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A *C. elegans*-Based Foam for Rapid On-Site Detection of Residual Live Virus

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Abstract

In the response to and recovery from a critical homeland security event involving deliberate or accidental release of biological agents, initial decontamination efforts are necessarily followed by tests for the presence of residual live virus or bacteria. Such "clearance sampling" should be rapid and accurate, to inform decision makers as they take appropriate action to ensure the safety of the public and of operational personnel. However, the current protocol for clearance sampling is extremely time-intensive and costly, and requires significant amounts of laboratory space and capacity. Detection of residual live virus is particularly problematic and time-consuming, as it requires evaluation of replication potential within a eukaryotic host such as chicken embryos. The intention of this project was to develop a new method for clearance sampling, by leveraging

Sandia's expertise in the biological and material sciences in order to create a *C. elegans*-based foam that could be applied directly to the entire contaminated area for quick and accurate detection of any and all residual live virus by means of a fluorescent signal. Such a novel technology for rapid, on-site detection of live virus would greatly interest the DHS, DoD, and EPA, and hold broad commercial potential, especially with regard to the transportation industry.

ACKNOWLEDGMENTS

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Mary Shi is greatly appreciated for her help in cloning *C. elegans* promoters. We are grateful to Creg Darby and the *C. elegans* Genetics Center for worm strains. We also thank Dr. Adolfo García-Sastre, Mount Sinai School of Medicine, for the *rVSV-luc* and *rVSV-GFP* viruses, and Dr. C.J. Peters, University Texan Medical Branch, for the *RVFV MP12* virus. Finally, we thank Dr. Benhur Lee, UCLA, for a full-length cDNA clone of the VSV genome (Indiana serotype 1), where the coding region of the G gene was replaced by the reporter gene renilla luciferase.

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I. Introduction

In the response to and recovery from a critical homeland security event involving deliberate or accidental release of biological agents, initial decontamination efforts are necessarily followed by tests for the presence of residual live virus or bacteria. Such "clearance sampling" should be rapid and accurate, to inform decision makers as they take appropriate action to ensure the safety of the public and of operational personnel. However, the current protocol for clearance sampling is extremely time-intensive and costly, and requires significant amounts of laboratory space and capacity ("Method Detection Limits and Non-Detects in the World of Microbiology," National Risk Management Research Laboratory, EPA, 2006). Large numbers of samples may be required to achieve a high degree of statistical certainty, depending upon the physical dimensions of the contaminated area. The samples must then be transported to the lab for measurement of biological activity. Detection of residual live virus is particularly problematic and time-consuming, as it requires evaluation of replication potential within a eukaryotic host such as chicken embryos.

The distinguishing feature of active ("live") vs. inactive ("dead") virus is the ability to proliferate. Whereas bacteria can proliferate autonomously given appropriate nutrients, viruses replicate only within a eukaryotic host. Standard practice for clearance sampling involves taking individual swab samples from a surface, transporting them to a laboratory, and *there* culturing them with a eukaryotic host in order to assess the replicative potential of the virus. This practice could be substantially improved in two critical ways: 1) by development of faster, cheaper, and more sensitive eukaryotic cell sensors of viral replication; and 2) by development of on-site methods of culturing virus, which would eliminate the need for large amounts of laboratory space and capacity. In theory, a *Caenorhabditis elegans (C. elegans)*-based foam technology could address both critical needs. The intention of this project was to develop a new method for clearance sampling, by leveraging Sandia's expertise in the biological and material sciences in order to create a *C. elegans*-based foam that could be applied directly to the entire contaminated area for quick and accurate detection of any and all residual live virus by means of a fluorescent signal. Such a novel technology for rapid, on-site detection of live virus would greatly interest the DHS, DoD, and EPA, and hold broad commercial potential, especially with regard to the transportation industry.

A number of characteristics make *C. elegans* attractive as a host organism for on-site clearance sampling. *C. elegans*, a non-parasitic nematode maintained in the laboratory under BSL-1 containment (Stiernagle, 2006), is an accepted model for studying eukaryotic host responses to viruses (Lu *et al*. 2005; Schott *et al*. 2005; Wilkins *et al*. 2005; Liu *et al*. 2006). The relative genetic simplicity of worms, the availability of an extensive collection of mutants, the ease of conducting random mutagenesis screens, enabling the isolation of gain-of-function as well as loss-of-function mutations, and the detailed genetic and physical maps enabling the straightforward identification of mutant genes makes the worm an attractive host for characterizing viral infectivity. Moreover, *C. elegans* are transparent, such that cellular responses to viral infection and replication can be monitored in living animals using fluorescence reporters (Figure 1). Finally, *C. elegans* are easily cultured on either agar plates or in large fluid volumes,

and could be suspended in an aqueous-based, oxygen-permeable gel for use in clearance sampling applications.

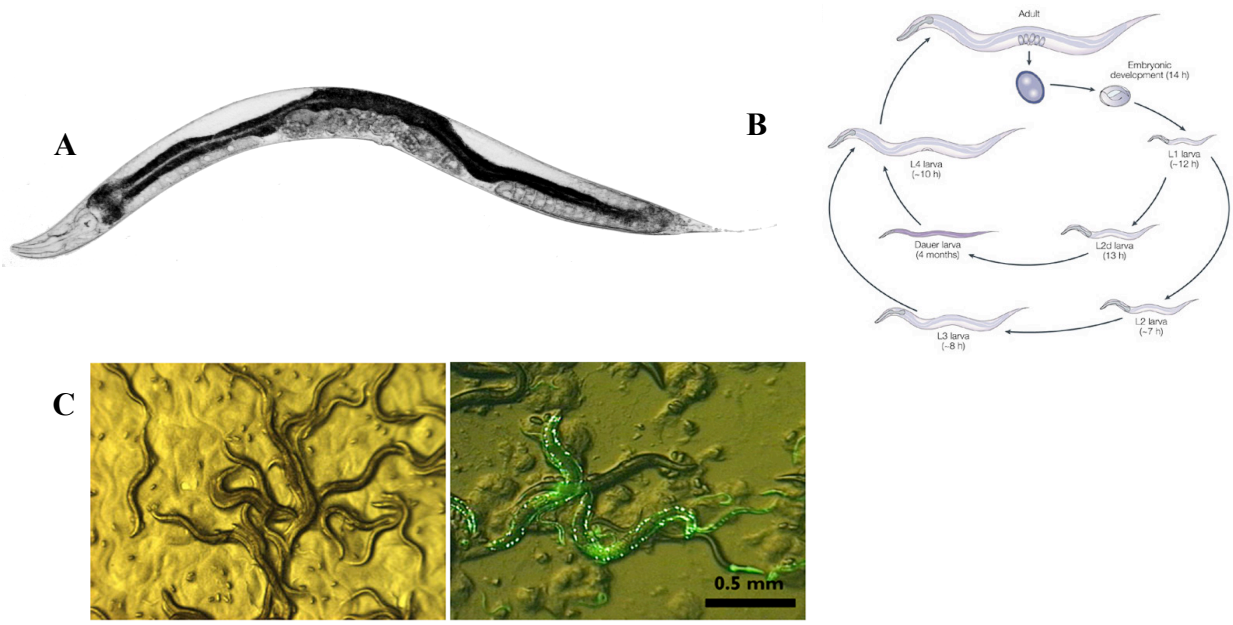


Figure 1: *C. elegans* are fully transparent at all stages of development, such that internal fluorescence can be detected easily. (A) Nomarski micrograph of an adult hermaphrodite; internal organs are visible to transparency of the cuticle; (B) Life cycle of *C. elegans;* (C) *C. elegans* swimming on agar plates; in the right-hand panel, some worms express GFP in a subset of their cells, visible due to the animal's transparency.

Generation of a strain of *C. elegans* that could be used for clearance sampling would require the following:

1) A *C. elegans* strain with increased susceptibility to viral infection;

2) Molecular sensors of viral infection that function to induce fluorescence expression in cells infected by virus,

3) A mechanism to amplify the fluorescent signal such that induced fluorescence in a single *C. elegans* nematode results in many *C. elegans* fluorescing, thereby facilitating detection; and

4) A gel with which to disperse an engineered strain of *C. elegans* on a surface for the purpose of clearance sampling The project concept is schematized in Figure 2.

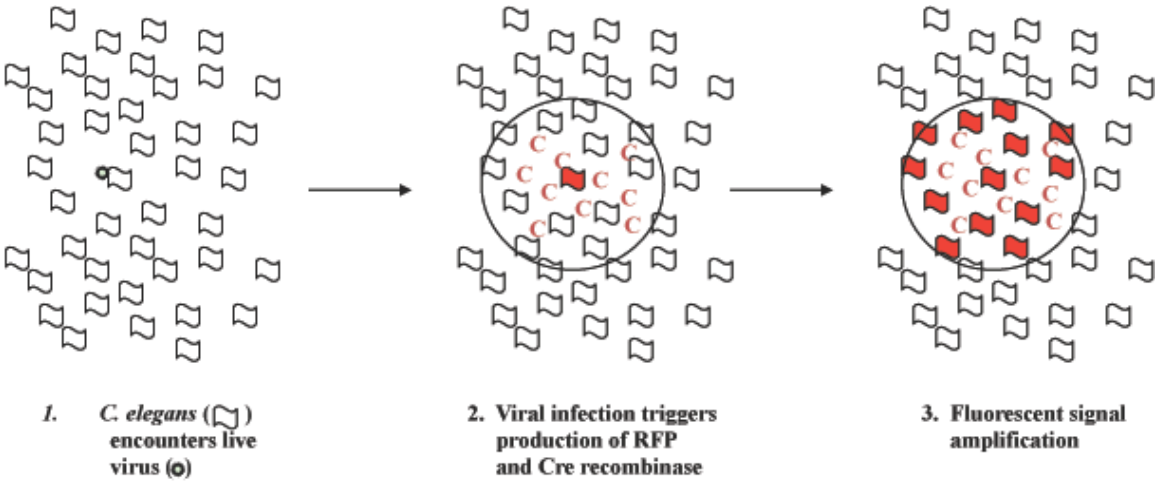


Figure 2: Project concept to use *C. elegans* for clearance sampling applications. RFP, red fluorescent protein.

Our progress towards each of these four goals is described below. While we made significant progress, we were not able to generate a *C. elegans* strain that approaches the sensitivity of current protocols for clearance sampling.

II. Generation of *C. elegans* with Increased Susceptibility to Viral Infection

II.1 Virus selection

We focused on three viruses in this project: *Rift Valley Fever Virus* (*RVFV*) *MP12*, *Vaccinia Virus* (*VV*), and *Vescicular Stomatitis Virus* (*VSV*). *RVFV MP12* and *VV* were chosen because of their relevance to NIAID Category A viral pathogens, and because of the availability of attenuated strains that could be worked with under BSL2 containment (Table 1, and Appendix A). *VSV* was chosen because it is one of the viruses known to infect *C. elegans* (Wilkins, *et al.* 2005), and because it is a model RNA virus with low human pathogenicity. Recombinant (r) viruses encoding GFP (for example, *rVSV-GFP*) are very useful tools for observing infection of *C. elegans* since the replicating virus can be observed directly in the host cells. We were grateful to obtain *RVFV-MP12* and *rRVFV-MP12-GFP* from Dr. George Bettinger, University Texan Medical Branch (UTMB); *rVSV-luc* and *rVSV-GFP* from Dr. Adolfo García-Sastre, Mount Sinai School of Medicine; and *rVV-GFP* from ATCC/BEI Resources.

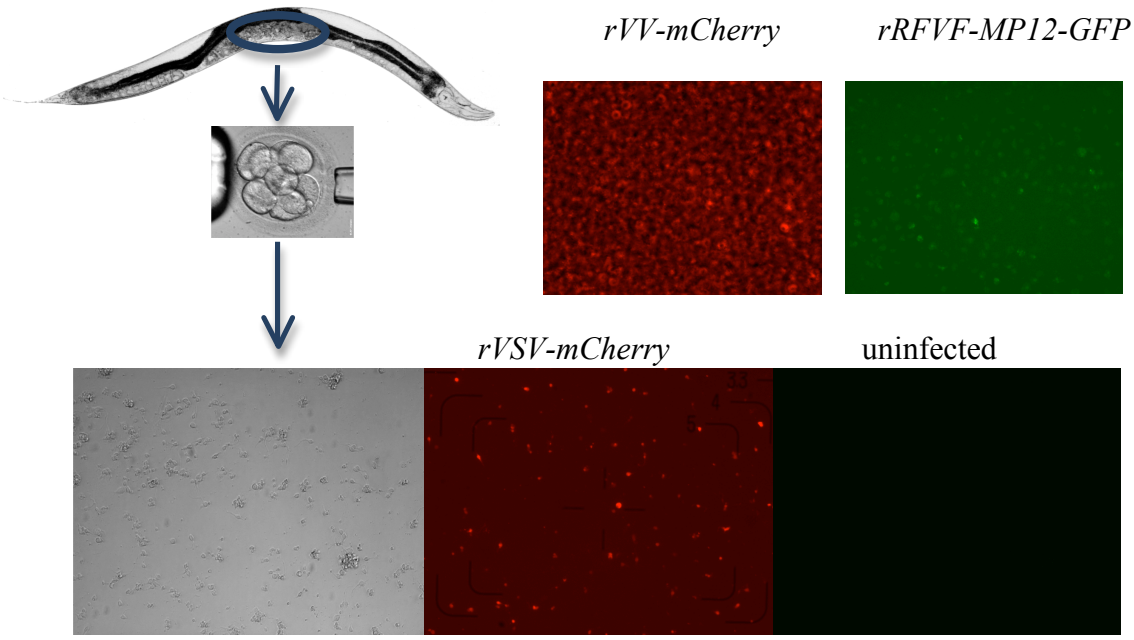
Table 1: NIAID Category A Viral Pathogens

Disease	Family	Virus	Type	Replication Site in Host	BSL2 Strain	Tagged Strain
Hemorrhagic fever	Arenaviridea	LCM	ssRNA	Cytoplasm	Yes	No
Hemorrhagic fever	Arenaviridea	Junin	ssRNA	Cytoplasm	Yes	No
Hemorrhagic fever	Arenaviridea	Machupo	ssRNA	Cytoplasm	No	No
Hemorrhagic fever	Arenaviridea	Guanarito	ssRNA	Cytoplasm	No	No
Hemorrhagic fever	Arenaviridea	Lassa	ssRNA	Cytoplasm	No	No
Hemorrhagic fever	Bunyaviridea	Hantaviruses	ssRNA	Cytoplasm	No	No
Hemorrhagic fever	Bunyaviridea	Rift Valley Fever	ssRNA	Cytoplasm	Yes	Yes
Hemorrhagic fever	Flaviviridae	Dengue	ssRNA	Cytoplasm	Yes	No
Hemorrhagic fever	Filoviridae	Ebola	ssRNA	Cytoplasm	No	No
Hemorrhagic fever	Filoviridae	Marburg	ssRNA	Cytoplasm	No	No
Smallpox	Poxviridae	Variola major	dsDNA	Cytoplasm	Yes	Yes

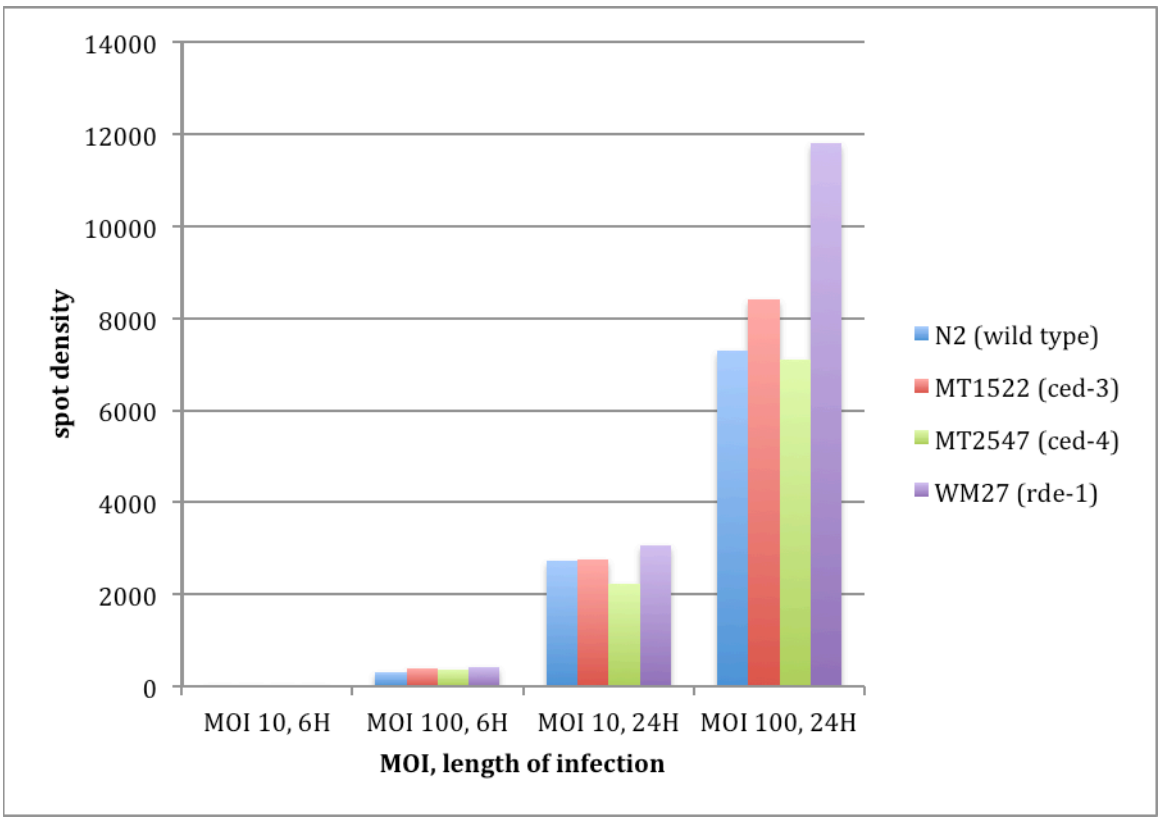
II.2 Viral infection of *C. elegans* embryonic stem cells

RVFV, *VV*, and *VSV* are known to have a wide host range, but to confirm that *C. elegans* cells can be infected by these viruses, we isolated embryonic stem cells from *C. elegans* and conducted infectivity assays (Figure 3). *C. elegans* are normally cultivated within a temperature range of 20-26^{o}C, however, viral infection of mammalian cells is typically conducted at 37^{o}C. We confirmed that *RVFV*, *VV*, and *VSV* infect *C. elegans* embryonic stem cells at 26^{o}C (experimental methods described in Appendix 2). Fluorescence indicative of replicativg virus can be observed within cells with all three viruses (Figure 3A). Results from the rVSV-luc experiment further confirmed that *C. elegans* can be infected with this virus, since luciferase activity (indicative of viral replication within the nematode) was significantly higher in WM27 animals than other strains infected (at 100 MOI, figure 3B).

