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Logical Circuits for Vaccinia Virus Vectors

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Logical Circuits for Vaccinia Virus Vectors

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Abstract

Vaccinia virus (VACV) is well known for its use as the vaccine in the successful campaign to eradicate smallpox and a powerful vector for vaccines, immunotherapies, and oncolytic viral therapies. Advancements in synthetic biology have recently led to the development of synthetic gene circuits, which can use recombinases to respond to inputs with logic and memory. We propose that this technology can be employed to make “logical” VACV vectors which could be programmed to change their actions based on sensory inputs for use in the development of safer vaccines or oncolytic viral therapy agents which selectively lyse cancer cells. In this project we tested the functionality of recombinases Bxb1 and PhiC31 in synthetic VACV circuitry. We developed simple synthetic circuits with VACV promoters wherein each recombinase can be induced under *lac* or *tet* operon elements to irreversibly invert a promoter, which switches fluorescent reporter expression from red to green and can be observed in VACV infection/transfection assays. We detected only red fluorescence with little to no green in cells transfected with *bxb1* plasmids, suggesting that no promoter inversion occurred and Bxb1 may not be functional in VACV-infected cells. Green fluorescence indicative of promoter inversion was detected in all cells transfected with *phiC31* plasmids. This occurred regardless of absence of inducer, suggesting PhiC31 is highly functional in VACV-infected cells but cannot be regulated by TetR or LacI repressor proteins, even when they are pre-expressed at high levels. Future recombinase-based synthetic circuitry in VACV will thus require tighter repressor systems.

INTRODUCTION

Vaccinia Virus Overview

Vaccinia virus (VACV) is a large, enveloped, double-stranded DNA virus prototypical of the family *Poxviridae* and the genus *Orthopoxvirus.* The VACV infectious cycle begins with attachment and entry of a virion into a susceptible cell. Entry occurs by fusion of the viral lipid membrane to the cell membrane, releasing the core into the cytoplasm (1), where replication occurs (2). Transcription is divided into early, intermediate, and late stages. Early transcription is coupled to the replication of the viral genome (3), which once completed proceeds to intermediate and late stages when genes for the synthesis of proteins for new viral particles are transcribed (4). Transmission of VACV between cells can take place by spread of intracellular mature virus after cell lysis, movement of cell-associated enveloped virus on actin tails to adjacent cells, and by extracellular enveloped virus traveling to proximate or distant cells (5).

Applications of Recombinant Vaccinia Virus Vectors

VACV was employed as the vaccine in the World Health Organization campaign to eradicate smallpox (6). As a result, the scientific community has extensive understanding of VACV. The VACV genome can accommodate over 25 kilobases (kb) of exogenous DNA (7), and such “recombinant” VACVs are

excellent expression vectors in eukaryotic cells. Additionally, recombinant VACVs have been shown to elicit strong humoral and cell-mediated immune responses both for vaccine and oncolytic therapy applications (8, 9). By expressing exogenous antigens, a recombinant VACV can train the immune response against different pathogens. Recombinant VACV vectors have accordingly been used extensively as both human and animal vaccines (10), including oral rabies vaccines for wildlife (11-13) and recently, promising HIV vaccines (14, 15). VACV has also been used for cancer vaccines, eliciting an immune response against cancers by expressing tumor-associated antigens (16). VACV has also shown great potential as an agent for oncolytic viral therapies since it can replicate in many mammalian tissues and destroys tumors both directly by virus-mediated cytotoxicity and by inciting the host cell-mediated effector immune response (17). Additionally, it has also been implemented in tumor-directed gene therapies, wherein viruses are targeted specifically to tumor cells and used to express enzymes which interact with prodrugs administered to the patient, increasing cure rate and prolonging survival (18).

The clinical use of these VACV-based therapies is limited by complications due to uncontrolled viral replication ranging from mild rash and fever, to rare but severe adverse reactions. These are usually limited to individuals with pre-existing susceptibilities, such as eczema in the case eczema vaccinatum and immune deficiency in the case of progressive vaccinia (19). As a result, many researchers have elected to use replication-deficient or attenuated VACV strains such as Modified Vaccinia virus Ankara (MVA) (20). However,

interest in using replication-competent and more immunogenic strains such as Western Reserve (WR) has prompted the development of regulatory systems for the situational control of vaccinia gene expression and replication, including inducible systems using elements of the *lac* or *tet* operons, and a repressible system using a reverse tet repressor (Hagen, Jasperse and Titong, unpublished), (21-23).

Lac and Tet Operons

The *lac* operon is the prototypical bacterial gene regulatory unit and its discovery and characterization are of great historical importance in molecular biology (24). Originally described by Jacob and Monod, it was the basis for the understanding of bacterial gene regulation, wherein the protein products regulate the expression of genes at the level of transcription depending on environmental conditions (25). In the *lac* operon, “structural genes” including β-galactosidase and a lactose transporter are downstream of an operator sequence, to which the lac repressor protein LacI binds, preventing transcription in the absence of the metabolite allolactose (26-28). An artificial analog for allolactose developed by Monod, 1-isopropyl-β-d-thiogalactoside (IPTG), can be used as a gratuitous inducer of the *lac* operon (26).

The *tet* operon was also discovered in *Escherichia coli*, in which the expression of TetA, a transporter protein enabling resistance to tetracyclines, is regulated analogously to the *lac* operon; repressor protein TetR binds tightly to the *tetO* operator sequence in the absence of tetracyclines, preventing

expression of *tetA*, but in the presence of tetracyclines a conformational change takes place which decreases affinity of TetR for the operator and allows for *tetA* transcription (29, 30). This regulatory system has been extensively used as a transactivator in both prokaryotes and eukaryotes to regulate both endogenous and transgene expression (31-33). Both the *lac* and *tet* repressor systems have been previously characterized in VACV vectors (22, 34, 35).

Applications of Current Tools in Synthetic Biology

Synthetic biology is a rapidly evolving field that is generally defined as the design and construction of novel biological systems or the re-design of existing biological systems to solve problems. In the former category, synthetic biologists develop artificial biomolecules which parallel known ones in the interest of creating artificial life, which although popular, exciting, and promising, is not the aim of our project and will not be discussed here (36). In the latter, scientists draw on a body of scientific knowledge about components of different biological systems and integrate them in various degrees of complexity to engineer a biological design (37). This technology can be used to assemble biological pathways for the synthesis of industrially important chemicals (38).

Of particular interest to us are synthetic regulatory gene networks. We believe that if VACVs could be developed with complex artificial regulatory systems that can interact with their environment, they would have myriad potential uses in medicine and research. Using well-studied and standardized

genetic elements, over the past decade and a half synthetic biologists have developed numerous varieties of genetic circuit elements capable of responding to environmental inputs. Using a pair of promoters and repressors, Gardner *et al.* developed a bistable genetic toggle switch to control gene expression (39). Transcriptional regulatory systems have also been built to oscillate on a timescale different from their host organism's replicative processes, allowing the state of the synthetic gene circuit to be transmitted and self-maintaining through generations (40). The implementation of recombinases, enzymes which catalyze the excision, inversion, and reinsertion of a target DNA sequence (41) in synthetic biology, or "recombinatorics", has opened the door to the development of computer-like logic in synthetic biology (42). At the simplest level, these recombinases catalyze irreversible inversions and allow for strict regulation of an inducible expression system (43). It is also possible to use recombinases that catalyze reversible inversion in conjunction with excisionases to create rewritable memory systems inside the genetic code of living cells (44). These technologies have demonstrated usefulness in bacterial and viral vectors with practical medical applications, including the engineering of bacteria to invade cancer cells or to destroy biofilms and thus serve as adjuvants for antibiotic therapies (45, 46). An excellent review on the current accomplishments and obstacles in the field of synthetic gene networks was written by Lu et al (47). Recently, Siuti *et al.* used recombinase-based synthetic gene circuitry to implement the sixteen Boolean logic gates in *E. coli* using two recombinases under different inducible

systems to integrate two signals into one logical output with “memory” which lasted through bacterial generations (48).

Bxb1 and PhiC31 Recombinases

For this project we sought recombinases that could carry out a unidirectional, irreversible inversion of a DNA segment flanked by recognition sequences specific to that enzyme, such that if a multi-recombinase logic system were ever developed, there would be no cross-reactivity between one recombinase and the recognition sites of another. Bacteriophage large serine recombinases catalyze viral integration by acting on a specific site on the viral genome (*attP*) and another on the host chromosome (*attB*), but can also catalyze the inversion of DNA segments resulting in gene expression (49). They do this by binding the DNA segments, cleaving them, repositioning their ends by subunit rotation, and ligating the segments together (50). We selected two large serine recombinases, Bxb1 (51) and PhiC31 (52) for use because they both have been thoroughly characterized (53), used by genetic engineers in eukaryotes (53-55), and were used by Siuti *et al.* in their Boolean logic circuits project (48), suggesting that there is no cross-reactivity between them and that they can be expressed as transgenes in eukaryotic cell lines.

Hypothesis

Recombinase-based synthetic gene circuits stand to greatly increase the potential uses and safety of VACV vectors. Because the VACV genome can accommodate 25 kb of exogenous genetic material, recombinant VACV vectors theoretically could be produced with multiple foreign or natural genes under a complex multi-recombinase regulatory system. We hypothesize that recombinase-based gene circuit regulation is possible in VACV. Because VACV replicates in the cytoplasm using its own transcriptional machinery, it is not possible to create a recombinant VACV with recombinase logic using existing synthetic circuitry because at the time of this paper none have included VACV promoters. The goal of this project was to demonstrate that recombinase-based regulation in a simple genetic circuit is possible in VACV. In the interest of ultimately building circuits with binary inputs, we sought to test two recombinases. We placed them under the control of a weak VACV promoter and inducible operator so their expression could be controlled by the administration of inducer molecules doxycycline (Dox) or IPTG. A reporter cassette was generated with a red and green fluorescence reporter on either side of a strong vaccinia promoter flanked by recombinase recognition sequences. If repression of recombinase activity with VACV transcription machinery is sufficient, then only red fluorescence reporter is produced. Induction of the recombinase results in inversion of the promoter, cessation of red fluorescence reporter expression and transcription of green fluorescence reporter (Figure 1).

