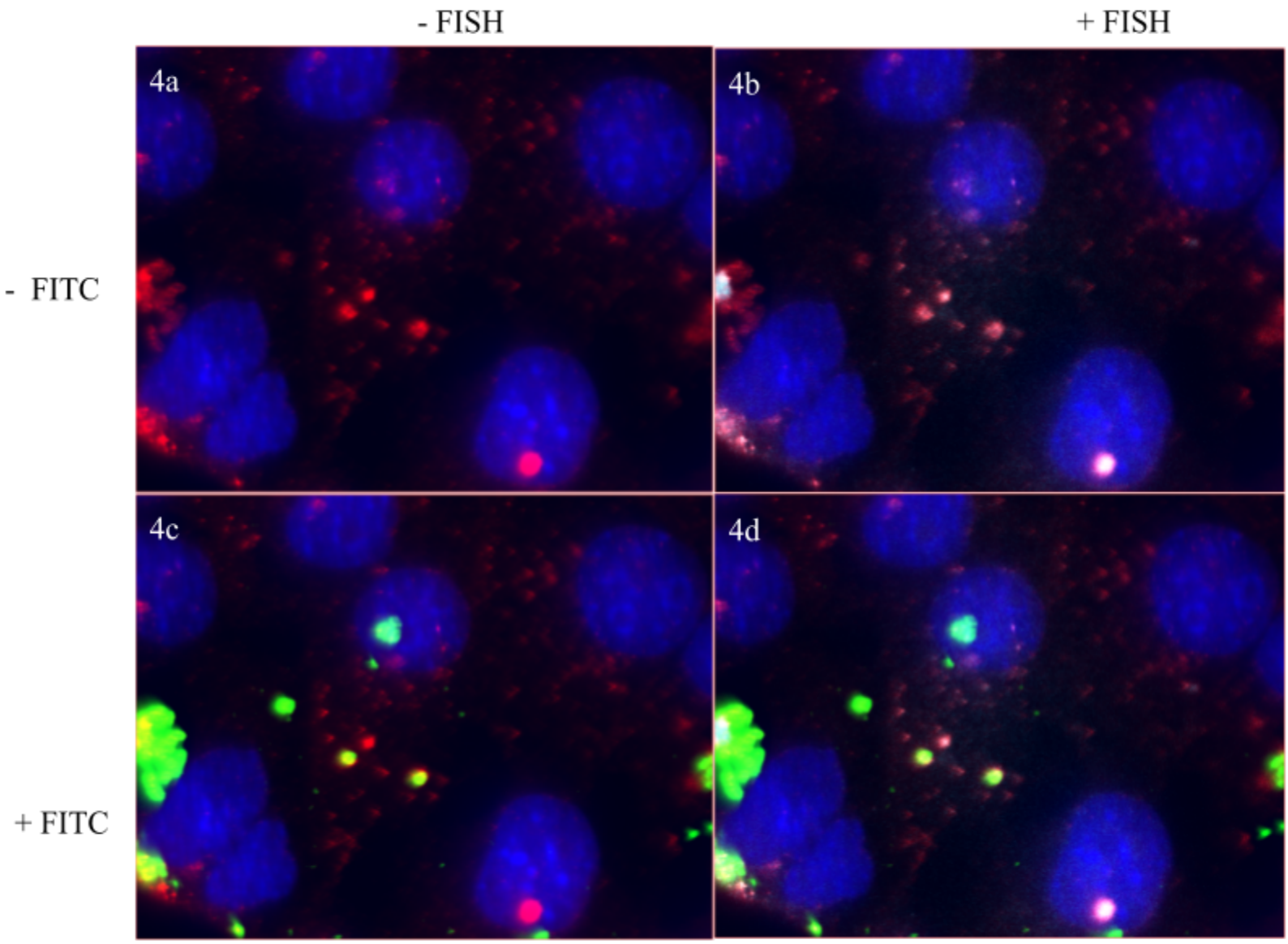

Figure 4. [Location 3] Fluorescence microscopy post FISH and immunohistochemistry of **Bla** cells infected with *salmonella* carrying SSEJlysE-tdtom-EGFP plasmid. All four frames represent cell microenvironment at 18 hour post infection in which DNA is stained with DAPI (blue), *salmonella* with FITC (green), SCV with LAMP1 (red), and plasmid using FISH probe (cyan).
4a. Frame including DAPI and LAMP1 staining
4b. Frame including DAPI, LAMP1, and FISH staining
4c. Frame including DAPI, LAMP1, and FITC staining
4d. Frame including DAPI, LAMP1, FITC, and FISH staining

Discussion

The expression level of EGFP is completely absent in cells infected with *salmonella* carrying the vector, suggesting that the nuclear localization of the plasmid did not occur. Most invaded *salmonella* are contained in a salmonella containing vacuole (figures 2-4), and only these undergo lysis; The bacteria present in the cytosol do not lyse. *Salmonella*'s T3SS system is activated along with SSEJ promoter, part of the lysis cassette, upon its invasion and endocytosis into SCV. Although the plasmid is released from *salmonella* as it lyses, it is hypothesized that it remains trapped in the SCV which prevents the nuclear localization of the vector. If the plasmid is released in the cytoplasm, it is expected to observe EGFP expression in any cells even if the efficiency of nuclear localization is poor. However, the complete absence of EGFP expression can be explained by trapped plasmid in SCV because it cannot integrate into the nucleus even during mitosis. In order to assess expression of the vector, *salmonella* carrying the plasmid should be transfected to the 4T1 cells using lipofectamine 3000 reagent. If the cells do not fluoresce green using this method, then the vector needs to be redesigned to ensure that EGFP can be expressed by the tumor cells.

Intravacuolar *salmonella* are indicated in region where FITC fluorescence overlap with LAMP1's. It is observed from figures 2 and 3 that most of the LAMP1 signal overlaps with FITC, however in figure 4, LAMP1 spot is visualized in the bottom right region of each frame where FITC signal is absent (figure 4c). The FISH probes's fluorescence localizes with the

LAMP1 fluorescence despite the absence of FITC signal. This indicates the presence of the vector in SCV where *salmonella* are absent, which seems unreasonable since *salmonella* are carrying the plasmid. It seems that The FISH signal colocalizes exactly with the LAMP1 signal as observed from comparing frames in the first row (figures 2,3 and 4). It is possible that LAMP1 fluorophore is bleeding through the probe's fluorophore. One way to validate the result is to infect 4T1 cells with *salmonella* carrying scrambled plasmid and perform FISH-immunostaining. Any observed signal from probe could indicate aspecific binding of the probe or bleed through the fluorophores. An infection assay and staining procedure without addition of the probe should be followed to distinguish the background fluorophore from actual signal.

Conclusion

The objective of the project is to optimize the nuclear localization of plasmid, delivered by *Salmonella Typhimurium* through their invasion in the 4T1 tumor cell line. Optimization of gene transfer and expression is significant in that it allows the development of an effective bacterial therapy that could potentially be a solution addressing the common challenges faced by clinicians and patients with current treatments. Moreover, such optimization not only opens the door to countless applications and may also provide useful background in the development of a new bacterial therapy. For example, the plasmid encodes protein product, which can silence oncogenes, increase expression of suppressor gene, mutate genes that control cell cycle or expression of other genes. Thus, increasing the efficiency of plasmid expression is important in

targeting tumor cell proliferation and developing a *salmonella* based gene therapy that can induce tumor suppression.

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