(A)



(B)



strain, MOI	density	average density
N2, 100	6816	7306
N2, 100	7796	
N2, 10	2895	2743.5
N2, 10	2592	
WM27, 100	10922	11816.5
WM27, 100	12711	
WM27, 10	3247	3074.5
WM27, 10	2902	
MT1522, 100	7769	8404.5
MT1522, 100	9040	
MT1522, 10	2919	2749
MT1522, 10	2579	
MT2547, 100	8050	7114.5
MT2547, 100	6179	
MT2547, 10	2403	2241.5
MT2547, 10	2080	

strain, MOI	average density
N2, 10	2743.5
N2, 100	7306
MT1522, 10	2749
MT1522, 100	8404.5
MT2547, 10	2241.5
MT2547, 100	7114.5
WM27, 10	3074.5
WM27, 100	11816.5

Figure 3: Embryonic stem cells isolated from gravid *C. elegans* hermaphrodites can be infected with *VV*, *VSV*, and *RVFV*. (A) Fluorescence expression is indicative of viral replication in the

cells. (B) Luciferase activity in cells 24 hours after being infected with *rVSV-luc*, titer 10^{10} PFU/mL, MOI 100 and 10.

II.3 High levels of *C. elegans* autofluorescence in liquid culture

The identification of *C. elegans* strains with high susceptibility to viral infection would be necessary for their practical use in biodefense applications such as clearance sampling, where live virus detection limits are desired to be as low as possible. In order to identify one or more strains of *C. elegans* with high susceptibility to infection, we first tested if we could infect *C. elegans* (intact, living nematodes) with *rVSV-GFP*. *C. elegans* were placed in 24 well plastic tissue culture dishes (100 L4s/well) with 100ul of either M9 (physiological saline) or M9 plus 10-100 MOI dilutions of *rVSV-GFP*. Wild type and three mutant strains were tested, including WM27 *rde-1* mutants deficient in the RNA interference (RNAi) pathway (Tabara, *et al*., 1999). We detected high levels of autofluorescence in both infected and uninfected populations (figure 4), suggesting that experimental conditions induced a stress response within the nematodes, characterized by high levels of autofluorescence. (Nematodes are normally cultivated on agar plates, but can be grown in liquid culture. We did not want to add virus solutions to agar plates as the liquid would absorb into the agar, making MOI uninterpretable.)

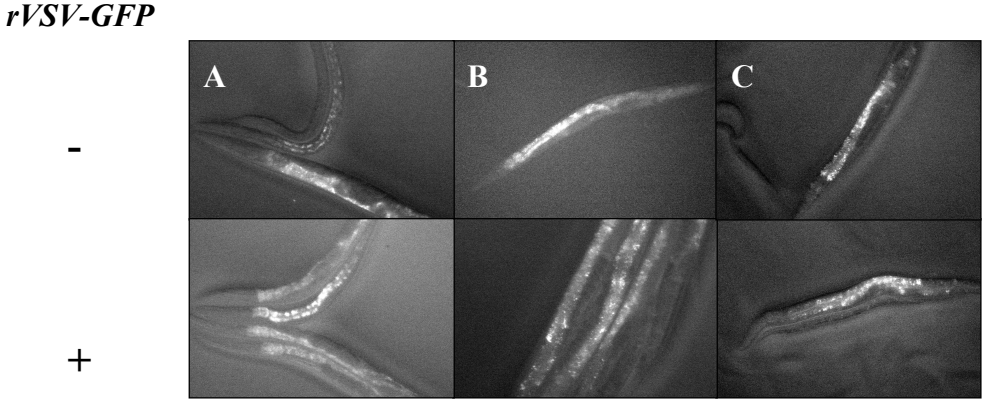


Figure 4: Autofluorescence detected with a GFP filter in *C. elegans* infected with *rVSV-GFP* (top) and uninfected animals (bottom). (A) wild-type N2; (B) MT1522 *ced-3* mutant strain; (C) WM27 *rde-1* mutant strain.

Analysis of the uninfected population revealed that the autofluorescence observed is excited predominantly by 450nm light (Figure 5), a wavelength that also excites the commonly used fluorophore green fluorescent protein (GFP; 488nm excitation, 507nm emission). As GFP expression is indistinguishable from *C. elegans* autofluorescence, we could not use, as planned, the GFP-labeled viruses to screen for *C. elegans* that are highly susceptibility to viral infection. To overcome this technical hurdle, we generated both VSV and RVFV that express the red fluorescent protein mCherry, characterized by 588nm excitation and 633nm emission spectra. Very little *C. elegans* autofluorescence is stimulated by 550nm light, and no autofluorescence is observed using an mKate2 emission filter. We found that the agar/OP50 substrate that *C. elegans* are cultivated on is also autofluorescent, and that this issue could be resolved as well with us RFP emissions filters (Figure 6).

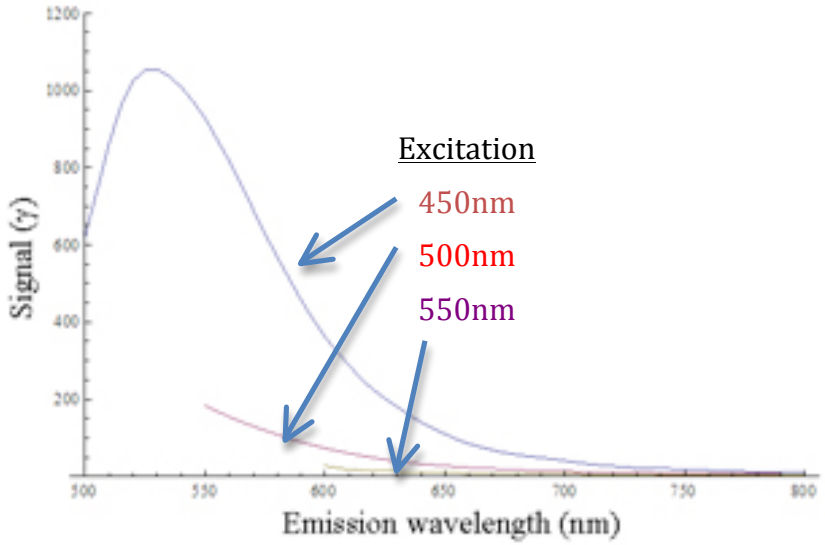


Figure 5: Fluorescence emission from homogenate *C. elegans* incubated in M9 at 26o for 24 hours.

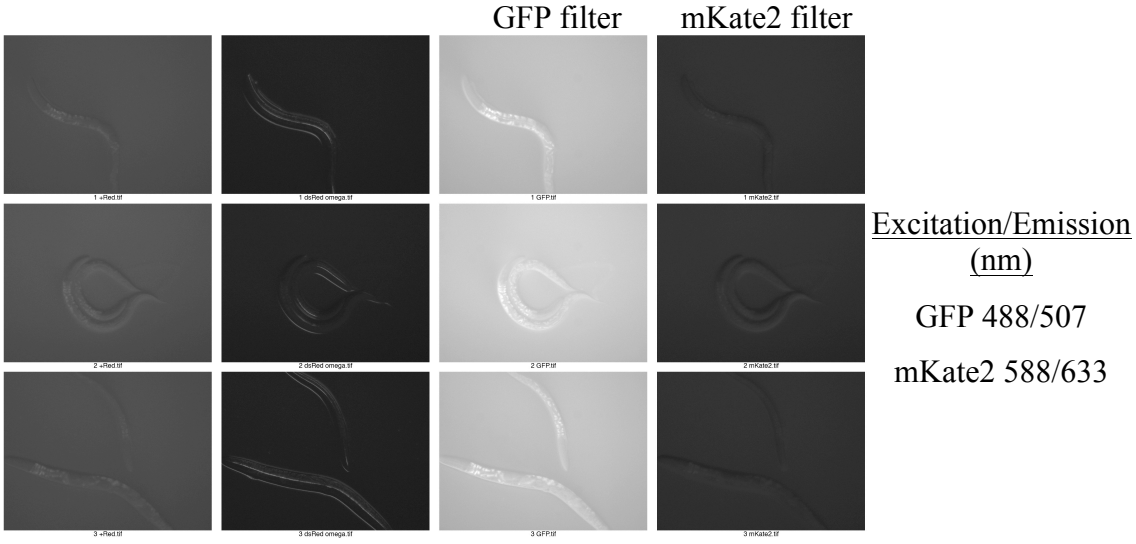


Figure 6: Autofluorescence from agar/OP50 plates, and from *C. elegans* incubated in M9 at 26^{o} for 24 hours can be removed using a RFP filter set such as mKate2, with peak excitation/ emission at 588nm/633nm.

II.4 Screen for C. elegans with increased susceptibility to viral infection

We generated recombinant VSV expressing the red fluorescent protein mCherry (*rVSV-mCherry*) (see figure 7a), and used this virus to screen multiple different mutant strains defective in either RNA interference (RNAi) (Schott *et al*. 2005), programmed cell death (Liu *et al*. 2006), or cuticle integrity (Darby *et al*. 2007, van der Keyl et al. 1994, Yang and Kramer, 1999), as these mutants have been shown to be, or are speculated to be, more susceptible to viral infection (Table 2). To further disrupt cuticle integrity in order to facilitate infection, we pretreated animals with dilute solutions of chitinase, collagenase or sodium hydroxide, combined with 10kpsi pressure. Collagen is a major component of the *C. elegans* cuticle and chitin a major component of the eggshell (Page and Johnstone, 2007). Susceptibility levels to viral infection in the mutants was scored in *C. elegans* by counting the number of animals that express RFP over time post-exposure to *rVSV-mCherry*. Strains for which the greatest number of RFP+ animals post exposure to *rVSV-mCherry* were observed were considered to be more susceptible to infection.

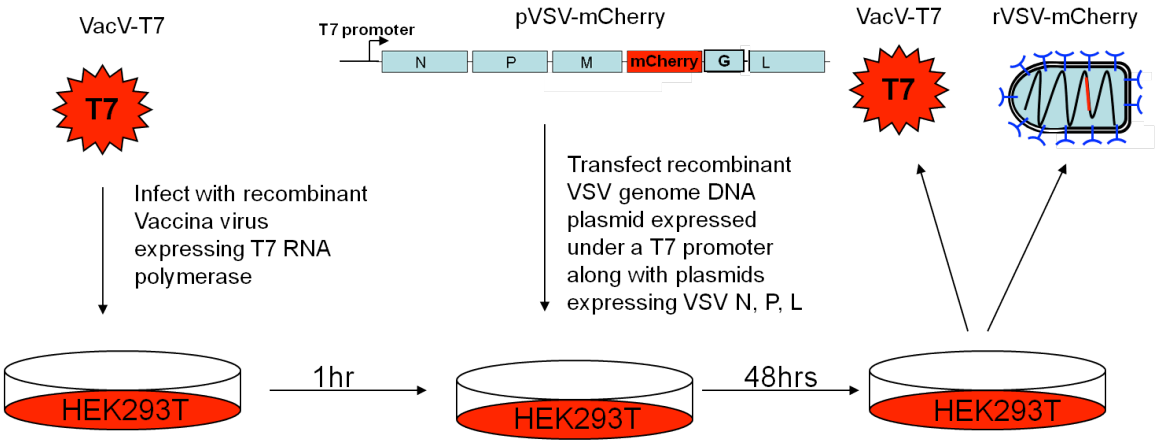


Figure 7a: A schematic representation of the procedure involved in creating recombinant *VSV-mCherry* (see also Appendix 2, Methods).

Table 2: Strains screened for susceptibility to *rVSV-mCherry* infection

strain	**genotype**
DC1	bah-1(br1)
DC1032	bus-4(br4);him-5(e1490)

DC1033	bus-12(br5);him-5(e1490)
DC1043	bah-2(br7);him-5(e1490)
DC1045	bah-1(br1);him-5(e1490)
DC1046	srf-2(br10);him-5(e1490)
DC1048	srf-3(br6);him-5(e1490)
DC1062	bah-3(br9);him-5(e1490)
DC1156	bah-4(br25);him-5(e1490)
DC19	bus-5(br19)
DC2	bus-17(br2)
DC20	br20
DC23	br23ts
DC24	br24dm
DC7	bah-2(br7)
DC9	bah-3(br9)
CB6055	bus-8(e2698) X
MT1522	ced-3(n717)
MT2405	ced-3(n717) unc-26(e205)
MT2547	ced-4(n1162)
MT2550	unc-79(e1068) ced-4(n1162)
N2	wild type
WM27	rde-1(ne219)
WM49	rde-4(ne301) III
MT2495	lin-15B (n744)
MT8189	lin-15B (n765)
CB6430	sqt-3(e2924)
BE3	sqt-2(sc3)
BE1	sqt-1(sc1)
CB24	sqt-3(e24)
CB1350	sqt-1(e1350)
BE16	bli-6 (sc16)
CB1255	vab-11 (e1255)
CB3241	clr-1 (e1745) 15C temp sens
CB518	bli-5 (e518)
CB767	bli-3 (e767)
CB769	bli-1 (e769)
ML514	che-14 (ok193)
CB3687	che-14 (e1960)

Two sample protocols used with results obtained are as follows:

***C. elegans* Infection Protocol Sample A**

Treat *C. elegans* with 1X, 5X, and 10X chitinase solution followed by 10K psi. 100X *S. griseus* chitinase (Sigma, #C6137) is defined as 1 vial resuspended in 500 µL M9 buffer. 10K psi pressure treatments administered using a PB Biosciences Barocycler.

1. Wash L3 WM27 off of plate using M9. Pellet worms at 1500 rpm, 5 min. Aspirate supernatant and continue washes until supernatant is clear of bacteria.
2. Resuspend worms in an appropriate volume so each chemical treatment gets 100-150 µL worms. Add the following chitinase solutions:
    a. 10X: 25 µL 100X chitinase + 225 µL M9
    b. 5X: 12.5 µL 100X chitinase + 237.5 µL M9
    c. 1X: 2.5 µL 100X chitinase +247.5 µL M9
3. Vortex briefly to mix. Incubate at 37°C, 100 rpm, 3 hours.
4. Wash worms with 10 mL M9 to remove chitinase. Pellet as in step 1. Repeat wash 2X.
5. Resuspend worms in 200-250 µL M9. Transfer to pressure tubes and treat at 10K psi.
6. Put 100 µL treated worms in 1 well of a 48-well plate. After all treatments are done, add the following to each well: 56 µL M9 + 4 µL DMSO + 40 µL VSV-mCherry (6-24-10 prep, amplified in HEK 293T, Titer: 4 x 10^9 pfu/mL)
7. Incubate in humidified 26°C incubator. After 24 hours, use Pasteur pipette to transfer worms to OP50-seeded plates. Check for viral infection after 24-48 hours.