MATERIALS AND METHODS

PCR of Recombinase Genes

Two recombinases (Bxb1 and PhiC31) and their recognition sites (53), were selected for use in our experiments on the basis of their previous use in mainstream synthetic biology projects with similar circuitry. *phiC31* was obtained from plasmid “pInt” (56) and *bxb1* was obtained from plasmid “Dual-Recombinase Controller” (57), both from Addgene (Cambridge, MA). Recombinase genes were PCR-amplified so that the final products would have 5’ and 3’ restriction sites to facilitate cloning into a vector backbone. Forward primers overlapped with the translation start site and included a 5’ spacer (6 bp) and restriction enzyme recognition site immediately preceding the start codon. Reverse primers overlapped with the sequence complementary to the 3’ end of the gene and included a 5’ spacer (6 bp) and restriction enzyme recognition site immediately preceding the complement of the stop codon.

Logical Circuit Plasmids

A vector backbone was synthesized to enable subcloning of red fluorescent reporter *DsRed* and green fluorescent reporter *EGFP*, recombinase *bxb1* or *phiC31*, and repressor gene *tetR* or *lacI*. Vector backbones were synthesized by a third party vendor DNA 2.0 (Menlo Park, CA) as high copy plasmids with a kanamycin resistance gene. Spacers of 25 bp from *ampR* were

included between restriction enzyme cut sites. Promoters were chosen based on the desired level of expression for each gene. Because even minimal expression of a recombinase could result in inversion of our target promoter, we sought to maximize expression of our repressor genes and have minimal but sufficient levels of expression of our recombinase when unrepressed. We also selected promoters with minimal sequence similarity to one another in order to avoid unwanted recombination events. We chose synthetic promoter $P_{E/L}$, the strongest known VACV promoter, for expression of *lacI* and *tetR*. The strong cowpox promoter P_{ATI}, as previously modified by the Verardi Lab (23), was selected for reporter gene expression. P_5, a weakened version of late promoter P_{11} (23) was chosen to drive recombinase expression. Restriction enzyme sites were selected based on the availability of genes from plasmids and enzymes in the Verardi Lab in order to minimize the need for PCR cloning and minimize the use of enzymes that would cut within the final transfer vector. Vector pPL189 was synthesized with recombinase recognition sites *bxb1 attB/attP* for use with *bxb1* under control of the *lac* operon (Figure 2A). Vector pPL190 was synthesized with recombinase recognition sites *phiC31 attB/attP* for use with *phiC31* under control of the *tet* operon (Figure 2B).

Inserts were cloned into vector backbones in parallel. Unless otherwise stated, plasmids were obtained from the Verardi Lab and enzymes were obtained from New England Biolabs (Ipswich, MA). Genes encoding respective repressor proteins were subcloned into the vector backbones first. The *lacI-wf* gene is a W200F mutant *lacI* that has been shown to produce a repressor with a 10-fold

greater decrease in leaky transcription (58). Our lab had previously synthesized *lacI-wf* with mutation in a TTTTNT to prevent early transcription termination by VACV transcriptional machinery. For cloning, *lacI-wf* was cleaved from pCO157 with PstI-HF and blunt cutting restriction enzyme SmaI and cloned into pPL189 cleaved with PstI-HF and blunt cutting restriction enzyme ScaI-HF to create pPL193. The *tetR* gene was obtained from pSMART10 because that version of the gene was also synthesized to lack a VACV terminator sequence TTTTTNT present in the wild type gene (14). pSMART10 was cleaved with SphI-HF and SmaI and cloned into pPL190 cut with SphI-HF and ScaI-HF to create pPL192 . *EGFP* was cloned into pPL192 and pPL193 from pCO191 with SacI-HF and NcoI-HF to create pPL194 and pPL195. *DsRed* was cloned into pPL194 and pPL195 from pCO191 with SpeI and NotI-HF to create pPL196 and pPL197. Recombinases were cleaved from their PCR amplicon into pPL196 and pPL197 with MfeI-HF and NotI-HF to create pPL198 and pPL199 for transient expression assays in VACV-infected cells (Figure 2C-D). To isolate the variables of each inducible system and recombinase, we then generated two more plasmids; in pPL200 *bxb1* is under control of the *tet* operon, and in pPL201 *phiC31* is under control of the *lac* operon. This was accomplished by digesting pPL198 and pPL199 with MfeI-HF and KpnI-HF and swapping out the inserts and vectors (Figure 2E-F).

Gene Cloning

Inserts were double digested from plasmids or PCR amplicons in the case of the recombinases, and vectors were double digested to remove spacers with restriction enzymes to create mutually compatible cleavage sites for directional cloning. Note that in the digestion of pPL198 with MfeI-HF and KpnI-HF, the insert and vector were of indistinguishable size, so to facilitate gel purification it was also incubated with NdeI or EagI-HF, digesting the unwanted fragment into smaller pieces. Vectors were dephosphorylated by incubation with Antarctic Phosphatase. Inserts and vectors were ligated with T4 DNA ligase and electroporated with a Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA) into NEB 5-alpha Electrocompetent *E. coli* (New England Biolabs), The electroporated suspension was plated onto low-salt LB agar plates with 50 µg/ml kanamycin and incubated for 16 hours at 37°C. Twenty well-isolated colonies were miniprepped and the DNA was evaluated for desired ligation and transformation results by diagnostic restriction enzyme digest and electrophoresis in an agarose gel containing ethidium bromide visualized by ultraviolet excitation. One culture verified to contain the desired plasmid was grown for a larger scale DNA isolation and use in subsequent cloning reactions. DNA isolation and purification was performed using a NucleoSpin® Plasmid kit (Macherey-Nagel, Bethlehem, PA). pPL198, pPL199, pPL200, and pPL201 were grown up, isolated, and purified on a larger scale using a NucleoSpin® Midiprep kit (Macherey-Nagel). After large scale-preparation with a commercial kit,

plasmids were reanalyzed with a restriction-enzyme digest and compared with the original vector to verify the identity of the product.

Cell Culture

African Green Monkey kidney epithelial cells (BS-C-1) and human HeLa-S3 cells were grown in adherent culture with Dulbecco's modified Eagle medium supplemented with glucose and amino acids (Invitrogen, Grand Island, NY) (cDME) and containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) at 37 °C in 5% CO_2 as described previously (22).

Viruses

L-variant Western Reserve (WR) VACV ATCC clone 9.2.4.8. (22) was used as our wild-type VACV strain and was previously amplified in HeLa-S3 cells, purified, and titered in BS-C-1 cells by the Verardi Lab. vP11IRG was previously developed, purified and amplified by our lab (Titong, Jasperse, Verardi unpublished data). It was generated by homologous recombination of plasmid pSP114 with the VACV thymidine kinase region. It contains *tetR* under a $P_{E/L}$ promoter, *lacI* under P_{11} and a *tet* operator, and *EGFP* under another P_{11} and a *lac* operator; so in the presence of Dox LacI is produced, preventing expression of EGFP. vS22 is a previously developed VACV ($vP_{E/L}$-Control) that expresses *tetR* constitutively under the $P_{E/L}$ promoter (21).

Transient Expression Assays

BS-C-1 or HeLa-S3 cells were seeded in 24 or 48-well cell culture plates to be 95% confluent at the time of the experiment. VACV stocks were sonicated in four 30-second bursts, vortexed between bursts, and diluted to achieve an MOI of 1 based on the desired infection volume and estimated number of cells per well. Cells were washed once with cDME, overlayed with virus dilution, and incubated for 1 hour post-infection during initial testing and 24 hours for subsequent testing when we wanted to pre-express LacI and TetR in vS22 and vP11IRG infected cells. Cells were then overlaid with cDME supplemented with 2.5% FBS +/- 1 mg/ml Dox or 1 mM IPTG and transfected with desired plasmids using FuGENE HD transfection reagent (Promega, Madison, WI). Cells were incubated at 37 °C in 5% CO_2. Results were obtained by fluorescence (Texas Red filter for DsRed and FITC filter for EGFP) and brightfield microscopy at 24, 48, and 72 hours post transfection using an Axio Observer D1 inverted fluorescence microscope (Carl Zeiss, Thornwood, NY). Images were captured using AxioVision software, release 4.8.1 (Carl Zeiss). Representative images were taken.

RESULTS

A pilot transient expression assay was performed in BS-C-1 cells using pPL198 and pPL199. Cells were infected with WR for one hour prior to transfection. 48 hours post transfection (p.t.), all wells transfected with pPL198 exhibited green fluorescence at similar levels to the transfection control with no detectable red fluorescence, which persisted for the course of the experiment. (Figure 3). Red fluorescence was detected in pPL199-transfected wells (Figure 3A) at levels similar to the transfection control (Figure 3C). After 72 hours, small amounts of green fluorescence were observed in the pPL199-transfected wells both with and without IPTG, which otherwise exhibited red fluorescence similar to transfection control levels (Figure 3B).

During the pilot experiment we observed that at 48 and 72 hours post infection, data collection became increasingly difficult due to the number of BS-C-1 cells that lysed or lost adherence to the plate, so subsequent assays were performed in HeLa-S3 cells. In order to confirm that the results of the pilot experiment were not due to improper insertion of fluorescent reporter genes or promoter inversion prior to transfection, we reanalyzed the placement and orientation of the recombinases in the plasmids via a different restriction digest and PCR, confirming our original determination that the plasmids were as designed.

Next we tested pPL198, pPL199, pPL200, and pPL201 (Figure 2) in HeLa-S3 cells infected with WR. To control for the possibility that in the first experiment PhiC31 was synthesized before sufficient repressor was produced to modulate

expression, we also tested *tet* operator plasmids pPL198 and pPL201 in cells infected with vS22, a recombinant VACV that constitutively expresses TetR, and *lac* operator plasmids pPL199 and pPL200 in cells infected with vP11IRG, a recombinant VACV that constitutively expresses LacI. Because vP11IRG expresses EGFP in a tetracycline-repressible fashion, cells infected with it were overlaid with media containing 1 mg/ml Dox, previously shown to repress EGFP expression to levels indistinguishable from WR (Titong et al., unpublished data). Our vP11IRG control (no plasmid transfected) results were consistent with this at 24 hours post infection (Figure 4A), however after 48 (Figure 4B) and 72 hours (Figure 4C), we noticed a few cells (less than 10 in a well of ~$9.4x10^4$ cells) with green fluorescence, suggesting that this virus may undergo mutation to constitutive EGFP expression. We also noted after 72 hours a detectable amount of EGFP fluorescence localized to every cell (Figure 6D), suggesting that a small amount of expression had occurred. These findings limit the power of our results from vP11IRG-infected cells.

The HeLa-S3 cells did maintain a better monolayer for fluorescence imaging than the BS-C-1 cells, even when infected with VACV, but once again we never observed any difference between groups with or without inducer (Figure 5). Cells infected with WR and transfected with pPL198 (Figure 5A) or pPL200 (Figure 5B) exhibited green fluorescence similar to the level of the transfection control (Figure 5E), with no detectable red fluorescence. This was also true for cells infected with rVACVs vS22 and vP11IRG to pre-express repressor proteins TetR and LacI respectively (Figure 5C-D). These data suggest

that PhiC31 is functional in our VACV synthetic circuitry, but it cannot be controlled by the *lac* or *tet* repressor systems.

Cells infected with WR and transfected with pPL199 (Figure 6A) or pPL201 (Figure 6B) exhibited red fluorescence at levels similar to the transfection control, with no detectable amount of green fluorescence. This is also true for cells infected with vS22 and transfected with pPL201 (Figure 6C). We did find a few green fluorescent cells in wells infected with vP11IRG and transfected with pPL199 (Figure 6D), however these were at levels indistinguishable from the vP11IRG control (Figure 6E), and this was not detected in cells infected with WR (Figure 6A), suggesting that this is more likely a result of vP11IRG mutation than Bxb1 activity. Taken together, these results support the conclusion that PhiC31 can function in VACV synthetic circuitry but is irrepressible by the *tet* operon, and that our Bxb1 is not functional in our VACV synthetic circuitry.

DISCUSSION

The implementation of reliable invertase-based logic gates or more complex logical circuitry in VACV vectors could greatly enhance their usefulness in both research and medicine. Eventually, VACVs could be engineered in which recombinases are inducible by well-studied cellular signals or transcription factors, either host-derived or exogenous genes carried by the virus. In oncolytic viral therapy, for example, important tumor suppressors such as p53 (59) or one of its downstream effectors could be considered an indicator of non-cancerous cells and accordingly used as an inducer of a recombinase that alters VACV circuitry in such a way that inhibits VACV replication. The reverse could be done with known oncogenes. Such an oncolytic VACV therapy would have to be specifically designed for a particular type of tumor. Such vectors could also include control switches allowing them to be induced or repressed within patients with drugs. Inducible (60) and repressible (Jasperse, O'Connell, Titong, Verardi, unpublished data) systems have already been shown to be efficacious in controlling VACV gene expression *in vivo*. Similarly, VACVs could be engineered to shut off essential gene expression in response to an adverse reaction. For example, patients with atopic dermatitis are susceptible to developing eczema vaccinatum. During this reaction a number of cytokines are produced, such as IL-3, IL-4, and IL-5, which do not normally play a role in controlling VACV infection (61). The cellular signaling pathways triggered by these interleukins could be used as regulators of recombinase expression to develop a responsive logic-gate.

In these preliminary tests we were not able to demonstrate effective recombinase-based logical circuitry in VACV vectors. In all of our experiments wherein VACV-infected cells were transfected with our plasmids containing *bxb1* (pPL199, Figure 2D and pPL201, Figure 2F), we did not detect any green fluorescence, indicating a lack of Bxb1 activity in our VACV synthetic circuitry (Figures 3, 5). This may be for a number of reasons. While PhiC31 has been well characterized in eukaryotes (53), to our knowledge Bxb1 has not, and neither of them have previously been studied in cytoplasmic DNA viruses, so the protein product may be different than in prokaryotic cells. Even if the post-transcriptional and post-translational modifications are correct in VACV-infected cells, *bxb1* may require codon optimization to be sufficiently expressed by VACV, however this is less likely due to the enzymatic nature of recombinases. It is also possible that a mutation occurred during our PCR cloning of *bxb1*, so there may be reason to repeat the cloning and reattempt this assay. However, since there are numerous other tools for altering DNA including other recombinases, transposases, and CRISPR-Cas systems (62) which could be used in logical VACV genetic circuitry (63), the need to invest significant resources beyond this in functionally expressing the *bxb1* transgene is limited.

In all of our assays wherein VACV-infected cells were transfected with our plasmids containing *phiC31* (pPL198, Figure 2C and pPL200, Figure 2E), both with and without inducer, we did not detect any red fluorescence but we did observe green fluorescence similar to the levels of the transfection control (Figure 3, 6). This suggests that our PhiC31-based circuitry poses the opposite

problem as it appears that the recombinase was active in every transfected cell regardless of the repressor system and absence of inducer. It is also possible that somehow the recombinase was expressed during cloning, prior to our transient expression assay, inverting the target promoter and causing all of our cells transfected with plasmids pPL198 and pPL200 to express green irresponsive to PhiC31 expression. This direction of the promoter in the final transfer vector will need to be verified by sequencing, possibly in conjunction with the parental vector pPL196 for comparison. If this is the case, then measures will be necessary to inhibit expression of this gene during cloning, even though it is downstream of a VACV promoter, not a bacterial one.

These results present the need for new controls to better verify that promoter inversion has occurred within the infection/transfection assay. Now that we know the fluorescence reporter genes on these plasmids work, in future experiments one could use the parental vectors, which lack recombinase genes, as negative controls to verify that red fluorescent reporter is expressed in the absence of recombinase. These or another *DsRed* plasmid should also be used as a control for comparison of DsRed expression during imaging. Finally, the best method to demonstrate promoter inversion would be to sequence the plasmid before the infection/transfection assay, and then collect, PCR, and sequence the plasmid DNA from wells in which green fluorescence was detected to compare the sequences and confirm inversion.

The possibility that PhiC31 is highly active in VACV-infected cells also warrants discussion. In this study we used the *lac* and *tet* repressors because

they had been thoroughly characterized previously in recombinant VACVs. However, these systems are prone to “leaky” expression, and since even one molecule of recombinase is theoretically sufficient to permanently alter the target sequence, a tighter method of gene regulation may be necessary. One possibility may simply be to decrease the strength of the promoter upstream of the recombinase. Moss *et al.* characterized the strength of many natural VACV promoters (64) and our lab recently developed a series of weakened P_{11} promoters (by systematically deviating from the consensus sequence) including the “P_5” we used in pPL198-pPL201. Perhaps using an even weaker promoter may increase repressibility of recombinase expression.

Based on the uncontrolled high efficiency of inversion exhibited by *phiC31* transfected cells, it may be more practical to implement a system which better inhibits gene expression, rather than being less capable of gene expression. Many genetic engineers control recombinases expression using “riboregulators”. Riboregulators are microRNAs (miRNAs) that post-transcriptionally repress the expression of a transgene that has been tagged with a 5’-complimentary sequence, allowing the miRNA to anneal to the transcript and form a secondary structure blocking the ribosomal binding domain (RBD) and preventing translation (65). Isaacs *et al.* originally developed both cis-repressor and trans-activator systems using these molecules, and they have since been demonstrated to have great utility as regulators in genetic switchboards (66, 67). Characterization of these riboregulators in VACV synthetic circuitry may be an important next step in the development of recombinase-based VACV vectors, as

well as useful for gene regulation in VACV vectors in general. Ultimately, in order to produce recombinant VACVs containing this sort of logical circuitry, we will face another hurdle in making sure that no undesired recombinase expression and activity occur during purification and amplification, further increasing the need for a much stricter mechanism for control of gene expression.

We believe that we have developed a transient expression system for testing the functionality of recombinases and corresponding regulators of gene expression in VACV. Although we did not succeed in demonstrating inducible-recombinase logic in VACV vectors, we did elucidate the problems that must be overcome in order to successfully develop this technology. Establishment of just two recombinases functional in VACV synthetic circuitry as well as an adequate regulatory system for the expression of these genes would allow for the implementation of binary logic gates in VACV vectors with tremendous potential for medical and research applications.