Results (strain listed only if fluorescence observed):

Strain	#infected/100 scored day 1	#infected/100 scored day 2
DC20	7	9
DC23	2	3
WM27	3	5
WM49	1	3
CB24	4	7
MT2550	1	3

C. elegans Infection Protocol Sample B

OP50 *E. coli* (for food): resuspend a 50mL LB pellet with 4mL M9 Buffer

Treatments:
(Ca i) NaOH 10mM infected 1:10 rVSV-Cherry
(Ca u) NaOH 10mM; uninfected
(Cb i) NaOH 100mM infected 1:10 rVSV-Cherry
(Cb u) NaOH 100mM; uninfected
(Cc i) NaOH 500mM infected 1:10 rVSV-Cherry
(Cc u) NaOH 500mM; uninfected
(1 i) NaOH 10mM; 10k psi; infected 1:10 rVSV-Cherry
(1 u) NaOH 10mM; 10k psi; uninfected
(2 i) NaOH 100mM; 10k psi; infected 1:10 rVSV-Cherry
(2 u) NaOH 100mM; 10k psi; uninfected

(3 i) NaOH 500mM; 10k psi; infected 1:10 rVSV-Cherry
(3u) NaOH 500mM; 10k psi; uninfected

1. Wash worms off plates (2 days old) with M9 buffer into 15mL tube.
2. Spin 1470 rpm, 5 min, 4C
3. Remove most of the supernatant
4. Wash with M9 and spin again. Remove supernatant
5. Add M9 so there is a total of 480uL.
6. Aliquot as follows:
   (Ca) 80uL worms, 400µL 10mM NaOH
   (Cb) 80uL worms, 400µL 100mM NaOH
   (Cc) 80uL worms, 400µL 500mM NaOH
   (1) 80uL worms, 400µL 10mM NaOH
   (2) 80uL worms, 400µL 100mM NaOH
   (3) 80uL worms, 400µL 500mM NaOH
7. Incubate on rotating platform in 26°C incubator for 2 hours.
8. Quick spin on table-top centrifuge. Remove most of supernatant from each tube. Wash worms 2X with 250uL M9. After second wash, resuspend worms in 250uL M9.
9. Transfer all samples except (C) into pressure tubes. Treat with 10K psi.
10. After pressure treatment, remove worms from pressure tube and transfer to eppendorf tube.
11. Measure yields from pressure tubes and split each condition in half for infected and uninfected conditions. Transfer uninfected sample directly to a 48-well plate (total volume of worms + 50µL food $\geq$ 150µL). For the infected sample, add VSV-Mcherry at a final dilution of 1:10, accounting for worm volume and 50µL food.
    a. Yields from pressure tubes:
        i. (C) 250µL → 125µL worms + 50µL food
        ii. (1) 240µL → 120µL worms + 50µL food + 18.9µL VSV-mCherry for infected condition
        iii. (2) 220 µL →110µL worms + 50µL food + 17.8µL VSV- mCherry for infected condition
        iv. (3) 200 µL →100µL worms + 50µL food + 16.7µL VSV- mCherry for infected condition
12. Transfer infected samples to 48-well plate
13. Feed all worm samples with 50µL OP50
14. Incubate the plate in a stationary 26°C incubator in a loosely covered Tupperware.

Results (strain listed only if fluorescence observed):

Strain	#infected/100 scored day 2
DC20	4
DC23	0

WM27	2
WM49	1
CB24	3
MT2550	0

While the number of worms that expressed RFP, indicative of rVSV-RFP infection, varied from experiment to experiment (within a window of 0-9%), one consistent finding was that fluorescence was only observed within a select number of strains, for example CB24 (figure 7b), which contains a mutation in the collagen gene *sqt-3*, and DC20, a novel mutant provided by our UCSF collaborator Dr. Creg Darby. Animals seemed to be infected when exposed to virus in the L4 stage of development. Moreover, the cells expressing RFP were consistently the same animal to animal, suggesting a particular path of viral entry (figure 8).

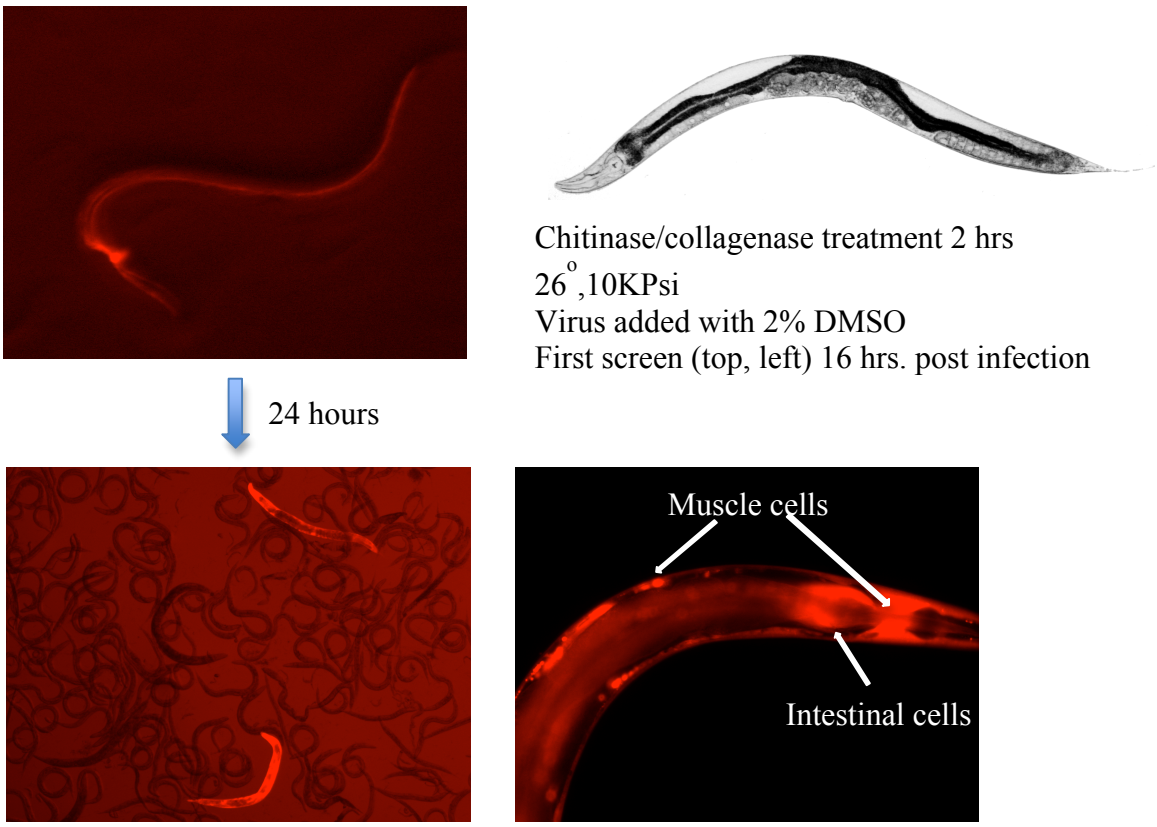


Figure 7b: *rVSV-mCherry* infection of a CB24 *sqt-3* mutant. RFP, indicative of viral infection is first observed in the excretory pore (top, left) and then spreads to other tissues, such as muscle and intestine.

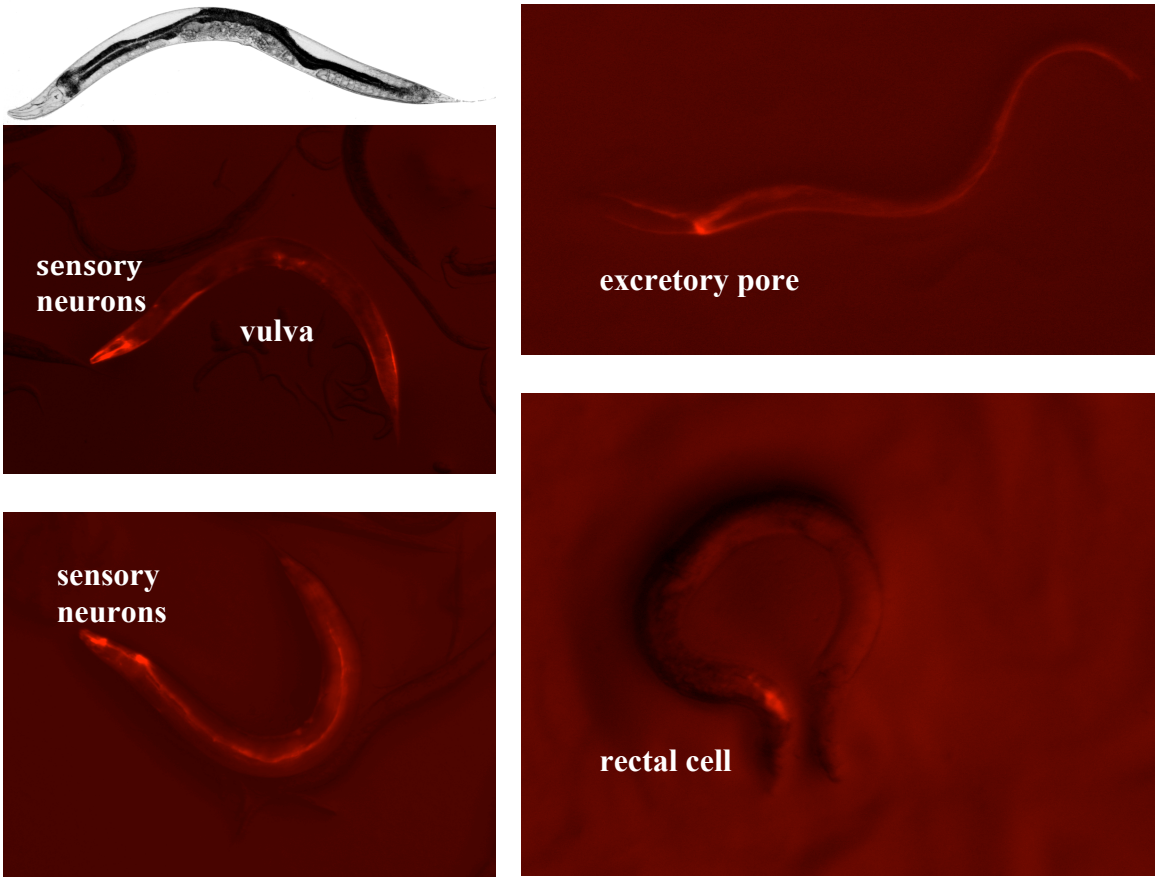


Figure 8: RFP, indicative of viral infection, was consistently observed initially in select cell types, labeled above, in all strains examined, suggesting particular routes of viral entry in *C. elegans*.

II.5 *C. elegans* infections as function of viral stock preparations

Unfortunately, we were not able to follow up on these initial findings, as after our initial stock of *rVSV-mCherry* was depleted, we rarely saw RFP expression in exposed *C. elegans* with any subsequent viral preparation, despite recording similar viral titres by plaque assay in mammalian cells. We speculated that some cellular factor either existed in our first batch of virus that promoted infection, or was present in subsequent preparations that inhibited infection. To try and address this hypothesis, we prepared *rVSV-mCherry* stocks using different mammalian cell types, purification methods, serum concentrations, and collection time-points, however, in all scenarios, RFP expression was rarely observed under any conditions (table 3).

Table 3: *rVSV-mCherry C. elegans* infections with varying methods of viral production

Virus	Cell Type	Purified*	[Serum]**	Time Point (hours)	# Infected Worms/100[#]
VSV-mCherry	HEK 293T	No	Low	24	1
VSV-mCherry	HEK 293T	No	High	24	0
VSV-mCherry	Vero	No	Low	24	4

VSV-mCherry	Vero	No	High	24	0
VSV-mCherry	BHK-21	No	Low	24	0
VSV-mCherry	BHK-21	No	High	24	0
VSV-mCherry	HEK 293T	Yes	Low	24	0
VSV-mCherry	HEK 293T	Yes	High	24	1
VSV-mCherry	Vero	Yes	Low	24	0
VSV-mCherry	Vero	Yes	High	24	1
VSV-mCherry	BHK-21	Yes	Low	24	2
VSV-mCherry	BHK-21	Yes	High	24	0
VSV-mCherry	Vero/HEK 293T	No	Low	48	1
VSV-mCherry	HEK 293T	No	High	48	1
VSV-mCherry	Vero	No	High	48	0
VSV-mCherry	BHK-21	No	Low	48	0
VSV-mCherry	BHK-21	No	High	48	0
VSV-mCherry	Vero/HEK 293T	Yes	Low	48	0
VSV-mCherry	Vero/HEK 293T	Yes	Low	48	0
VSV-mCherry	HEK 293T	Yes	High	48	0
VSV-mCherry	Vero	Yes	High	48	1
VSV-mCherry	BHK-21	Yes	Low	48	0
VSV-mCherry	BHK-21	Yes	High	48	0

* Viral stocks were purified through a sucrose gradient. The non-purified stocks were separated from mammalian cells via centrifugation.
**Two different sera concentrations were used in the mammalian cell culture media. Low concentration is defined at 2.5% FBS while high concentration is defined as 10% FBS.
Each virus stock was tested for infection in *C. elegans* strain DC20 following a chemical treatment with chitinase and a pressure treatment at 10K psi.

We also did a series of experiments to determine if experimental conditions were relevant to infection rate; for example, see table 4.

Table 4: Sample of experimental conditions tested in *rVSV-mCherry C. elegans* infection studies

Strain	**Stage**	**Chem.**	**10K psi**	**4% DMSO***	**Virus**	**# infected**
WM27	L4	Chitinase 2X	Y	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L4	Chitinase 2X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L4	Chitinase 5X	Y	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L4	Chitinase 5X	N	N	Vero/HEK Low serum, 48 hr, non-purified	

WM27	L4	Collagenase 2X	Y	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L4	Collagenase 2X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L4	Collagenase 5X	Y	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L4	Collagenase 5X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L3	Chitinase 2X	Y	N	Vero/HEK Low serum, 48 hr, non-purified	1
WM27	L3	Chitinase 2X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L3	Chitinase 5X	Y	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L3	Collagenase 2X	Y	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L3	Collagenase 2X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L3	Collagenase 5X	Y	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L4	Chitinase 1X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L4	Collagenase 1X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
WM27	L3	Chitinase 1X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Chitinase 1X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Chitinase 1X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L4	Chitinase 1X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	1
DC20	L4	Chitinase 1X	N	Y	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L4	Chitinase 2X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Chitinase 2X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L4	Chitinase 2X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Chitinase 2X	N	Y	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L4	Chitinase 5X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Chitinase 5X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	

DC20	L4	Chitinase 5X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Chitinase 5X	N	Y	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L4	Collagenase 1X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Collagenase 1X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L4	Collagenase 1X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Collagenase 2X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Collagenase 2X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L4	Collagenase 2X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	1
DC20	L4	Collagenase 2X	N	Y	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L4	Collagenase 5X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L4	Collagenase 5X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L4	Collagenase 5X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Chitinase 1X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Chitinase 1X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L3	Chitinase 1X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Chitinase 1X	N	Y	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L3	Chitinase 2X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Chitinase 2X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L3	Chitinase 2X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Chitinase 5X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Chitinase 5X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L3	Chitinase 5X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Chitinase 5X	N	Y	Vero/HEK Low serum, 48 hr, PURIFIED	

DC20	L3	Collagenase 1X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Collagenase 1X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L3	Collagenase 1X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Collagenase 2X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Collagenase 2X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L3	Collagenase 2X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Collagenase 2X	N	Y	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L3	Collagenase 5X	N	N	Vero/HEK Low serum, 48 hr, non-purified	
DC20	L3	Collagenase 5X	N	N	Vero/HEK Low serum, 48 hr, PURIFIED	
DC20	L3	Collagenase 5X	N	Y	Vero/HEK Low serum, 48 hr, non-purified	

*4% DMSO has been reported to facilitate assembly of viral particles (Scholtissek and Muller, 1988)

Note that although polyethylene glycol (PEG) was reported to increase *C. elegans* susceptibility to viral infection (Liu et al, 2006, PNAS, 103:4174), we did not observe any differene in infection efficiency of *rVSV-GFP* in *C. elegans* exposed to different PEG molecular weights and concentrations.