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Figures

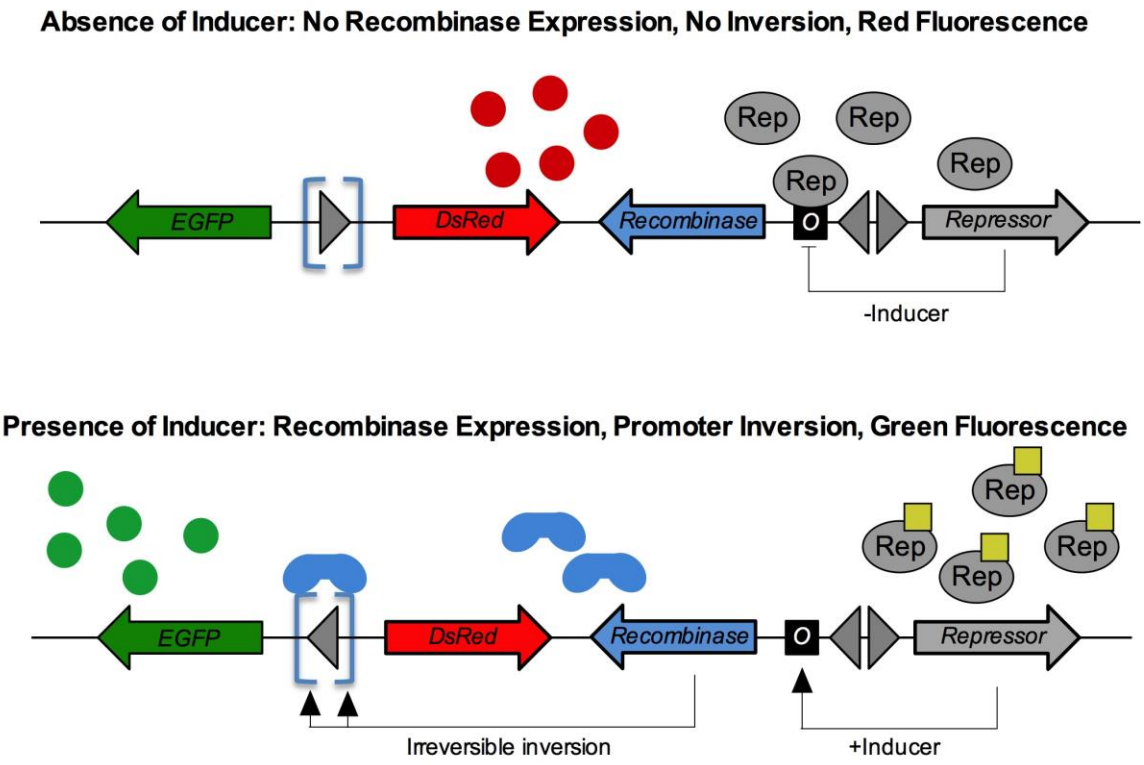


Figure 1. Recombinase test vector concept. To test the functionality and repressibility of recombinases in VACV synthetic circuitry, we developed constructs that constitutively express a repressor (TetR or LacI, here "Rep"), which in the absence of the inducer binds to an operator, blocking transcription of the recombinase. In the presence of inducer (yellow squares), a conformational change occurs in the repressor protein, releasing it from the operator, permitting transcription of the recombinase. This catalyzes the inversion of a promoter, switching fluorescence reporter expression from red to green.

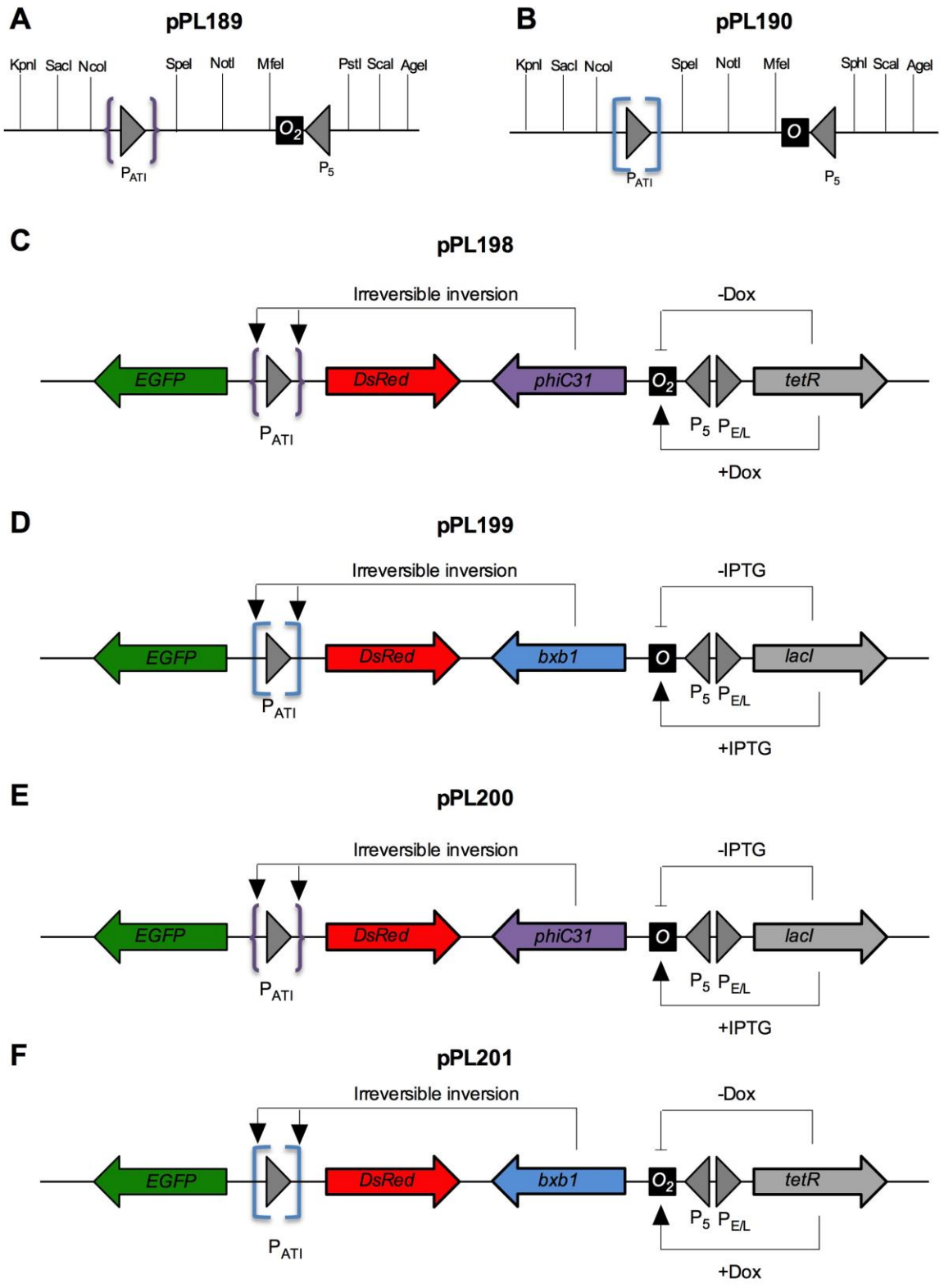
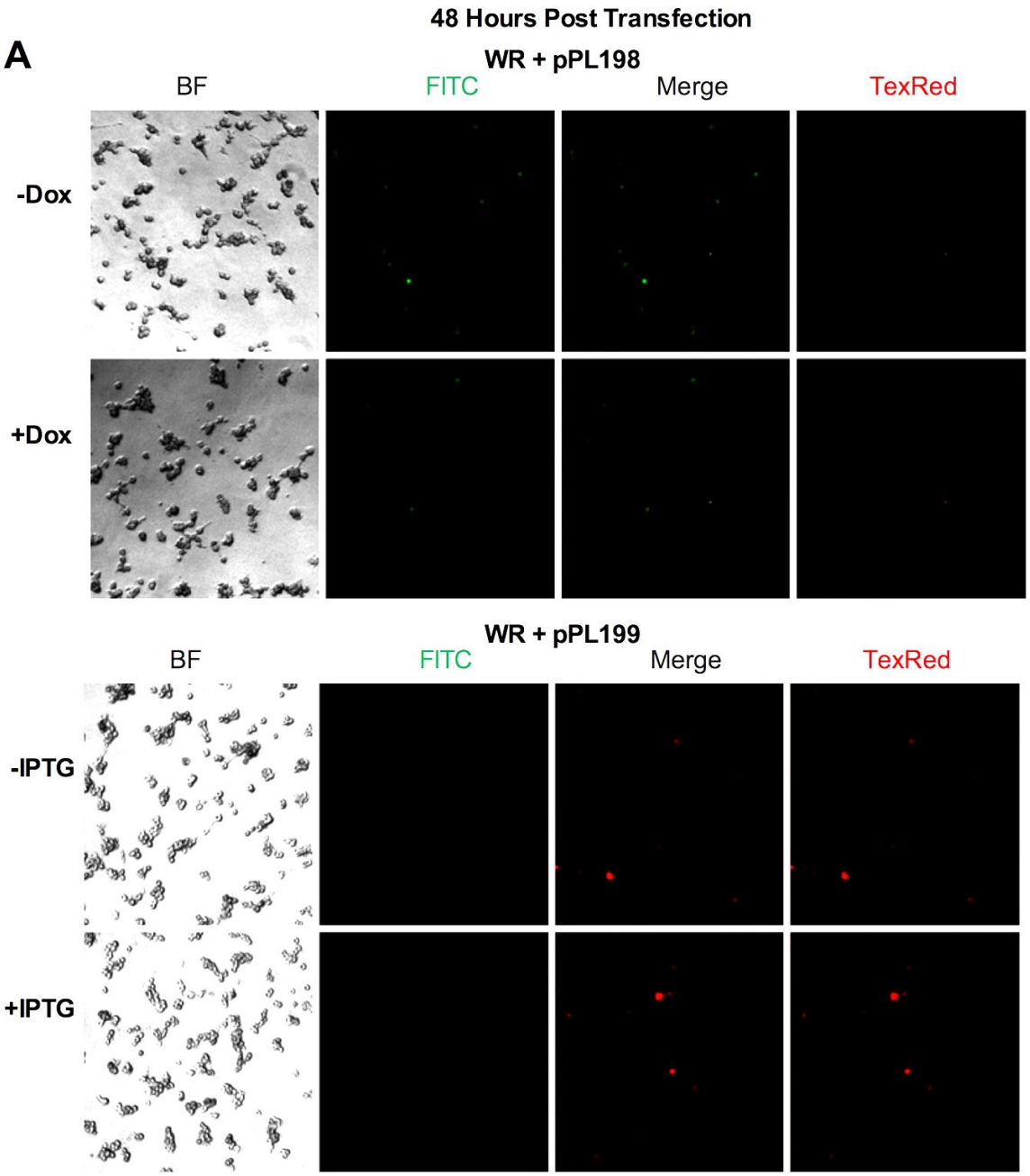
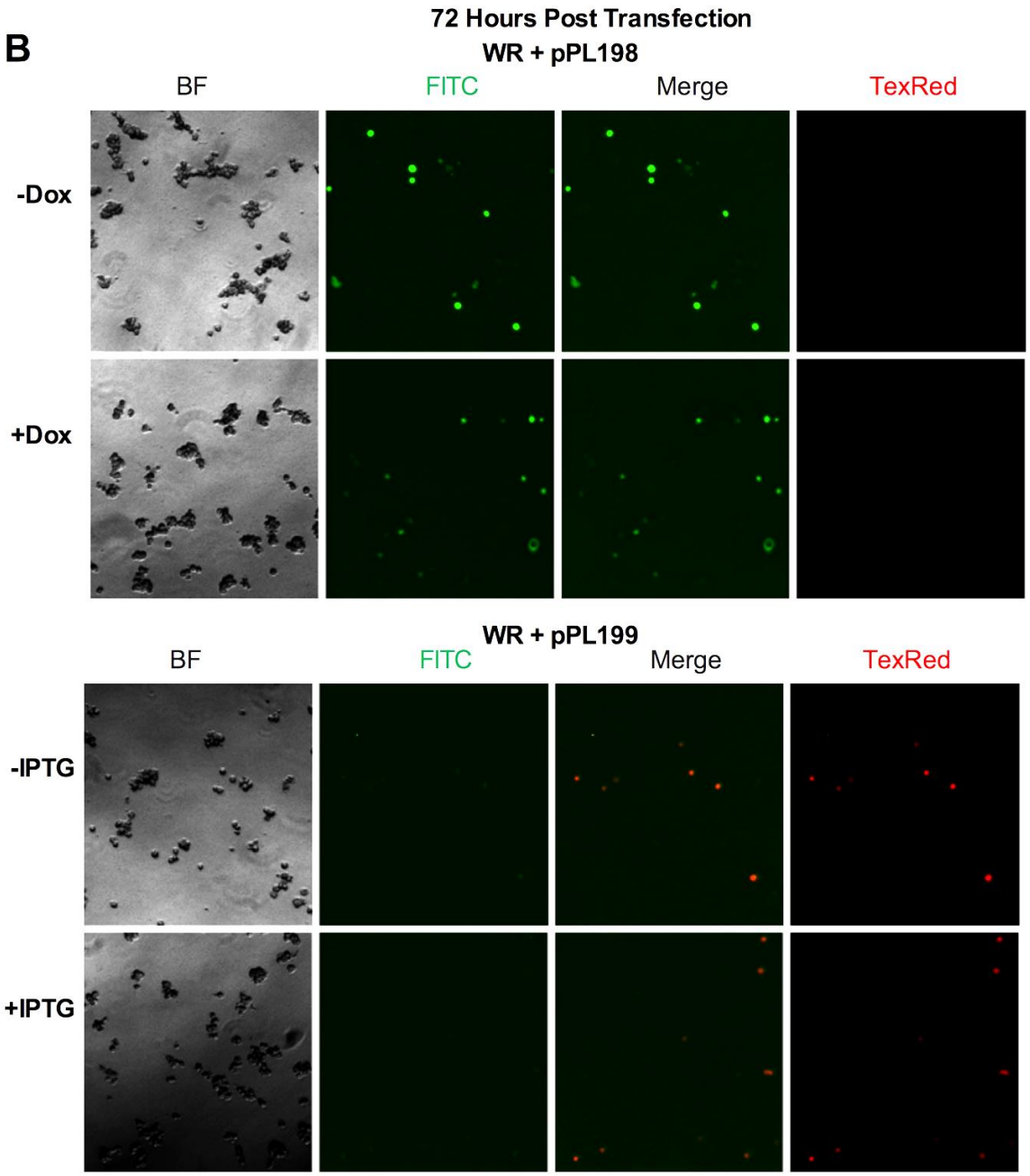

A
pPL189
KpnI SacI NcoI SpeI NotI MfeI PstI ScaI AgeI
O2
PATI
P5
B
pPL190
KpnI SacI NcoI SpeI NotI MfeI SphI ScaI AgeI
O
PATI
P5
C
pPL198
Irreversible inversion
-Dox
EGFP
DsRed
phiC31
O2
tetR
PATI
P5 PE/L
+Dox
D
pPL199
Irreversible inversion
-IPTG
EGFP
DsRed
bxb1
O
lacI
PATI
P5 PE/L
+IPTG
E
pPL200
Irreversible inversion
-IPTG
EGFP
DsRed
phiC31
O
lacI
PATI
P5 PE/L
+IPTG
F
pPL201
Irreversible inversion
-Dox
EGFP
DsRed
bxb1
O2
tetR
PATI
P5 PE/L
+Dox

Figure 2. Recombinase test plasmid maps. Plasmid vector backbones pPL189 **(A)** and pPL190 **(B)** were synthesized by DNA 2.0 with a high-copy *E. coli* origin of replication and kanamycin resistance gene. *EGFP*, *DsRed*, repressors, and recombinases were cloned into backbones to produce recombinase test plasmids pPL198 **(C)** pPL199 **(D)** pPL200 **(E)** and pPL201 **(F)** for use in transient expression assays.


48 Hours Post Transfection
A
WR + pPL198
BF
FITC
Merge
TexRed
-Dox
+Dox
WR + pPL199
BF
FITC
Merge
TexRed
-IPTG
+IPTG


B
72 Hours Post Transfection
WR + pPL198
BF
FITC
Merge
TexRed
-Dox
+Dox
WR + pPL199
BF
FITC
Merge
TexRed
-IPTG
+IPTG