II.6 Expression of the EphB2 Nipah viral receptor in *C. elegans*

While some viruses have a broad host range and naturally infect both nematodes and humans, such as VSV, VV, and RVFV, other viruses infect with a limited host range due to a requirement for cellular entry through specific membrane receptors. To determine if *C. elegans* could be made susceptible to viral infection through the generation of recombinant strains expressing exogenous receptors, we generated several strains of *C. elegans* expressing the EphB2 receptor, the Nipah virus receptor, not normally found in *C. elegans*. Figure 9A pictures a recombinant strain of *C. elegans* that expresses EphB2 in the intestine under control of the *act-5* promoter. Constructs were also generated to drive EphB2 in the sensory amphid neurons of the head, which are exposed to the environment, using the *odr-10*, *tax-4*, and *unc-119* promoters (for example, figure 9B).

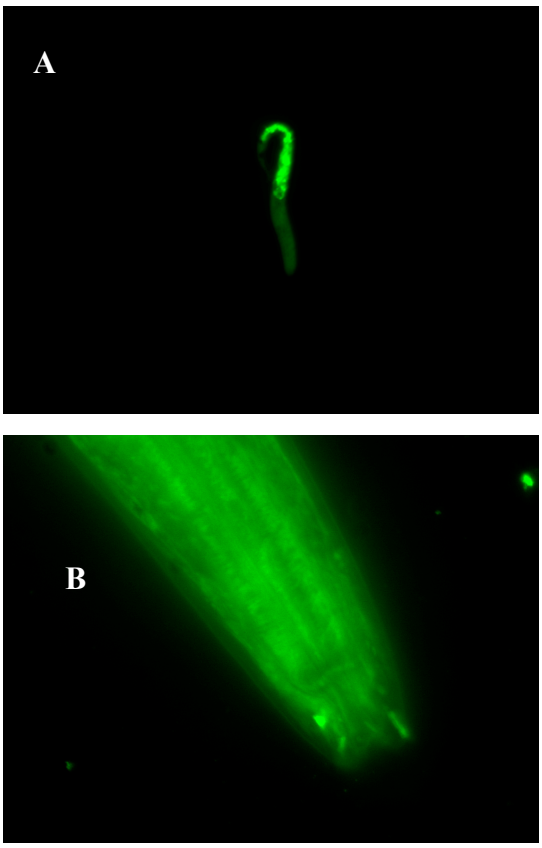


Figure 9: *C. elegans* expressing the mammalian Nipah viral receptor, EphB2, in the intestine, under control of the *act-5* promoter, or in the amphid neurons, under control of the *tax-4* promoter. Development of *act-5::ephB2* transgenic *C. eleagns* was arrested in the first larval stage (L1, pictured in A).

Nipah virus is a deadly risk group 4 agent; therefore, to determine in our (BSL2) lab if Nipah could utilize the EphB2 receptor exogenously expressed in C. elegans, we generated recombinant VSV virus expressing the Nipah viral coat protein, termed VSVΔG/NiVFG (figure 10). Table 5 lists all the recombinant viruses generated for this project.

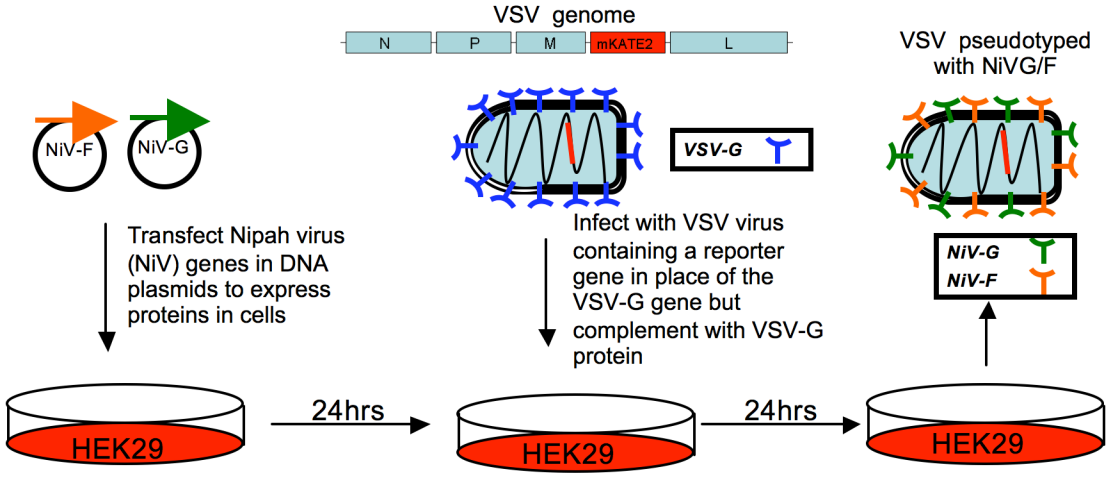


Figure 10: Method used for the production of VSV pseudotyped with Nipah virus envelope protein

Table 5: Recombinant viruses generated from genomic elements expressing mCherry

Virus	**Type**	**Single cycle* or Infectious**	**Abbreviation**
Vescicular Stomatitis Virus	RNA	Infectious	rVSV-RFP,
Vescicular Stomatitis Virus	RNA	Single cycle	rVSVΔG-mCherry
Vescicular Stomatitis Virus	RNA	Single cycle	rVSVΔG/NiVFG-mCherry#

* Single-cycle viruses lack the genomic element required to make envelope protein (G), so don't replicate to produce new viral particles.
# This single-cycle virus has the Nipah (not VSV) protein envelope

We encountered both a technical hurdle as well as a surprising result in the course of conducting these experiments: first, we found that high levels of EphB2 expression can be toxic (using, for example, the *unc-119* promoter), restricting our work to promoters with relatively low levels of expression, and second, we found, surprisingly, that a (used to study Nipah virus cell entry since intact), can infect a subset of intestinal cells in wild-type *C. elegans* (data not shown).

III. Production of molecular sensors of viral infection to trigger fluorescence expression in *C. elegans* cells infected by virus

III.1 Reporter of VSV infection

Viral ssRNAs are flanked by 3' and 5' untranslated regions (UTRs) that are required for ssRNA replication mediated by viral RNA polymerases. Figure 11 depicts a novel reporter system we constructed that would harness viral UTRs to drive fluorescent protein expression upon viral infection of the host cell.

Figure 11: Schematic of the reporter we designed to detect cellular infection by VSV. Viral ssRNAs are flanked by 3' and 5' untranslated regions, the leader and trailer sequences, respectively, that are required for ssRNA replication, or GFP in the case of the reporter, mediated by viral RNA polymerases. The construct is constitutively expressed in cells under control of the RNA polymerase 1 promoter; we used this promoter so that the resulting RNA would be unmodified, ie lack a cap and poly A tail, and thus look as similar as possible to a viral RNA.

We built and tested this reporter but consistently did not detect a fluorescent signal above background in infected mammalian tissue culture cells. We made a number of alterations in efforts to improve upon our original design; for example, we tried using the better defined T7 promoter/terminator sequences, a luciferase reporter (more sensitive), M segment UTRs, and brought in a collaborator, Ramon Flick, Ph.D., CSO, BioProtection System, for advice. However, we were not able to construct a functional VSV reporter system, so instead focused efforts on a RVFV reporter system.

III.2 Reporter of RVFV infection

Figure 12 depicts our design and test results for a cellular sensor of RVFV infection. We designed this sensor according to the published sequence of MP12; however, the 3' and 5' UTRs of all RVFV sequenced to date, including pathogenic strains, are fully conserved and thus mostly like could be applied to outbreak strains.

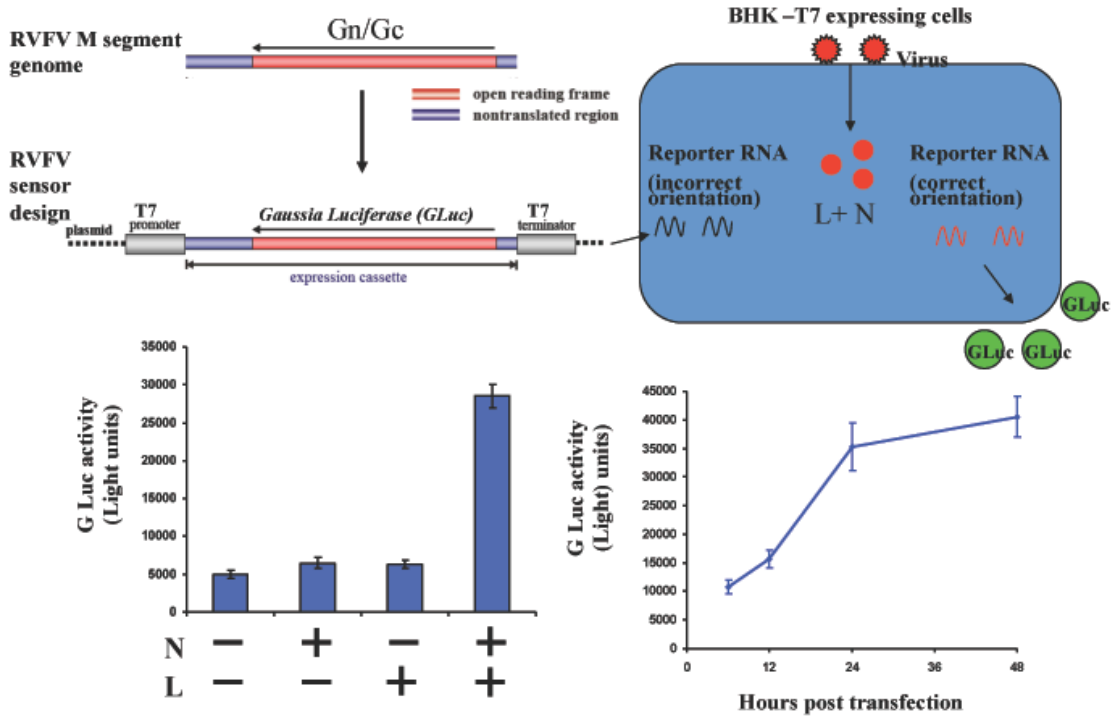


Figure 12: RFVF sensor design and function in BHK-T7 cells. Expression of RVFV L and N protein is required to detect luciferase activity above background.

In all cases, unacceptibly high background expression from the sensor has been observed, with only modest increases in fluorescence observed following infection with the RVFV MP12 strain.

III.3 Reporters of VV infection

III.3.1 Novel Cre-based sensor for VV infection

The NIAID Category A Viral Pathogen list includes one dsDNA virus, Variola major, the causative agent of Smallpox. Variola major and other Poxviridae family viruses replicate exclusively in the cytoplasm, despite having dsDNA genomes (other dsDNA viruses, such as HIV, insert their genomes into the host chromosomes, which are located in the nucleus).

We have designed a novel reporter for poxvirus infection (Figure 13). Viral proteases, which normally mediate the maturation of viral molecules, are harnessed for processing of a membrane tether that otherwise restricts the movement of site-specific recombinase Cre; once the tether is cleaved, Cre translocates to the nucleus and activates transcription of GFP.

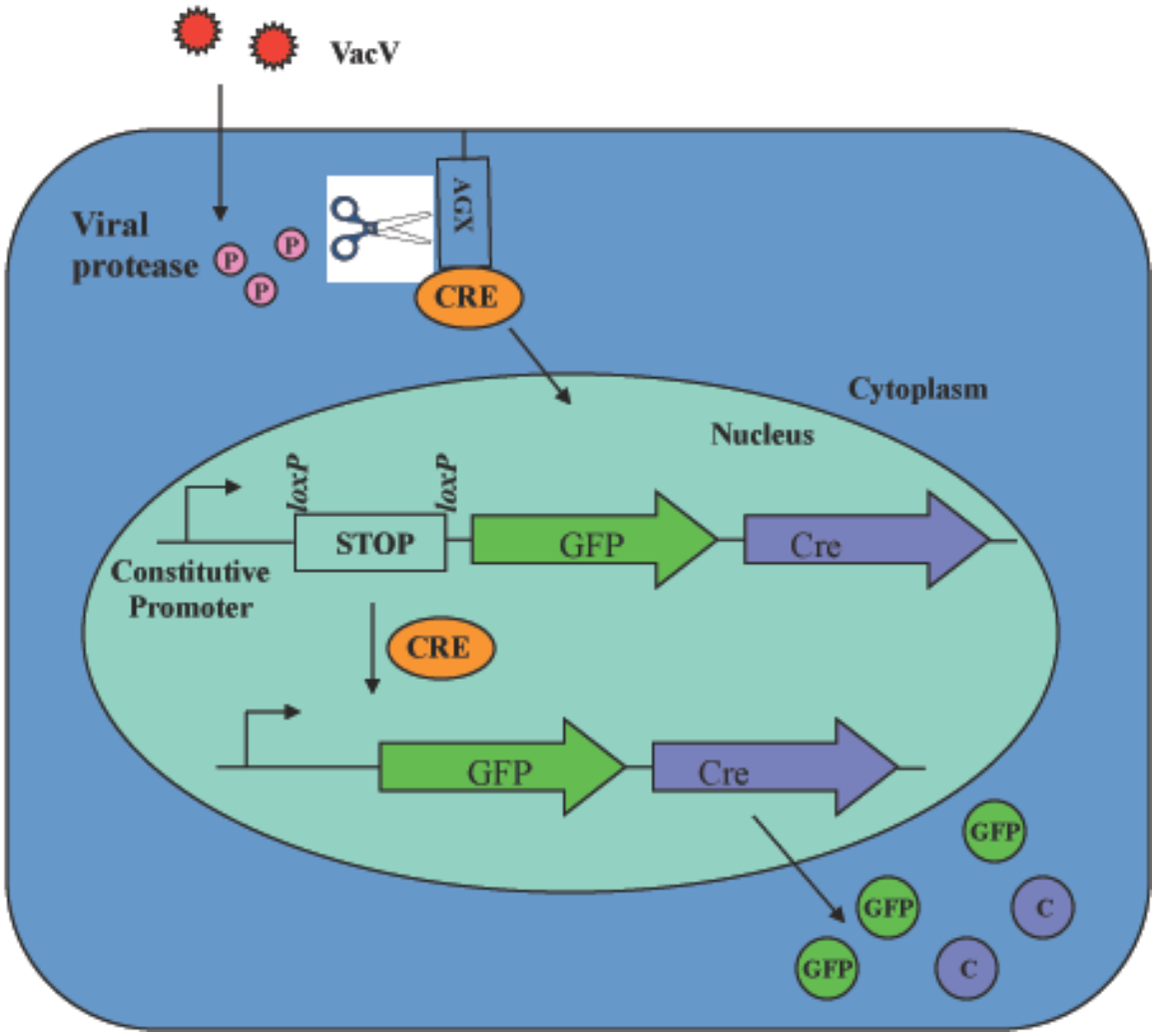


Figure 13: VV infection results in expression of viral proteases, such as A12L, which specifically cleaves AGX peptide sequences (Yang, 2007). A12L cleavage of a tether maintaining the site-specific recombinase Cre in the cytoplasm permits Cre to move to the nucleus, where it will excise a transcriptional stop cassette (Branda and Dymecki, 2004), and enables production of GFP (or RFP) and more Cre.

Toward production of this VV sensor, we focused on I7L, a cysteine proteinase required for viron assembly (Byrd and Hruby, 2005; Byrd et al., 2003). I7L cleaves the major core protein precursors P4a, P4b, and P25K, products of the A10L, A3L, and L4R open reading frames respectively, at a novel Ala-Gly-X aa cleavage site with cleavage occurring after the glycine residue. I7L also is likely to be responsible for cleavage of the A17 membrane protein, at an Ala-Gly-Ala site. We synthesized through Epoch Biolabs the following 8 constructs:

1. pAcGFP-C1-Src-P4A-Cre (Src myristoylation sequence-P4A-Cre fusion)
2. pAcGFP1-C1-Src-P4B-Cre
3. pAcGFP1-C1-Src-A17L-Cre
4. pAcGFP-C1-Src-p25K-Cre

5. pAcGFP1-C1-I7L
6. pAcGFP1-N1-I7L

All of the VV coding sequences were mammalian codon optimized. The Src myristoylation sequence includes Nhe1 and HIII sites on the ends and was cloned into the corresponding sites of the pAcGFP-C1 vector to remove GFP from the vector. The two IL7 sequences have XhoI and KpnI sites on the ends and were cloned into either pAcGFP-C1 or pAcGFP-N1, to generate GFP fusions on either the N or C terminus of the IL7 protein, respectively. The first four constructs were transfected into HeLA cells for IHC using AbCam Rabbit polyclonal to cre recombinase (#ab40011), and goat polyclonal secondary to rabbit IgG-H&L FITC. Cre appeared to be expressed in the cytoplasm in the transfected cells, but these results need to be repeated.

7. CMV-HTNC (Peitz, et al., 2002)
8. CMV-HSTNC (adds EGL-17 signal sequence for Cre cell secretion)

These two Cre expressing constructs were synthesized by Epoch Biolabs and terned pTriEx-HTNC (TATCre) Nhe-Not1and pTriEx-HSTNC (SS-TATCre).

III.3.2 VV promoter::mCherry sensor

In another VV sensor design, we used a VV-specific promoter to drive mCherry expression only in cells infected with VV (Figure 14).

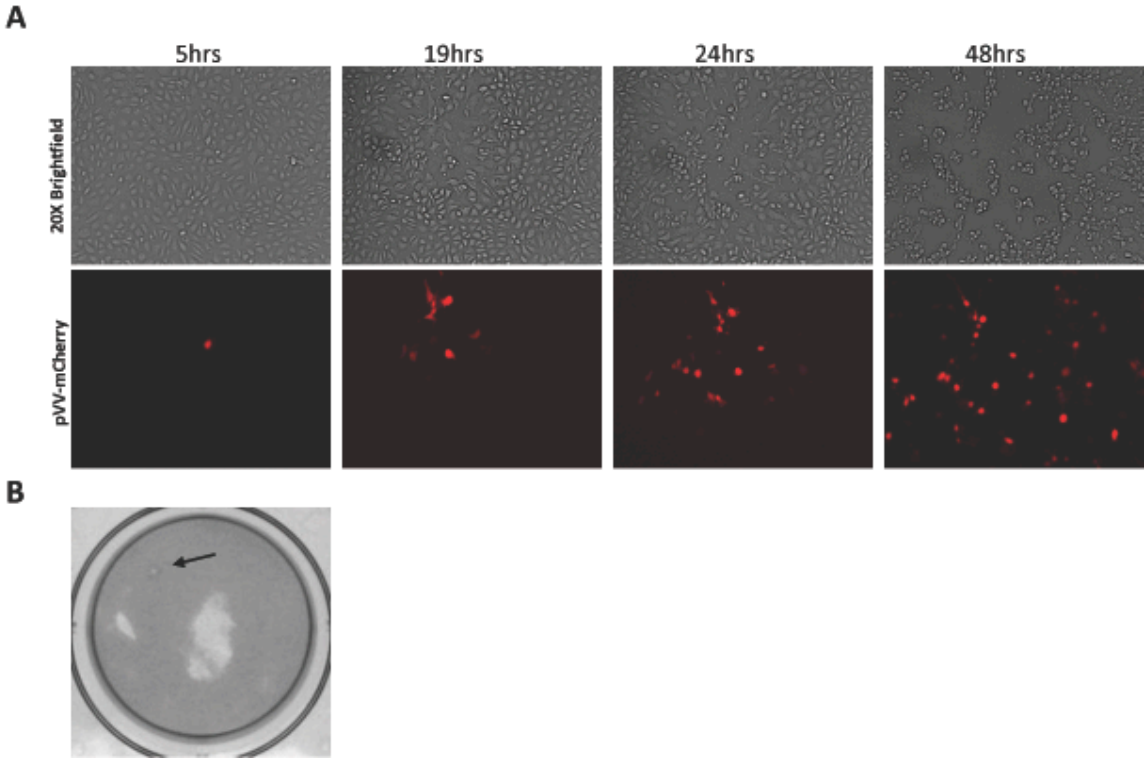
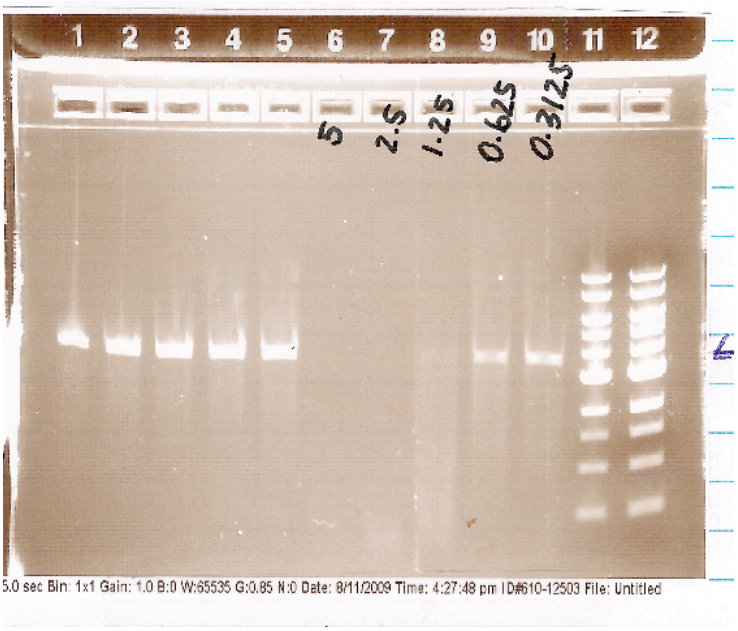


Figure 14: Early detection of Vaccinia virus infection through a DNA sensor. A) BSC40 cells were transfected with a sensor plasmid expressing mCherry under the p7.5 Vaccinia virus

promoter. Twenty four hours post-transfection, Vaccinia virus was used to infect the transfected cells. Brightfield and fluorescent images were taken at indicated time points to measure sensor-based fluorescent detection versus onset of cytopathic effects (CPE) B) One 12 well image containing a plaque (arrow) formed from a single infectious Vaccinia virus 48hrs post-infection was positive for mCherry expression.

IV. A mechanism to amplify the fluorescent signal, such that viral infection of a single cell in a *C. elegans* nematode would result in multiple *C. elegans* fluorescing, thereby facilitating detection

For clearance sampling applications, we expect that fluorescent expression from an optimized molecular sensor for viral infection will need to be amplified in order to be reliably detected. For this purpose, we used a cell permeable Cre recombinase-based system for activating fluorescence production in cells neigboring a virally-infected cell. A membrane permeable Cre construct (Peitz, et al., 2002) was obtained from Adgene, and control pLox2+ DNA with which to assess DNA recombination activity was obtained from New England Biolabs. We purified this Cre protein and demonstrated its activity in vitro (Figure 15).



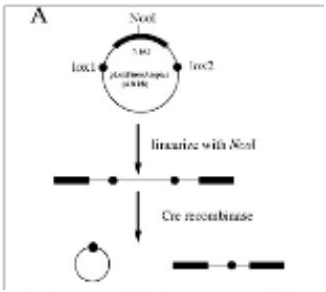


Figure 15: Purification of functional membrane-permeable Cre protein. lanes 1-5: 2, 1.5, 1, 0.5, 0.25 units Cre from NEB per reaction (250 ng pLox2+) lanes 6-10: 5, 2.5, 1.25, 0.625, 0.3125 uL HTNCre per reaction (250 ng pLox 2+) lanes 11-12: 5, 10 uL 1 kb ladder Linearized pLox2+ is 3.7 kb. Recombination of control pLox2+ DNA is observed with 0.02 and 0.004 ul Cre protein.

A CMV::loxP-GFP-loxP-mKate DNA sequence (with or without introns) was synthesized by Epoch Biolabs and cloned into the pPD49.26 *C. elegans* vector containing the *unc-54* 3' end. Figure 16a illustrates a design for Cre-based amplification of fluorescence expression, and Fig16b shows the potential of a membrane permeable Cre protein to enter cells to activate RFP expression.

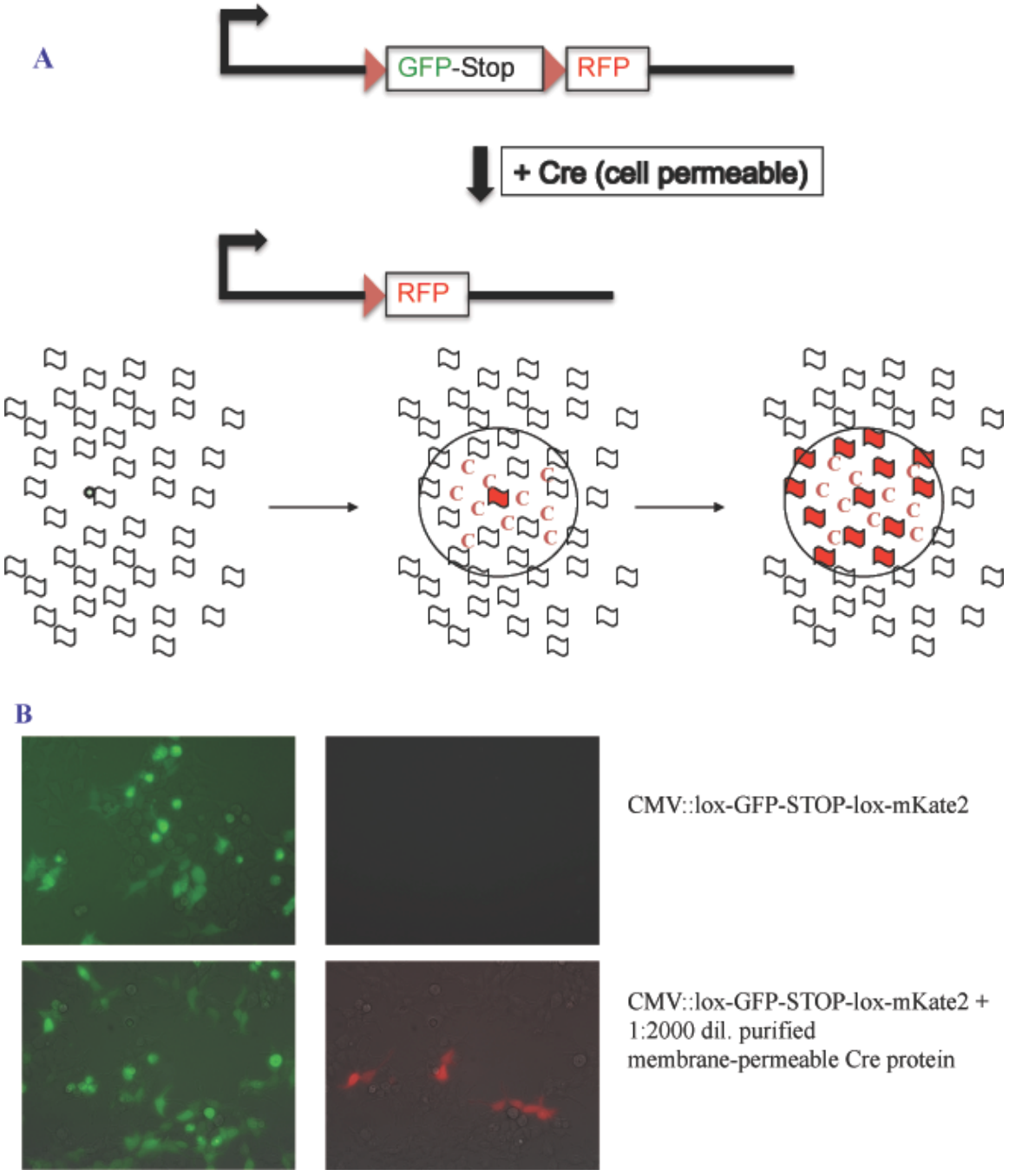


Figure 16: **Cre-recombinase-based mechanism for amplifying a fluorescent signal from cell to cell. (A)** The CMV::loxP-GFP-loxP-mKate construct contains the constitutively-active CMV promoter upstream of two *loxP* sites (triangles) flanking coding sequence for GFP plus a transcriptional STOP sequence, followed by coding sequence for the RFP mKate2. Cre recombinase will excise DNA between its target *loxP* sites. In the presence of Cre protein, the GFP-STOP cassette is excised, enabling CMV to drive expression of mKate2. **(B)** Cell-permeable Cre enters cells to activate mKate2 expression. Untreated transfected HEK293 cells express GFP but not mKate2 (top). By contrast, cells treated with a 1:2000 dilution of cell-permeable Cre express both GFP and mKate2 (bottom).

V. Production of a gel with which to disperse our engineered strains of *C. elegans* on a surface for the purpose of clearance sampling

A gel with which to disperse our engineered strains of *C. elegans* on a surface for the purpose of clearance sampling would need to:

- be non-toxic and contain non-corrosive ingredients (silica gel, polymers, etc.)
- be deployed through off-the-shelf equipment (e.g., paint sprayers)
- contain wetting agents to help penetrate into cracks and crevices
- remain in position on all surfaces (horizontal, downward facing, vertical) for several hours
- be easy to clean-up by vacuuming, brushing, or drying up
- incorporate crystallizing polymers (similar to carpet cleaners) to enable the gel to dry to small, non-sticky particles and not adhere to a surface
- be transparent to enable detection of a fluorescent signal

Appendix 3 lists a variety of gel candidate materials and relevant characteristics.

Since low pH is a trigger for many enveloped viruses to fuse with the eukaryotic host cell (and thereby initiate viral infection), we tested if lowering the pH of the media in which *C. elegans* embryonic stem cells were cultured affected the cells' rate of infectivity. Preliminary results suggest that a low pH shock (of pH 5.1) does indeed result in a higher rate of viral infection of the cultured cells (Figure 17).

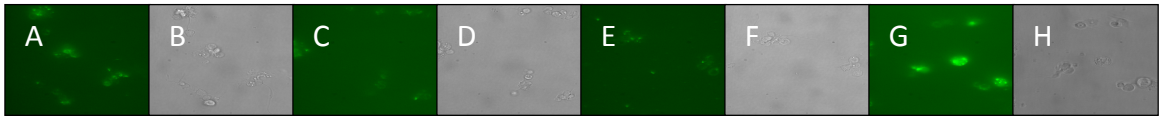


Figure 17: Effect of pH on uptake of VSV-GFP into embryonic cells prepared from the *C. elegans* N2 strain. Cells with bound virus at an MOI=10 were pulsed with pH 7.0 growth medium (A-B), or buffer at pH 6.6 (C-D), pH 5.7 (E-F), or pH 5.1 (G-H) for 1 minute and then were allowed to resume growth in culture medium until imaging.

We also confirmed that C. elegans cells, like mammalian cells, contain low pH vesicles within the cells (Figure 18).

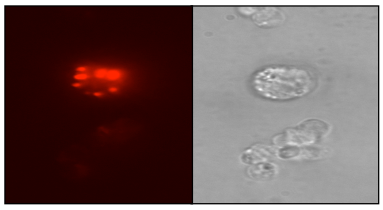


Figure 18: Lysotracker red staining of *C. elegans* wild type strain N2 embryonic cells indicates the presence of low pH vesicles within the cells.

Our finding that a low pH pulse increases *C. elegans* susceptibility to viral infection enables us to better define the characteristics required for the gel for on-site clearance sampling.

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APPENDIX A: VIRUSES USED IN THIS STUDY

Vescicular Stomatitis Virus (VSV), a member of the rhabdovirus family, is a single-stranded negative-sense RNA virus. The two most clinically-relevant serotypes of VSV are Indiana and New Jersey (BMBL 5th Edition). The epidemiology of VSV is poorly understood although it is known that VSV is spread through direct contact, fomites, and insects. VSV is endemic in horses, mules, donkeys, cattle, and swine throughout Mexico and Central and South America; outbreaks occurred in the US in 2004, 2005, and 2006. VSV causes vesicular stomatitis, a disease in cattle resembling foot-and-mouth disease, though with a milder course. A USDA permit is required to receive VSV (below). Because VSV is a RG2 human pathogen, registration with ATCC/BEI is required for ordering this strain from this resource. We received recombinant VSV expressing either the luciferase or GFP genes (rVSV-luc and rVSV-GFP) from Drs. Richard Cadagan and Adolfo García-Sastre, Mount Sinai School of Medicine.

Rift Valley Fever virus (RVFV) causes viral disease endemic throughout Africa and, more recently, the Arabian Peninsula. RVFV infections of livestock are often recognized by the onset of abortion "storms" and mortality in livestock (primarily sheep and cattle), with newborn mortality approaching 100%. Human infections typically occur due to the bites of infected mosquitoes or aerosol exposure during the handling of diseased animals. In most human cases, the disease is characterized by a self-limiting febrile illness (2-5 days), which progresses to more serious complication in 1-2% of infected individuals.