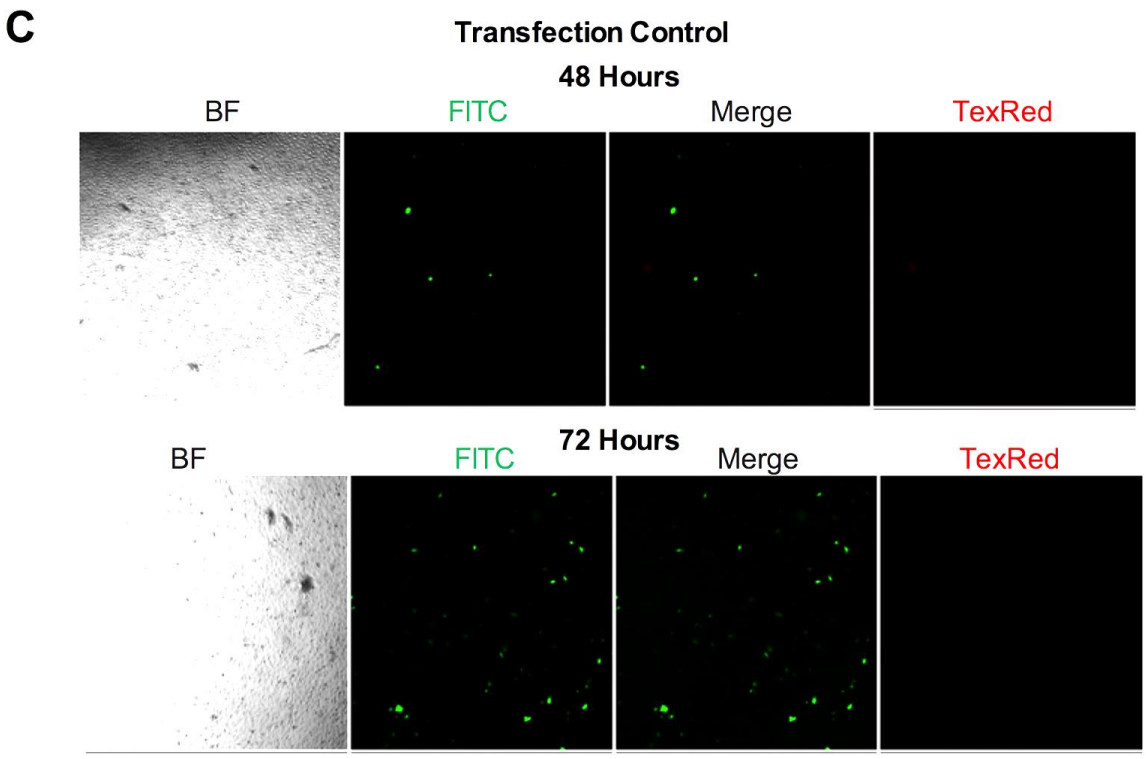
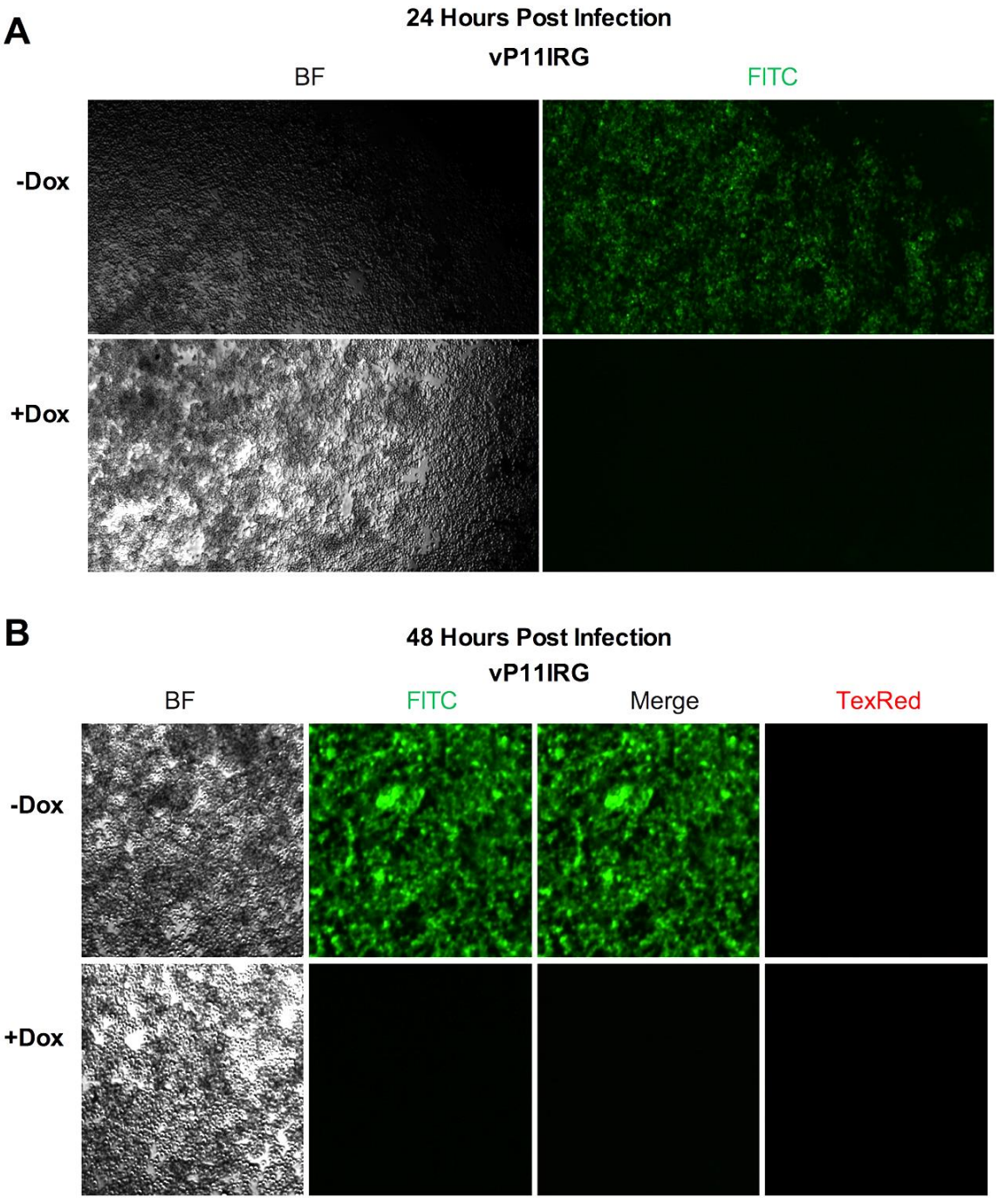
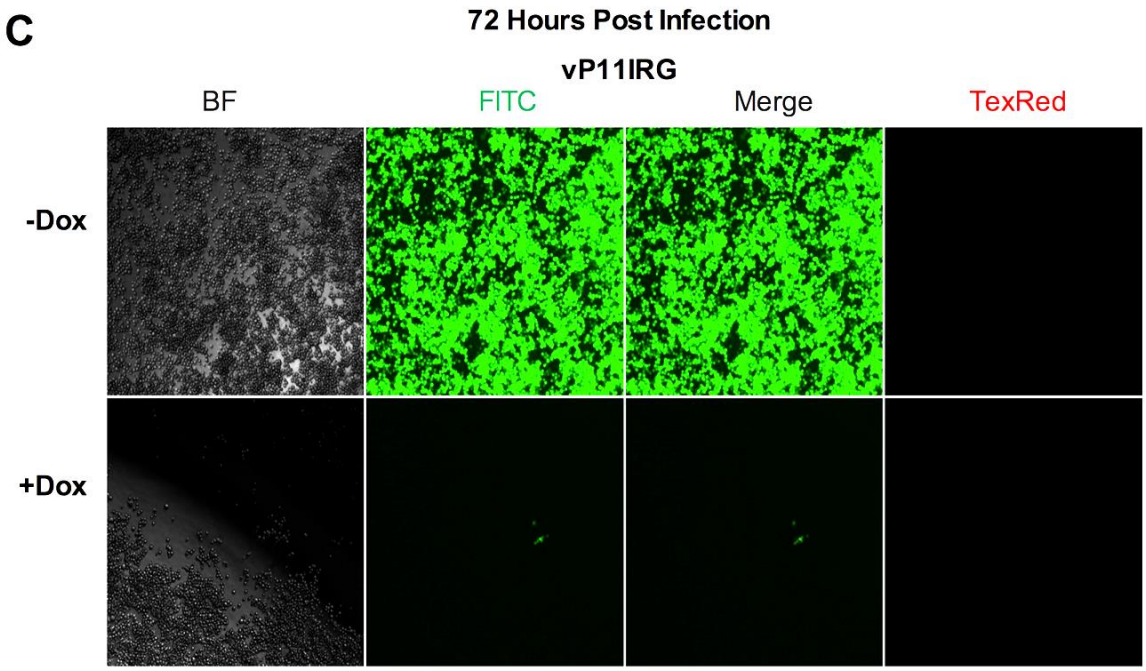


Figure 3. Initial test of logical circuits transient expression assay.

Representative images of our pilot transient expression assay. BS-C-1 cells were infected with WR at an MOI of 1 and after 1 hour overlaid with complete DME supplemented with 2.5% FBS, with or without 1mg/ml doxycycline or 1mM IPTG and transfected with pPL198, pPL199. Wells were imaged using FITC (Green) and Texas Red (Red) fluorescent filters, and brightfield (BF) after 24 **(A)** and 48 **(B)** hours p.t. As a transfection control, an uninfected monolayer was transfected with a plasmid coding for EGFP under a constitutive CMV promoter **(C)**.


A
24 Hours Post Infection
vP11IRG
BF
FITC
-Dox
+Dox
B
48 Hours Post Infection
vP11IRG
BF
FITC
Merge
TexRed
-Dox
+Dox