The MP12 attenuated strain of Rift Valley fever virus (RVFV) was obtained by 12 serial passages of a virulent isolate ZH548 in the presence of 5-fluorouracil. Strain ZH548 was originally isolated from the serum of an uncomplicated human febrile case of RVF that occurred during the Egyptian outbreak of 1977. RVFV has a tripartite single stranded RNA genome and characterization of the MP12 virus indicated that determinants for attenuation were present in each of the three segments. (Vaccine 8: 369). These findings suggest that reversion to virulence is unlikely, and further indicate that genetic reassortment with wild-type viruses would also be expected to yield attenuated variants.

We will obtain the MP12 strain of RVFV from Drs. C.J. Peters and George Bettinger, University of Texas Medical Branch (UTMB). The exact virus preparation to be sent by UTMB is a reconstitution from culture of a clinical trial vial used for human vaccination. There is documentation that describes the safety testing done on the bulk vaccine virus prior to filling and lyophilization, however, this information is confidential. The USDA regulates the transport of MP12 and therefore the shipper and the receiver must have permits to exchange this virus. The USDA makes the shipper liable for correctly sending the MP12 strain.

Vaccinia virus (VV) from American Type Culture Collection (ATCC)/BEI Resources *Vaccinia virus* is the prototype of the genus Orthopoxvirus, whose members include cowpox, monkeypox and variola virus (smallpox). It is a double-stranded DNA virus that has a broad host range under experimental conditions but is rarely isolated from animals outside the laboratory. We will use the Western Reserve (WR) standard strains of *VV* and a recombinant *VV* derived from the WR strain, VV.NP-S-EGFP, expressing GFP for infection studies if necessary. The VV.NP-S-EGFP

contains a chimeric gene encoding the influenza virus nucleoprotein, the ovalbumin peptide and EGFP.

U.S.DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES
RIVERDALE, MARYLAND 20737

UNITED STATES VETERINARY PERMIT FOR IMPORTATION AND TRANSPORTATION OF CONTROLLED MATERIALS AND ORGANISMS AND VECTORS

PERMIT NUMBER
107385
Research

DATE ISSUED
09/23/2008

DATE EXPIRES
09/23/2009

NAME AND ADDRESS OF SHIPPER(S)

Mr. Richard Cadagan
Mount Sinai School of Medicine
Department of Microbiology
One Gustave L. Levy Place, Box 1124
New York, New York 10029
UNITED STATES

CC:
AVIC, VS, CA (Sacramento, CA)

NAME AND ADDRESS OF PERMITTEE INCLUDING ZIP CODE AND TELEPHONE NUMBER

Oscar Negrete
Sandia National Laboratories
7011 East Avenue, Biological Receiving
Bldg 968, Rm.132
Livermore, California 94550
925-294-6359

U.S. PORT(S) OF ARRIVAL
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MODE OF TRANSPORTATION AIR

AS REQUESTED IN YOUR APPLICATION, YOU ARE AUTHORIZED TO IMPORT OR TRANSPORT THE FOLLOWING MATERIALS

Recombinant vesicular stomatitis virus expressing a fluorescent reporter gene

RESTRICTIONS AND PRECAUTIONS FOR TRANSPORTING AND HANDLING MATERIALS AND ALL DERIVATIVES

THIS PERMIT IS ISSUED UNDER AUTHORITY CONTAINED IN 9 CFR CHAPTER 1, PARTS 94,95 AND 122. THE AUTHORIZED MATERIALS OR THEIR DERIVATIVES SHALL BE USED ONLY IN ACCORDANCE WITH THE RESTRICTIONS AND PRECAUTIONS SPECIFIED BELOW (ALTERATIONS OF RESTRICTIONS CAN BE MADE ONLY WHEN AUTHORIZED BY USDA, APHIS, VS).

- **Adequate safety precautions shall be maintained during shipment and handling to prevent dissemination of disease.**
- *** THIS PERMIT IS INVALID WITHOUT PERMITTEE'S SIGNATURE ***. "I, Oscar Negrete, certify that this material will be used in accordance with all restrictions and precautions as are specified in this permit,
  ***
- *** signed: Oscar Negrete . " ***
- This permit DOES NOT authorize direct or indirect exposure of or inoculation into laboratory and domestic animals, including poultry, cattle, sheep, swine, horses, etc. Work shall be limited to IN VITRO uses only.
- Packaging, containers, and all equipment in contact with these materials shall be sterilized or considered a biohazard and be disposed of accordingly.
- This permit is valid only for work conducted or directed by you or your designee in your present U.S. facilities. (MATERIALS SHALL NOT BE MOVED TO ANOTHER U.S. LOCATION, OR DISTRIBUTED WITHIN THE U.S., WITHOUT USDA, APHIS, VS, NCIE AUTHORIZATION.)

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SIGNATURE	TITLE	NO. LABELS
Waleid Muhmed Waleid I. Muhmed	Senior Staff Veterinarian National Center - Import - Export	

VS FORM 16-6A (MAR 95) Replaces VS Form 16-3A and 16-28 which are obsolete Page 1 of 2

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UNITED STATES VETERINARY PERMIT FOR IMPORTATION AND TRANSPORTATION OF CONTROLLED MATERIALS AND ORGANISMS AND VECTORS

PERMIT NUMBER
105772
Research

DATE ISSUED
04/03/2008

DATE EXPIRES
04/03/2009

NAME AND ADDRESS OF SHIPPER(S)

Ms. Beverly Foghtman
United States Army Medical Institute of Infectious Disease [see attached list]
1425 Porter St
Fort Detrick, Maryland 21702
UNITED STATES

CC:
AVIC, VS, TX (Austin, TX)

NAME AND ADDRESS OF PERMITTEE INCLUDING ZIP CODE AND TELEPHONE NUMBER

Dr. George E. Bettinger
University of Texas Medical Branch
301 University Blvd
Mail Stop 0926
Galveston, Texas 77555-0926
409-392-5049

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AS REQUESTED IN YOUR APPLICATION, YOU ARE AUTHORIZED TO IMPORT OR TRANSPORT THE FOLLOWING MATERIALS

Rift Valley fever virus MP-12 vaccine strain

RESTRICTIONS AND PRECAUTIONS FOR TRANSPORTING AND HANDLING MATERIALS AND ALL DERIVATIVES

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- **Adequate safety precautions shall be maintained during shipment and handling to prevent dissemination of disease.**

- *** THIS PERMIT IS INVALID WITHOUT PERMITTEE'S SIGNATURE ***. "I, Dr. George E. Bettinger, certify that this material will be used in accordance with all restrictions and precautions as are specified in this permit,

- ***

- *** signed: George E. Bettinger . " ***

- All studies must be conducted in Biosafety Level 2 laboratory facilities located in "Libbie Moody Thompson" Basic Science Building #54 , rooms 3-104A and 3-104B, and Medical Research Building #59, room 3-134, and Animal Biosafety Level 2 facility located in Keiller Building #2, rooms G-142-A, -B, -C, -D, -E, -F, -G, and -H, which have been inspected and approved by the USDA. These facilities shall be reinspected every 3 years.

- Importing facility is subject to routine inspection by USDA, APHIS personnel during normal business hours to ensure compliance with permit restrictions.

- Permittee shall notify USDA, APHIS, Veterinary Services within 48 hours of failure of receipt of a shipment of materials. Permittee to contact [1-866-994-LOST(5678)].

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SIGNATURE	TITLE	NO. LABELS
Thomas Letonja	Staff Veterinarian National Center - Import - Export	

VS FORM 16-6A (MAR 95) Replaces VS Form 16-3A and 16-28 which are obsolete

01 October,2008

Portia Cortes
USDA,APHIS,VS
10365 Old Placerville Rd.,Suite 210
Sacramento, CA 95827

Dear Portia,

I have inspected the facilities, viz. Building 968 on the Sandia National Laboratories site at 7011 East Ave., Livermore, CA as BL2 laboratories to use Rift Valley Fever Virus MP-12 vaccine strain and future use of Vesicular Stomatitus and Vaccinia Viruses.

I have met David Brekke, the Facility Manager, Joe Schoeniger, Biosafety Officer, Cathy Branda, Principal Investigator, Aden Jackson, Manager Health and Safety and Malin Young, Manager Biological Sciences.

Rooms 132 and 134 are the primary initial rooms for the use of the Rift Valley Fever Virus and Rooms 117,121,122,125,126,127,129 and 129a are also BL2 inspected/approved. However, there are other rooms in Building 968 that are not BL2 approved and are used otherwise. Therefore, the entire Building 968 is not BL2 approved.

Additional questions that are usually asked are answered as following:

1. Hazardous materials are autoclaved in an inlab autoclave before disposal.
2. Personnel are experienced and trained in proper procedures.
3. Personnel do not appear to have contact with susceptible species. There is no animal work involved.
4. The Biosafety Officer was present at the inspection.
5. I do not know if a confirming letter from the State Veterinarian is necessary.

I had no recommendations on corrections except that all the BL2 facilities be clearly marked (and separated) from the nonBL2 facilities in the building.

Security arrangements were excellent.

I would recommend these facilities as meeting the criteria for level 2 biosafety.

Gary F.L. Chun, VMD
Port Veterinarian
San Francisco

United States Department of Agriculture

Animal and Plant Health Inspection Service

Veterinary Services

National Center for Import and Export

4700 River Road
Unit 2, Mailstop 22,
Cub. 1A07
Riverdale, MD 20737

(301) 734-5960
FAX (301) 734-3652

Dr. Oscar Negrete
Sandia National Laboratories
7011 East Ave, MS 9292
Livermore, CA 94550

Friday, May 29, 2009

Dear Dr. Oscar Negrete:

Your USDA Veterinary Permit to import and/or transport controlled materials, organisms and vectors accompanies this cover letter.

Please note that your signature is required to validate the permit.

As the permittee, your signature is required to certify that you understand and will abide by all restrictions and precautions that are specified on the permit. Without your signature the permit is invalid and will not be acceptable to USDA inspectors at a Port of Entry into the U.S. Imported materials that require a permit but are presented for import without a valid permit are subject to additional import compliance fees and delays. This certification statement is not a new requirement. It occurs on the application form, USDA VS Form 16-3. The certification statement is used on the permit to allow faster processing and distribution.

Review the permit carefully before signing, and contact us with any questions or concerns.

Please be advised that effective April 29, 2009 user fees associated with permit applications have increased. The new fees are as follows:

New permit application $133, Renewal permit $86, Amended permit $66, FBS inspection $455 (all fees are per application) and the Import compliance fee is $514 per shipment.

Sincerely,

Waleid I. Muhmed

Dr. Waleid I. Muhmed
Senior Staff Veterinarian, AOVSA
National Center for Imports & Exports

re: application number 09121024

To apply for a permit on-line and check the status of a permit, go to the following web page: https://epermits.aphis.usda.gov/epermits or www.aphis.usda.gov/vs/ncie

U.S.DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES
RIVERDALE, MARYLAND 20737

UNITED STATES VETERINARY PERMIT FOR IMPORTATION AND TRANSPORTATION OF CONTROLLED MATERIALS AND ORGANISMS AND VECTORS

PERMIT NUMBER
109556
Research

DATE ISSUED
05/29/2009

DATE EXPIRES
05/29/2010

NAME AND ADDRESS OF SHIPPER(S)

American Type Culture Collection/BEI Resources
10801 University Boulevard
Manassas, Virginia 20110
UNITED STATES

CC:
AVIC, VS, CA (Sacramento, CA)

NAME AND ADDRESS OF PERMITTEE INCLUDING ZIP CODE AND TELEPHONE NUMBER

Dr. Oscar Negrete
Sandia National Laboratories
7011 East Ave, MS 9292
Livermore, California 94550
925-294-6359

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AS REQUESTED IN YOUR APPLICATION, YOU ARE AUTHORIZED TO IMPORT OR TRANSPORT THE FOLLOWING MATERIALS

Vaccinia virus (ATCC VR-2153, BEI Resources NR-55 and NR-624)

RESTRICTIONS AND PRECAUTIONS FOR TRANSPORTING AND HANDLING MATERIALS AND ALL DERIVATIVES

THIS PERMIT IS ISSUED UNDER AUTHORITY CONTAINED IN 9 CFR CHAPTER 1, PARTS 94,95 AND 122. THE AUTHORIZED MATERIALS OR THEIR DERIVATIVES SHALL BE USED ONLY IN ACCORDANCE WITH THE RESTRICTIONS AND PRECAUTIONS SPECIFIED BELOW (ALTERATIONS OF RESTRICTIONS CAN BE MADE ONLY WHEN AUTHORIZED BY USDA, APHIS, VS).

- **Adequate safety precautions shall be maintained during shipment and handling to prevent dissemination of disease.**

- *** THIS PERMIT IS INVALID WITHOUT PERMITTEE'S SIGNATURE ***. "I, Oscar Negrete, certify that this material will be used in accordance with all restrictions and precautions as are specified in this permit,
  ***

- *** signed: _____ . " ***

- This permit DOES NOT authorize direct or indirect exposure of or inoculation into laboratory and domestic animals, including poultry, cattle, sheep, swine, horses, etc. Work shall be limited to IN VITRO uses only.

- Packaging, containers, and all equipment in contact with these materials shall be sterilized or considered a biohazard and be disposed of accordingly.

- This permit is valid only for work conducted or directed by you or your designee in your present U.S. facilities. (MATERIALS SHALL NOT BE MOVED TO ANOTHER U.S. LOCATION, OR DISTRIBUTED WITHIN THE U.S., WITHOUT USDA, APHIS, VS, NCIE AUTHORIZATION.)

- This permit does not exempt the permittee from responsibility for compliance with any other applicable federal, state, or local laws and regulations.

- A copy of this permit must be included with the shipping documents.

TO EXPEDITE CLEARANCES AT THE PORT OF ENTRY, BILL OF LADING, AIRBILL OR OTHER DOCUMENTS ACCOMPANYING THE SHIPMENT SHALL BEAR THE PERMIT NUMBER

SIGNATURE	TITLE	NO. LABELS
Waleid Muhmed Waleid I. Muhmed	Senior Staff Veterinarian National Center - Import - Export	

VS FORM 16-6A (MAR 95) Replaces VS Form 16-3A and 16-28 which are obsolete

APPENDIX B: METHODS

A. Preparation of C. elegans Genomic DNA

Solutions

- Worm lysis buffer; 0.1M Tris-Cl pH 8.5, 0.1M NaCl, 50 mM EDTA pH 8.0, 1% SDS. Store at RT. There could be some precipitation after long-term storage. If there is any precipitate then put it at 37C before use.
- CTAB/NaCl solution; 10 % CTAB in 0.7M NaCl. Dissolve 4.1 g NaCl in 80 ml distilled water and slowly add 10 g CTAB (Sigma M-7635) while heating and stirring. If necessary, heat to 65C to dissolve. Adjust final volume to 100 ml.
- Protease K; 20 mg/ml in TE pH 8.0. Store at -20C in single use aliquots. DO NOT FREEZE-THAW.

Worm preparation

1. Seed 4 x 10 cm plates with 15 young adult hermaphrodites and grow 1 week to have a lot of starved L1
2. Harvest starved L1 from plates and do 200 ml size liquid culture for 3-4 days at 20C until the majority of worms reach the adult stage
3. Harvest and do 30% sucrose float twice to remove E. coli and other contaminants.
4. Aliquot worms in 500ul volumes in 1.5 ml Epp. tubes and freeze at -80C.

DNA preparation

1. Add 4.5 ml of worm lysis buffer to a frozen 500 ul aliquot of worms. (transfer to 15 ml Falcon tube)
2. Add 200 ul of 20 mg/ml Protease K to worms and vortex
3. Incubate at 62C for 60 minutes. Vortex 4-5 times during the incubation. The solution should clear as the worms disintegrate
4. Add 800 ul of 5M NaCl. Mix thoroughly by inversion (important)
5. Add 800 ul CTAB solution. Incubate 10 minutes at 37C.
6. Add 7 ml chloroform, mix and spin. Recover aqueous phase.
7. Add 7 ml phenol/chloroform/isoamyl alcohol (saturated with TE pH 8.0), mix and spin. Recover aqueous phase.
8. Add 0.6 volume of -20C isopropanol. Invert to mix. The stringy white DNA should be obvious. Spin at 4C for 5 minutes.
9. Wash twice with 70 % EtOH
10. Dry and resuspend DNA in 340 ul TE.
11. Add 10 ul of RNase A (10 mg/ml heat-treated to kill DNase) and incubate 2 hours at 42C.
12. Add 20 ul of 20 % SDS, 10 ul of 0.5 M EDTA pH 8.0, 20 ul of Protease K and incubate 65C for 2 hours
13. Add 40 ul of 10M Ammonium Acetate
14. Extract twice with phsnol/chloroform/isoamyl alcohol (saturated with TE pH 8.0), once with chloroform. Add 1 ml of EtOH, mix and spin at 4C for 10 minutes.
15. Wash twice with 70% EtOH.

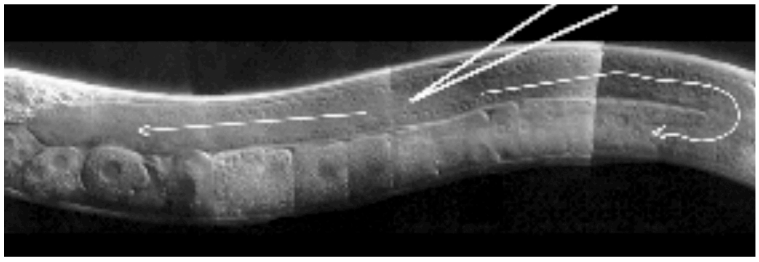
16. Dry and resuspend DNA in 200 - 500 ul TE. (usually get 2 to 5 mg/ml genomic DNA. Should run agarose gel to make sure the size is big)

B. Staging *C. elegans* (to synchronize larval stage of population)

1. Wash gravid adults from plates using M9 Buffer. Pellet worms at 1500 rpm, 5 min., 4°C. Aspirate supernatant. Repeat washes with M9 until supernatant is clear of bacteria.
2. Aspirate supernatant and resuspend worms in 8 mL of M9. To each strain, add 0.5 mL 5M KOH + 1.2 mL 20% bleach in M9.
3. Mix worms and incubate on rotator for 15 minutes at room temperature.
4. Monitor worms on dissecting scope. When 50-75% of worms have broken open (may have to vortex worms), centrifuge at 3000 rpm for 5 min. to pellet eggs.
5. Remove supernatant and wash 3X with M9. After the final wash, resuspend in 10 mL M9 and incubate on rotator overnight at room temperature. The following morning, pellet L_1 worms at 1500 rpm, 5 min, and plate on OP50-seeded agar plates.

C. DNA Microinjection for production of transgenic C. elegans

Stable integration of recombinant DNA will be achieved following microinjection of small amounts (< 2ul, ~100ng) of the recombinant DNA molecules into the gonads of *C. elegans* hermaphrodites. The DNAs of interest can be injected individually or together, in addition to an injection marker used for the visual identification of transformants. A number of dominant DNA markers can be used; my preference is to use a rescuing plasmid for the dumpy-20 gene that rescues the stubby phenotype of dumpy-20 mutant animals. Briefly, *C. elegans* microinjection is performed as follows: *C. elegans* young adults are submerged in a droplet of Series 700 Halocarbon Oil placed on a thin 2% agarose pad on a 24x50mm coverslip. The *C. elegans* are then pressed onto the agarose to immobilize them temporarily. A fine injection needle (generated using glass capillaries pulled on a P-97 Sutter needle puller) containing the DNA plasmid cocktail is penetrated into the distal gonad of the hermaphrodite (see image below). The *C. elegans* distal gonad contains a central core of cytoplasm shared by many germ cell nuclei. DNA injected into this cytoplasmic region is incorporated into a small percentage of germ cell nuclei. Following injection, the *C. elegans* are rescued in M9 buffer and moved to freshly seeded plates. Transformant progeny are identified as those animals carrying the dominant injection marker, for example, by rescue of the dumpy-20 mutation by wild-type dumpy-20 cDNA. In most cases, the transgenic *C. elegans* will contain the recombinant DNA as extrachromosomal array.



D. Growing worms in liquid culture

Wash worms from 1-2 plates into a 500 mL flask containing 100 mL S medium (S basal + additives) and OP50 as food. Grow at desired temperature, 18-23°C, depending on how soon you need to worms to be ready, with shaking at 200 RPM. The culture will become clear as the food is depleted. Monitor stage of worms by looking at a few uL under the microscope.
Note: A convenient way to supply food is to grow a 1 liter culture of OP50 in LB, aliquot into 50 mL tubes, pellet, and freeze pellets at -80° for later use. Just thaw a pellet and add to S media when you need to feed the worms. Give a 100 mL culture 2-3 pellets to start out.

E. Cell culture: BHK-21 and Vero cell lines

Culture medium:
MEM-alpha
10% FBS
pen/strep (0.5% of 10,000 U/mL)

Freezing medium:
Culture medium
20% FBS
5% DMSO

Conditions: 5% CO_2, 37°C humidified chamber, adherent

To passage cells:

1. Gently rinse monolayer once with 5-10 mL PBS.
2. Add 1 mL 0.25% trypsin-EDTA and let cells detach. 5 min at RT is probably sufficient for BHK21; 5 min at 37°C is sufficient for Vero. You should be able to tap the flask or plate, and see that the cells have detached.
3. Add about 8-10 mL culture media, and pipet cells up and down to gently resuspend. Avoid making bubbles in the media.
4. Transfer an appropriate volume of cells to the new vessel, and add enough culture media; gently mix, and then place back into incubator.
    a. for 10 cm dishes, 10 mL final volume cells + culture media is appropriate
    b. for T75 flasks, ~10-15 mL
    c. for T225 flasks, ~25-30 mL
5. Generally I split Vero 1:4 on Monday and Wednesday, and then 1:8 on Friday. I split BHK21 1:8 Monday and Wednesday, and 1:20 on Friday. These are estimates; adjust depending on cell density. Don't let cells get too crowded.

To freeze cells:

1. Follow steps 1-3 above. Collect cells in 50 mL centrifuge tube.
2. Count cells with hemacytometer; usually counting a 1:5 or 1:10 dilution is sufficient.
3. Spin 3 min at 1200 RPM to pellet cells.
4. Remove supernatant. Gently tap closed tube on work surface to loosen the pellet.
5. Resuspend in appropriate volume of freezing medium, so you will have the cell concentration you want. I generally like to freeze $5X10^6$ cells per mL per vial. Aliquot into cryovials, and place into room temperature Mr. Frosty. Then place Mr. Frosty into -80°C freezer overnight. Transfer to liquid nitrogen storage in the next few days, as cells will lose some viability at -80°C.

To thaw cells:

1. Wearing safety glasses and face shield, remove vial from liquid nitrogen storage and quickly transport to lab in a sealed secondary container. Note that if the vial was submerged in liquid nitrogen, it could have leaked and could contain liquid nitrogen, creating the possibility that the tube may explode upon thawing.
2. Thaw quickly in 37°C water bath, keeping the o-ring and cap out of the water.
3. Spray off tube with 70% ethanol and blot dry with a Kimwipe.
4. In BSC, transfer contents of vial to a 15 mL tube containing culture medium, and gently mix. At this point, you can just transfer mix to culture vessel or you can spin down the cells before plating to remove residual DMSO. Note that Vero and BHK21 cells are generally robust don't require the spin.
5. To wash: pellet cells at 1200 RPM, 3 minutes. Resuspend in culture medium, and then transfer to vessel and place into incubator.

F. Production of recombinant viruses expressing red fluorescent protein

Generation of Recombinant VSV-mCherry: Vesicular stomatitis virus (VSV) is an enveloped virus of 100nm size that contains a single strand RNA genome in negative orientation. The VSV RNA genome is approximately 11kb in length and encodes five major proteins: G protein (G), large polymerase protein (L), phosphoprotein (P), matrix protein (M) and nucleoprotein (N). A full-length cDNA clone of the VSV genome (Indiana serotype 1), where the coding region of the G gene was replaced by the reporter gene renilla luciferase, was received from Dr. Benhur Lee (UCLA). The renilla luciferase gene was then replaced by the reporter mCherry, and the gene encoding G was inserted between the mCherry and L genes by standard molecular cloning techniques. This plasmid was designated pVSV-mCherry and its expression was under the control of a T7 polymerase promoter. To generate infectious VSV-mCherry particles, HEK293T cells were seeded in a 6-well poly-lysine coated tissue culture plate with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The next day, the HEK293T cells were infected at a multiplicity of 10 with recombinant vaccinia virus expressing T7 (VacV-T7) (ATCC) for 1 hr at 37C. The infected cells were then cotransfected with the pVSV-mCherry, the VSV-N, -P and –L expressing plasmids at a ratio of 5ug, 1.5, 2.5, 1 mg, respectively. The supernatant was harvested 48 hr after transfection and was

then filtered through a 0.2mm membrane pore size to remove the vaccinia virus (>400mm particles). The remaining rVSV-mCherry particles were further passaged through Vero cells and titers were determined by plaque assay and fluorescence focal units.

Generation of Recombinant Vaccinia virus containing the mCherry gene: Vaccinia virus promoters are required to regulate transcription of the DNA which is introduced by homologous recombination into the 185-kb double stranded DNA genome. To generate a vaccinia virus strain expressing the mCherry gene, a plasmid was obtained from ATCC containing a vaccinia virus promoter (p7.5), with restriction sites for insertion of foreign DNA, and flanking vaccinia TK sequences to direct recombination into the TK locus of the genome. This plasmid containing the mCherry gene was designated p7.5-mCherry. HEK293T cells were then seeded in a 6-well poly-lysine coated tissue culture plate with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The next day, the HEK293T cells were infected at a multiplicity of 10 with recombinant vaccinia virus expressing T7 (VacV-T7) (ATCC) for 1 hr at 37C. The infected cells were then transfected with the p7.5-mCherry. The intracellular recombinant VacV-mCherry particles were obtained 48 hr post-transfection through trypsin digestion of the infected cells and plaque purified on BSC-40 cells.

G. Purifying Virus through Sucrose

1. Remove virus-containing supernatant from cells. Centrifuge at 1250rpm, 5 min, 4°C.
2. Make a 20% sucrose solution in PBS. Filter sterilize.
   *Note: You can also layer 65% sucrose and 20% sucrose. The virus will accumulate at the interface, not passing through the 65% solution.
3. Use the swinging-bucket rotor (SW41-Ti) for the ultracentrifugation of virus. The tubes for this rotor hold about 11-12mLs total, so fill the bottom of the tube with 3mLs of the sucrose/PBS solution.
4. Overlay the virus supernatant very slowly to not disrupt the interface. Set the pipet-aid on "slow". Slowly add media to top of virus for a total volume of 11mL. Seal tubes in rotor with screws before removing from hood.
5. Balance the tubes to 0.01 with media and spin at 30,000rpm for 1.1 hrs.
   Settings should read as follows: 30,000rpm
   1hr, 10min
   4°C
   Max acceleration
   Max deceleration
6. When run is completed, press vacuum button. Temperature will decrease into the negatives. When it starts to increase, the centrifuge can be opened. Log run number in log book.
7. Bring rotor back to the biosafety cabinet and remove tubes. Aspirate all liquid in the tube, but do not touch the very bottom of the tube where the invisible virus pellet is located.
8. Invert the tube for 2min and aspirate all the residual liquid from the tube.

9. Add 500µL of whatever media was used for infection to the pellet (can also use opti-MEM or PBS). Cover tube with parafilm and leave at 4°C for at least 1hr.
10. Vortex tube and pipet up and down while scraping the bottom of the tube. Transfer virus to a cryovial and store at -80°C.

H. Determining virus titer with plaque formation assay

(This general protocol works for a wide variety of paramyxoviruses; you may need to try a different overlay such as carboxymethylcellulose or agarose for some viruses)

1. Plate cells on 6 well plates, such that you will have a nearly confluent monolayer on the day of the plaque assay. BHK21 and Vero cells are good cell lines to use to plaque many different viruses. BHK21 divide much more quickly than Vero, so be careful that they aren't too confluent at the start, as they will overgrow, and the monolayer will not last the duration of the assay. In general, you'll want about $1x10^6$ cells/well on the day of the assay. Prepare 2x6 well plates per virus sample you will be titering.
2. Prepare dilutions of virus in tissue culture medium, as below:

a.	undiluted	(1)	g.	1:10 of f	($1X10^{-6}$)
b.	1:10 of a	($1X10^{-1}$)	h.	1:10 of g	($1X10^{-7}$)
c.	1:10 of b	($1X10^{-2}$)	i.	1:10 of h	($1X10^{-8}$)
d.	1:10 of c	($1X10^{-3}$)	j.	1:10 of i	($1X10^{-9}$)
e.	1:10 of d	($1X10^{-4}$)	k.	1:10 of j	($1X10^{-10}$)
f.	1:10 of e	($1X10^{-5}$)	l.	medium only, no virus	

3. Remove media from cells. Pipet 200 uL of each virus dilution gently onto the monolayer, such that it covers the entire well. Place into incubator for 1 hour for virus binding. Rock plates every 10 min or so, so the wells don't dry out.
4. Add 3 mL overlay per well. Overlay is 0.8% tragacanth in MEM (mix equal volumes of sterile 1.6% tragacanth and 2X MEM containing 4% FBS, 2% pen/strep). Note: to make tragacanth, mix with appropriate volume of water, autoclave with a stir bar in bottle, and then refrigerate. The cooling will help the blobs go into a smooth viscous solution. Store in fridge.
5. Incubate 1-3 days, until plaques form. Plaques usually form more quickly on BHK21 than on Vero.
6. Gently pipet off overlay into bleach solution to inactivate virus. To each well, add 1 mL of 0.25% crystal violet stain in 10% paraformaldehyde fixative to each well, and gently swirl to distribute evenly throughout well and mix with residual tragacanth media. Leave stain/fix for about 30 minutes at RT in BSC.
7. Remove stain/fix into chemical waste container. Add 3-4 mL water per well, and swirl to rinse cells. Remove water, and wash again until plaques are visible and easily counted. Remove water, and leave plates to air dry in BSC.
8. The expected result is that the samples will range from too many plaques to count and a completely destroyed monolayer to no plaques at all and an intact monolayer in the uninfected well. Somewhere in between these two extremes, count the plaques, and calculate titer of virus stock.

   e.g. if well H has 30 plaques, then the titer is

   30 PFU x $\frac{1/1X10^{-7}}{}$ dilution / 0.2 mL =

   30 PFU x $\frac{1X10^{7}}{}$ dilution / 0.2 mL =

   $1.5X10^9$ PFU/mL

I. Primary culture of *C. elegans* embryonic cells

(modification of Kevin Strange and Rebecca Morrison's protocol)

Isolation of *C. elegans* eggs

1. Generate cultures of gravid adult worms on 10 cm plates or liquid culture (at least 8 plates or 25-50 mL liquid culture).
2. These steps can be carried out on the benchtop, with autoclaved or filter sterilized solutions.
3. Wash adult worms off agar plates with cold di water into a 15 or 50 ml conical tube on ice. Pellet worms by centrifugation at 1470 rpm (~450g) for 5 min at 4ºC in a swinging bucket rotor in a tabletop centrifuge. These rpm are based on the Sorvall super T-21 in 134 or 129. Wait another minute of two after the rotor has stopped to remove the tubes; the pellet is very delicate, and handling the tube immediately following centrifugation will disturb the pellet. Using chilled solutions and refrigerated centrifuge also makes the worms less wiggly and yields better pellets.
4. Add a small volume of di water, and transfer worms to a 15 mL tube, if you started with a 50 mL tube, before subsequent washes. Worms that are very "clean" will stick to the inside of a blue tip, and you'll lose quite a few in the transfer. Wash worms with di water 1-3 more times, until the supernatant is clear of bacteria.
5. Next to the dissection microscope, lyse the worm pellet with the following fresh mixture of bleach and NaOH (1.0 ml bleach, 0.25 ml 10 N NaOH, 3.75 ml sterile H_2O) prepared immediately prior to use.
6. Rock worms gently during the lysis. The progress of the lysis reaction is monitored by viewing the worm suspension with a dissecting microscope (through the tube, or look at a few uL on a microscope slide), and the lysis reaction should be stopped when ~50% of the worms are lysed. Lysis time is critical. Under no conditions should the lysis reaction exceed 5 min.
7. Stop the lysis reaction by filling the tube with egg buffer (118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM Hepes, pH 7.3, 340 mOsm) and immediately pellet the eggs and lysed worms by centrifugation at 1470 rpm for 3 min.
8. Remove the supernatant using a sterile plastic pipette and wash 3X with egg buffer. Make sure the pellet is completely re-suspended in the egg buffer during each wash.
9. After the last centrifugation, carefully remove the buffer by sterile plastic pipette.
10. Eggs are separated from debris by centrifugation in a 30% sucrose solution. Resuspend the pelleted eggs and lysed worms in 5 ml of sterile water and then add 5 ml of a sterile 60% sucrose stock. Mix this solution well.
11. Centrifuge the suspension at 1470 rpm (~450g) for 10 min using a swinging bucket rotor in a tabletop centrifuge.
12. Eggs should float in the 30% sucrose solution and will collect at the solution meniscus. Some eggs may appear in the region just below the meniscus. Using a sterile plastic blue tip (eggs will stick to glass pipets), transfer the eggs at the meniscus into a sterile 15 ml conical tube. Eggs that stick to the tube can be recovered by gently washing down the sides with a small volume of the sucrose solution and then removing them with a plastic transfer pipette. If you mess up and disturb the eggs too much, re-centrifuge and try again.
13. Collect no more than 3–4 ml of the egg/sucrose suspension. Fill the tube with sterile water and re-pellet to remove the sucrose (10-15 min 1470 RPM). If you collect too much of the egg/sucrose suspension, the solution will be too dense to pellet the eggs.