C
72 Hours Post Infection
vP11IRG
BF
FITC
Merge
TexRed
-Dox
+Dox

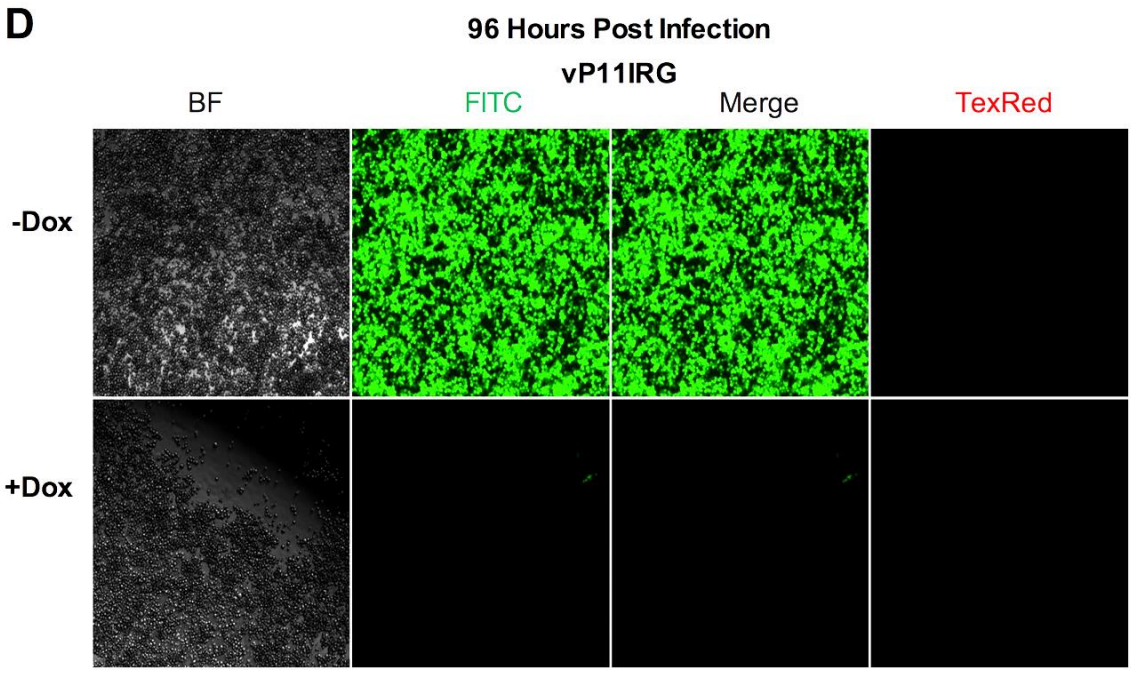
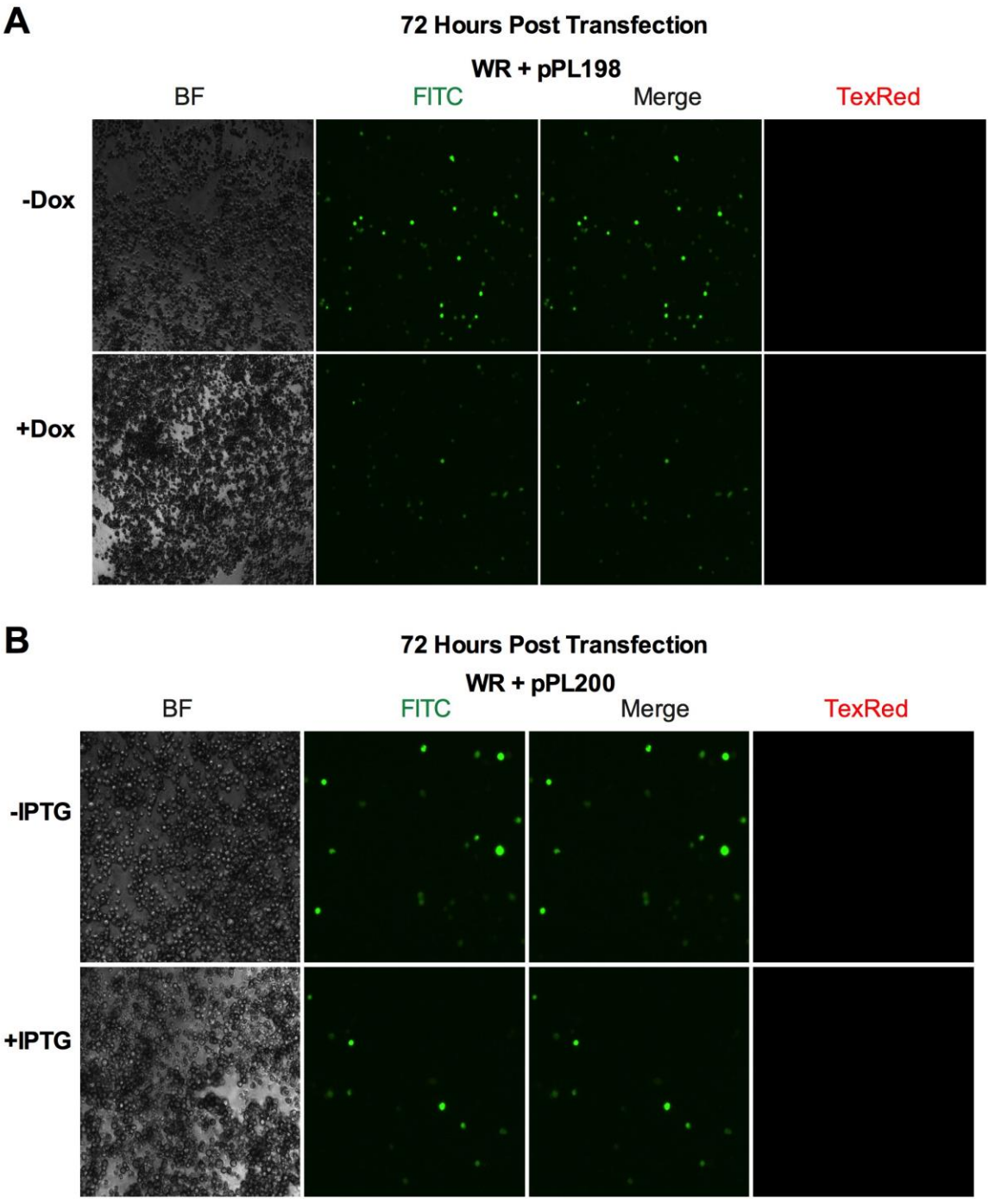
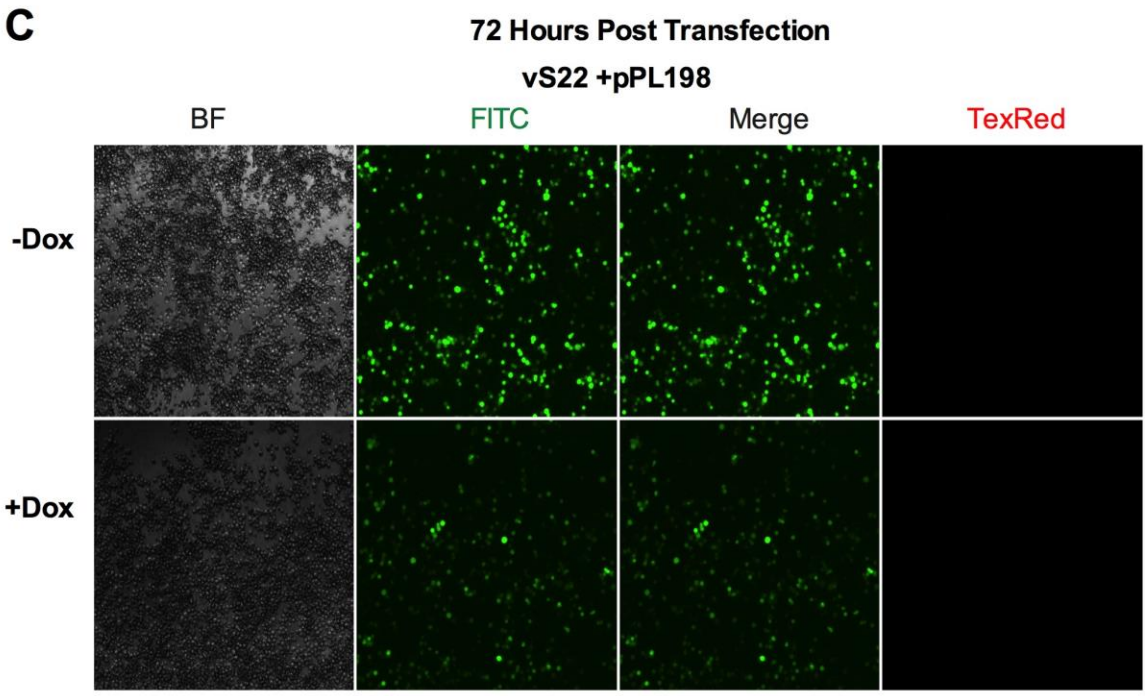

D
96 Hours Post Infection
vP11IRG
BF
FITC
Merge
TexRed
-Dox
+Dox

Figure 4. Repressibility of EGFP expression in vP11IRG-infected HeLa-S3 cells. HeLa-S3 cells were infected with vP11IRG at an MOI of 1 and overlaid with 1 mg/ml Dox. Wells were imaged using FITC (Green) and Texas Red (Red) fluorescent filters, and brightfield (BF) after 24 **(A)**, 48 **(B)**, 72 **(C)** and 96 **(D)** hours p.t.


A
72 Hours Post Transfection
WR + pPL198
BF
FITC
Merge
TexRed
-Dox
+Dox
B
72 Hours Post Transfection
WR + pPL200
BF
FITC
Merge
TexRed
-IPTG
+IPTG


C
72 Hours Post Transfection
vS22 +pPL198
BF
FITC
Merge
TexRed
-Dox
+Dox

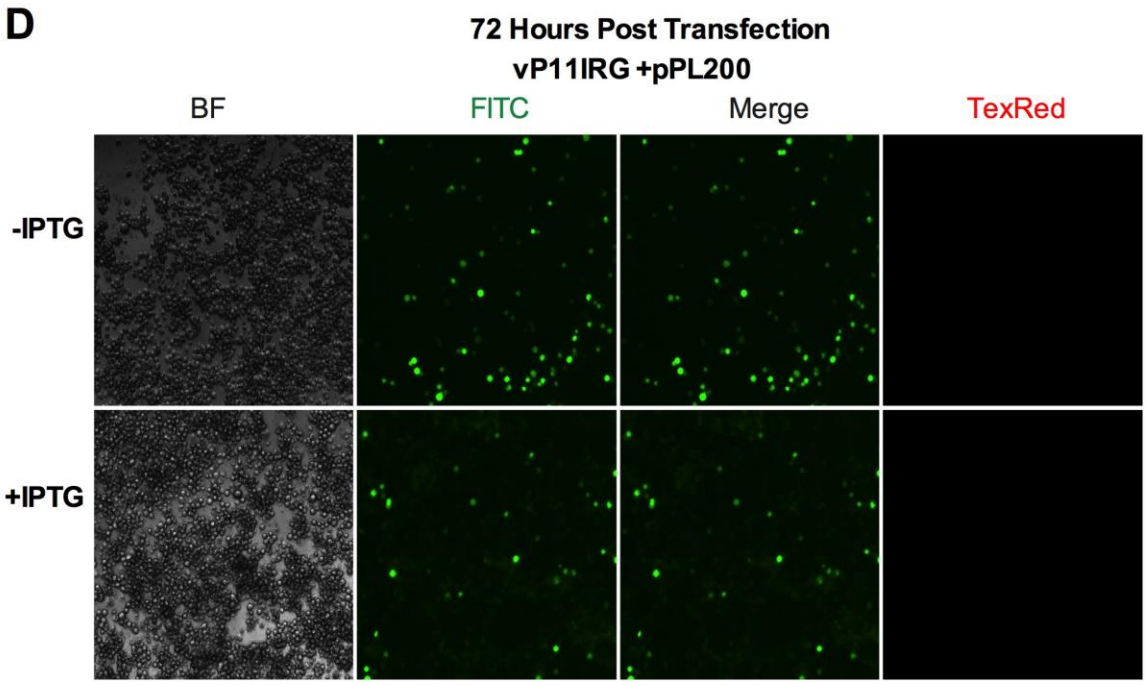
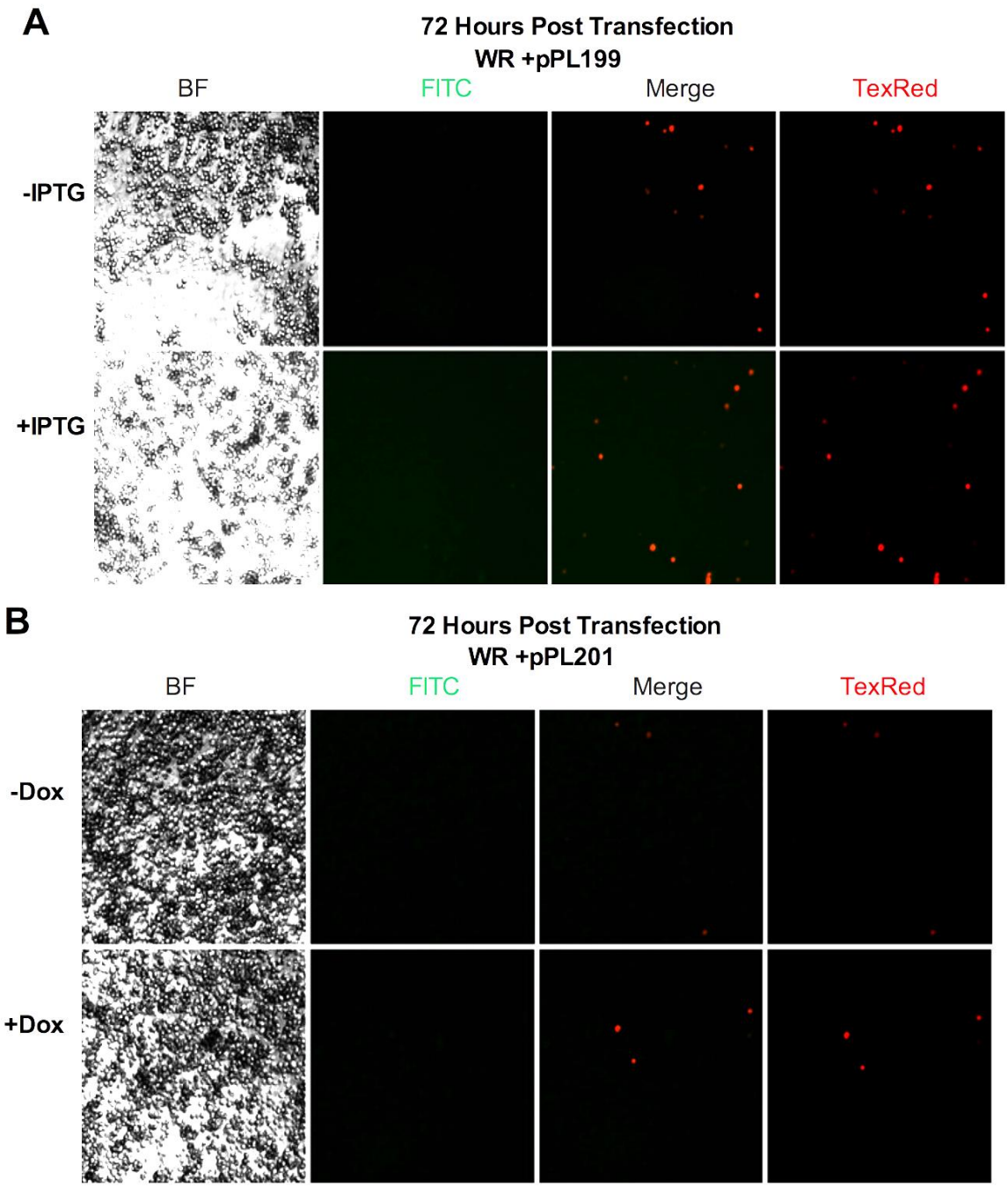

D
72 Hours Post Transfection
vP11IRG +pPL200
BF
FITC
Merge
TexRed
-IPTG
+IPTG

Figure 5. Expression of PhiC31 under Tet and Lac repressor systems.

HeLa-S3 cells were infected with WR for 24 hours, overlaid with or without 1mg/ml Dox or 1mM IPTG and transfected with pPL198 **(A)** and pPL200 **(B)**. In order to pre-express TetR or LacI in transfected cells, we repeated this assay using cells infected with vS22 for pPL198 **(C)** and vP11IRG for pPL200 **(D)**. Images shown are representative of 72 hours p.t. and taken using brightfield (BF), FITC (Green), or Texas Red (Red) fluorescent filters.


A
72 Hours Post Transfection
WR +pPL199
BF
FITC
Merge
TexRed
-IPTG
+IPTG
B
72 Hours Post Transfection
WR +pPL201
BF
FITC
Merge
TexRed
-Dox
+Dox

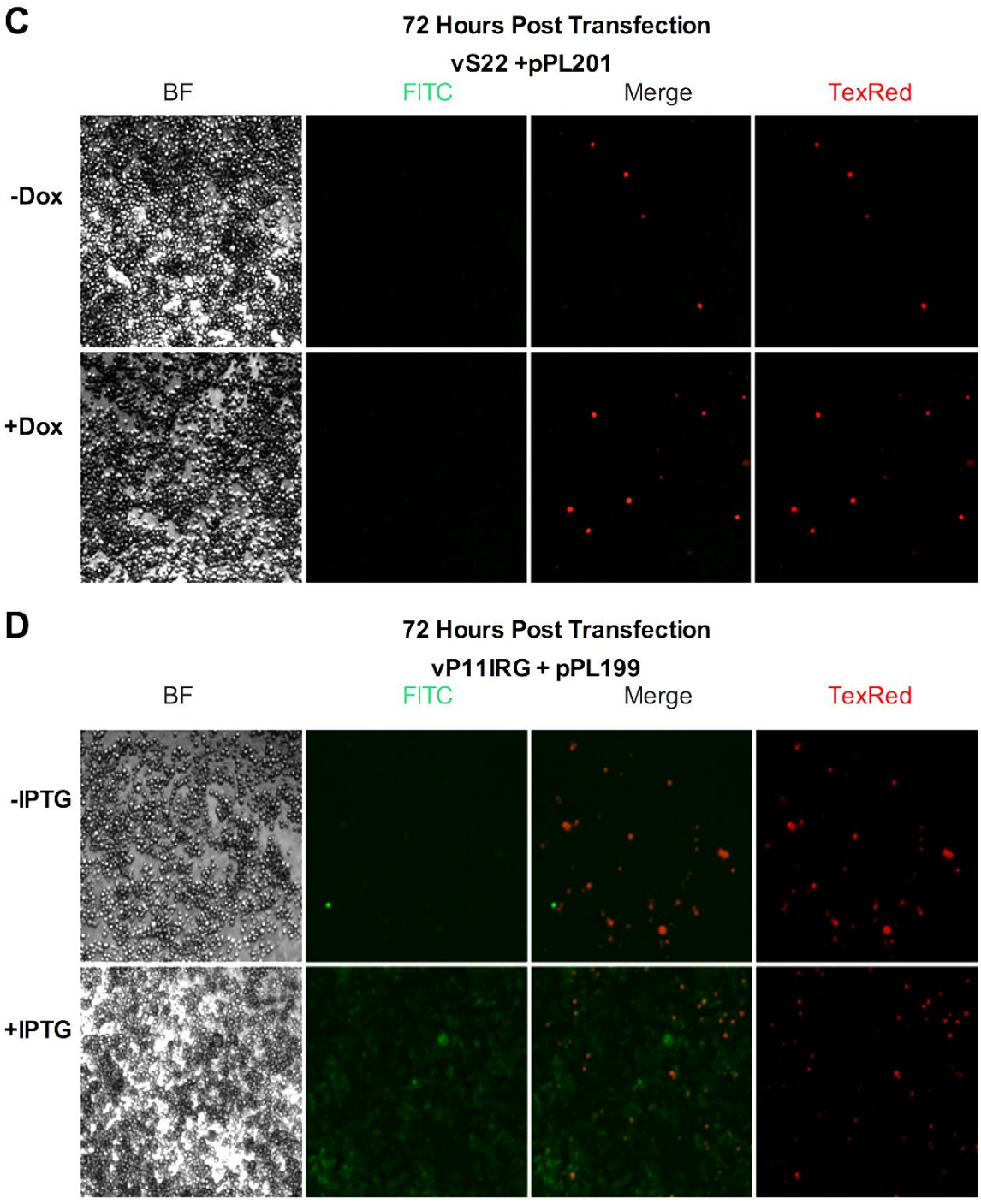

C
72 Hours Post Transfection
vS22 +pPL201
BF
FITC
Merge
TexRed
-Dox
+Dox
D
72 Hours Post Transfection
vP11IRG + pPL199
BF
FITC
Merge
TexRed
-IPTG
+IPTG

Figure 6. Expression of Bxb1 under Tet and Lac repressor systems.

HeLa-S3 cells were infected with WR for 24 hours, overlaid with or without 1mg/ml Dox or 1mM IPTG and transfected with pPL199 **(A)** and pPL200 **(B)**. In order to pre-express TetR or LacI in transfected cells, we repeated this assay using cells infected with vS22 for pPL200 **(C)** and vP11IRG for pPL199 **(D)**. Images shown are representative of 72 hours p.t. and taken using brightfield (BF), FITC (Green), or Texas Red (Red) fluorescent filters.