Preparation of dissociated embryo cells

14. All subsequent steps should be carried out in a laminar flow biosafety cabinet under sterile conditions.
15. Remove embryo eggshells by incubating approximately 50–200 µl of pelleted eggs in 500 µl of chitinase (Sigma Chemical Co., St. Louis, MO) solution (1U/ml in sterile egg buffer) in a sterile 15 mL conical tube.
16. Mix the egg suspension by rocking gently at room temperature for 20–80 minutes. Note that each lot of chitinase tends to vary in potency. Consequently, incubation times will have to be assessed for each lot. The progress of eggshell digestion should be monitored by viewing a few uL of the egg suspension under a microscope.
17. Isolated cells are washed and cultured in L-15 cell culture medium (Life Technologies, Grand Island, NY) containing 10% heat inactivated fetal bovine serum (HYCLONE, Logan, UT), 50 U/ml penicillin and 50 µg/ml streptomycin. The osmolality of the culture medium should be adjusted to 340 mOsm with sucrose and filter sterilized. Osmolality can be measured with a vapor pressure or freezing point osmometer. If these instruments are not available, the osmolality of the L-15 cell culture medium is listed on the bottle label. As a first approximation, 1 mM sucrose ≈ 1 mOsm. Therefore, osmolality can be increased to ~340 mOsm by adding an equimolar amount of sucrose.
18. When approximately 80% of the eggshells have been lysed, add 800 µl of L-15 cell culture medium to the tube. Pellet the eggs by centrifugation at ~2080 rpm (~900g) for 5 min at 4°C.
19. Carefully remove the supernatant and add 800 µl of fresh L-15 cell culture medium.
20. Gently dissociate the cells by repeatedly pipetting the cell suspension using a 1 ml pipettor and sterile blue tip. Monitor the degree of dissociation by periodically placing a few uL of the suspension on a microscope slide and viewing at 20X. Continue the dissociation until you have a large number of single cells present. The preparation will also contain some undissociated embryos, clumps of cells and hatched larvae. I generally pipet up/down 20 times, monitor the dissociation, and then proceed as appropriate. If you don't dissociate the cells enough, your yield will suffer, as the clumps will be filtered out and lost in later steps.
21. Pellet the dissociated cell suspension by centrifugation at ~2080 rpm (~900g) and 4°C for 5 min. Remove the supernatant, which may be somewhat cloudy, and resuspend the pellet in 500 µl of L-15 cell culture medium.
22. The cell suspension is filtered to remove hatched larvae, cell clumps and very large cells. Filtration is carried out using a 5.0 micron Durapore filter (Millipore Corporation, Bedford MA) and a 3 ml sterile syringe as follows:
    a. Remove plunger from syringe. Secure 5 micron filter onto syringe. Pipet cell suspension into syringe, and use plunger to filter the suspension; do not force air through the filter.
    b. Remove filter from syringe, keeping the tip sterile. Remove plunger, and reconnect filter. Pipet 3 mL L-15 medium into syringe, and use plunger to rinse the medium through filter; avoid forcing air through the filter.
23. Pellet cells by centrifugation at ~3500 rpm (~900g) and 4°C for 3 min.
24. Remove the supernatant and resuspend the cells in 25–200 µl (the actual volume depends on the size of the cell pellet) of L-15 cell culture medium.
25. Prepare appropriate dilutions of the cell suspension in L-15 medium and determine cell density using a hemacytometer. Dilution will vary, depending on the yield. For our experimental purposes, we count only "large" and "medium" cells and ignore any very small cells. To make counting easier, take a photo of the field, and count cells on the computer screen, rather than through the microscope eyepiece.

26. Cells must adhere tightly to the growth substrate in order for differentiation to occur. Cells are grown on glass chambered coverslips or in plastic dishes with glass coverslip bottoms. The coverslips are coated with peanut lectin (Sigma) to promote cell adhesion.
27. Prepare a peanut lectin solution in sterile water at a concentration of 0.5 mg/ml. Pipette a small volume of the peanut lectin solution onto the coverslip so that it covers about 80% of the surface. Incubate the coverslips with the lectin solution for 10–20 min and then remove completely with vacuum aspirator and sterile pipet tip. It is important to completely remove the lectin solution. Excess lectin on the coverslips causes cell clumping.
28. Plate the cells onto the coverslips in a minimal volume of L-15 medium. Plate cells at a density of ~250,000-500,000 cells /cm^2. Allow the cells to settle and attach for 2 h and then add additional L-15 medium.
29. Culture vessels are kept in small sealed Tupperware containers with a small folded wet paper towel. Prevention of medium evaporation is critical. The Tupperware containers are kept in a sterile, humidified incubator at room temperature and ambient air.
30. In our hands, morphological differentiation of cells is largely complete within 24 h. Use for infection experiments within 5 days. Note from original protocol: Cell survival is excellent for 2–3 weeks. However, these are primary cultures and they may dedifferentiate with time. We typically do not use cultures for physiology experiments that are older than 5–8 days.

Solutions

Egg Buffer:

118 mM NaCl	6.89 g per 1 liter
48 mM KCl	3.58 g per 1 liter
2 mM $CaCl_2$	0.294 g per 1 liter ($CaCl_2$-$2H_2O$)
2 mM $MgCl_2$	0.407 g per 1 liter ($MgCl_2$-$6H_2O$)
25 mM Hepes, pH 7.3	25 mL of 1 M

Chitinase:

4 mg/mL	21 mg + 5.25 mL water, filter sterilize, aliquot, and freeze

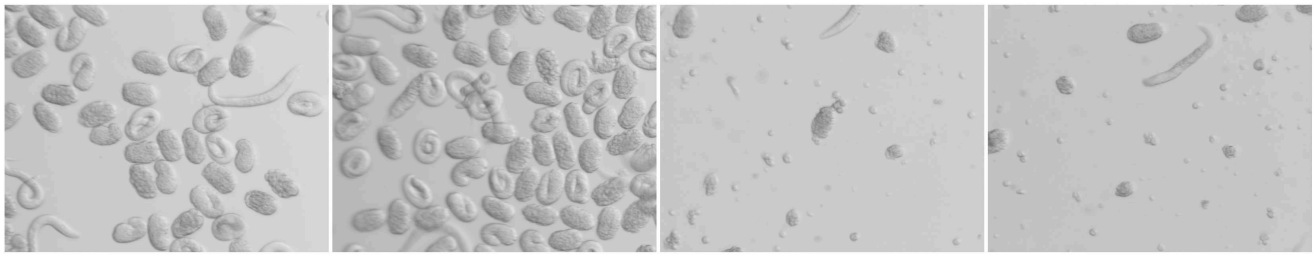
Complete L-15:

L-15 from Invitrogen	500 mL
FBS	50 mL
sucrose	7.7 grams (45 mosm)
pen/strep (10,000)	2.5 mL

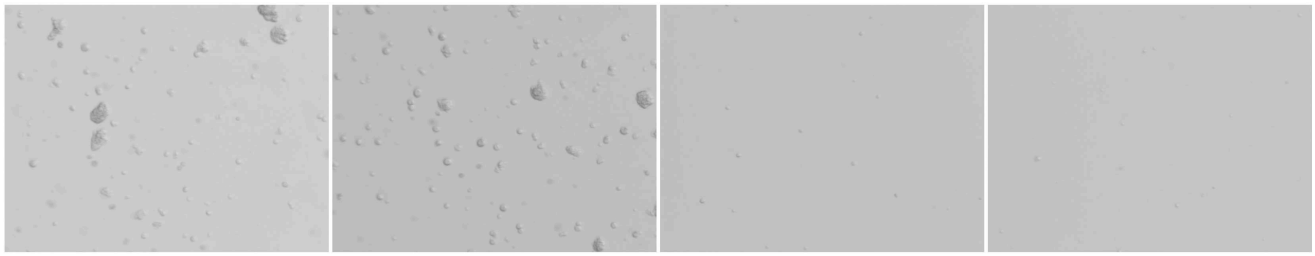
60% sucrose:
120 grams sucrose + water to 200 mL
filter sterilize and aliquot

Peanut Lectin:
25 mg peanut lectin (Sigma) + 50 mL water
filter sterilize, aliquot, and freeze

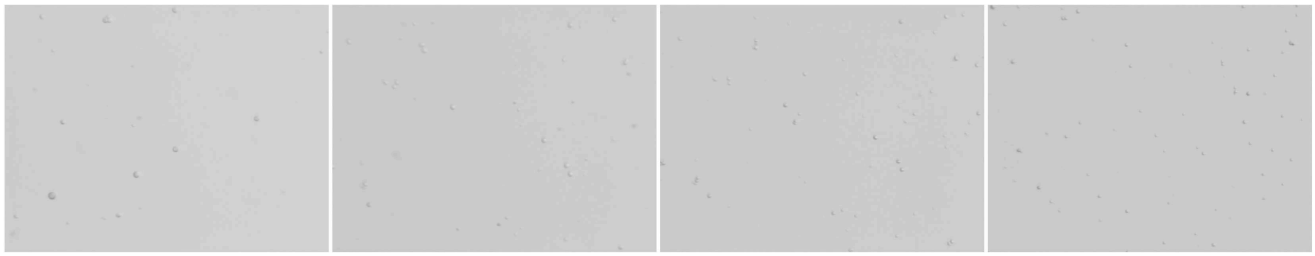
Dissociation of the embryonic blastulas for isolation of embryonic stem cells is picture below:



001-chitinase treated eggs.tif | 002-chitinase treated eggs.tif | 003-after 1st dissociation.tif | 004-after 1st dissociation.tif



005-after 2nd dissociation.tif | 006-after 2nd dissociation.tif | 007-after 5 um filter.tif | 008-after 5 um filter.tif



009-after concentration.tif | 010-after concentration.tif | 011-after concentration.tif | 012-after concentration.tif

J. Infection of C. elegans embryonic cells

1. Prepare dilutions of virus in complete L-15 medium, according to desired MOI and surface area of culture vessel. For 8 well chambered coverslips, use 200 uL inoculum per well.
2. Remove supernatant from cells. Gently add inoculum by pipetting the liquid onto the side of the vessel well. If you pipet directly onto the adherent cells, you may disturb and/or detach them. Incubate at 26°C for 1 hour.
3. Remove inoculum and gently wash cells once with complete L-15.
4. Gently, replace fresh medium, and incubate at 26°C until the timepoint you wish to image.
5. Remember to include an uninfected control, so you can gauge any autofluorescence that may occur. Image directly through the coverslips at 100X magnification, and keep the lid on the sample while it is outside the BSC.

K. *C. elegans* Infection Protocol Sample A

Treat *C. elegans* swith 1X, 5X, and 10X chitinase solution followed by 10K psi. 100X *S. griseus* chitinase (Sigma, #C6137) is defined as 1 vial resuspended in 500 µL M9 buffer. 10K psi pressure treatments administered using a PB Biosciences Barocycler.

8. Wash L3 WM27 off of plate using M9. Pellet worms at 1500 rpm, 5 min. Aspirate supernatant and continue washes until supernatant is clear of bacteria.
9. Resuspend worms in an appropriate volume so each chemical treatment gets 100-150 µL worms. Add the following chitinase solutions:
    a. 10X: 25 µL 100X chitinase + 225 µL M9
    b. 5X: 12.5 µL 100X chitinase + 237.5 µL M9
    c. 1X: 2.5 µL 100X chitinase +247.5 µL M9
10. Vortex briefly to mix. Incubate at 37°C, 100 rpm, 3 hours.
11. Wash worms with 10 mL M9 to remove chitinase. Pellet as in step 1. Repeat wash 2X.
12. Resuspend worms in 200-250 µL M9. Transfer to pressure tubes and treat at 10K psi.
13. Put 100 µL treated worms in 1 well of a 48-well plate. After all treatments are done, add the following to each well: 56 µL M9 + 4 µL DMSO + 40 µL VSV-mCherry (6-24-10 prep, amplified in HEK 293T, Titer: 4 x 10^9 pfu/mL)
14. Incubate in humidified 26°C incubator. After 24 hours, use Pasteur pipette to transfer worms to OP50-seeded plates. Check for viral infection after 24-48 hours.

L. <u>C. elegans Infection Protocol Sample B</u>

OP50 *E. coli* (for food): resuspend a 50mL LB pellet with 4mL M9 Buffer

Treatments:
- (Ca i) NaOH 10mM infected 1:10 rVSV-Cherry
- (Ca u) NaOH 10mM; uninfected
- (Cb i) NaOH 100mM infected 1:10 rVSV-Cherry
- (Cb u) NaOH 100mM; uninfected
- (Cc i) NaOH 500mM infected 1:10 rVSV-Cherry
- (Cc u) NaOH 500mM; uninfected
- (1 i) NaOH 10mM; 10k psi; infected 1:10 rVSV-Cherry
- (1 u) NaOH 10mM; 10k psi; uninfected
- (2 i) NaOH 100mM; 10k psi; infected 1:10 rVSV-Cherry
- (2 u) NaOH 100mM; 10k psi; uninfected
- (3 i) NaOH 500mM; 10k psi; infected 1:10 rVSV-Cherry
- (3u) NaOH 500mM; 10k psi; uninfected

7. Wash worms off plates (2 days old) with M9 buffer into 15mL tube.
8. Spin 1470 rpm, 5 min, 4C
9. Remove most of the supernatant
10. Wash with M9 and spin again. Remove supernatant
11. Add M9 so there is a total of 480uL.
12. Aliquot as follows:
    - (Ca) 80uL worms, 400µL 10mM NaOH
    - (Cb) 80uL worms, 400µL 100mM NaOH
    - (Cc) 80uL worms, 400µL 500mM NaOH

(1) 80uL worms, 400µL 10mM NaOH
(2) 80uL worms, 400µL 100mM NaOH
(3) 80uL worms, 400µL 500mM NaOH

7. Incubate on rotating platform in 26°C incubator for 2 hours.
15. Quick spin on table-top centrifuge. Remove most of supernatant from each tube. Wash worms 2X with 250uL M9. After second wash, resuspend worms in 250uL M9.
16. Transfer all samples except (C) into pressure tubes. Treat with 10K psi.
17. After pressure treatment, remove worms from pressure tube and transfer to Eppendorf tube.
18. Measure yields from pressure tubes and split each condition in half for infected and uninfected conditions. Transfer uninfected sample directly to a 48-well plate (total volume of worms + 50µL food $\geq$ 150µL). For the infected sample, add VSV-Mcherry at a final dilution of 1:10, accounting for worm volume and 50µL food.
    a. Yields from pressure tubes:
        i. (C) 250µL → 125µL worms + 50µL food
        ii. (1) 240µL → 120µL worms + 50µL food + 18.9µL VSV-Mcherry for infected condition
        iii. (2) 220 µL →110µL worms + 50µL food + 17.8µL VSV-Mcherry for infected condition
        iv. (3) 200 µL →100µL worms + 50µL food + 16.7µL VSV-Mcherry for infected condition

19. Transfer infected samples to 48-well plate
20. Feed all worm samples with 50µL OP50
21. Incubate the plate in a stationary 26°C incubator in a loosely covered Tupperware.

M. Cloning of C. elegans receptors upstream of the EphB2 Nipah receptor

The *tax-4*, *odr-10*, and *act-5 C. elegans* promoter sequences were PCR amplified from C. elegans genomic DNA using primers that added SphI and Sal1 to the 5' and 3' ends of the sequences, respectively. The Fire Lab promoterless cloning vector pPD95.79 was acquired from Adgene. The EphB2 coding sequence was synthesized by Epoch Biolabs and cloned Sal1-SmaI and cloned into this vector. The promoters were then individually cloned into the SphI and Sal1 sites of the EphB2-95.79 vector to create the *C. elegans* promoter::ephB2 constructs.

N. Construction and testing of a Cre-responsive reporter construct

APPENDIX C: SPRAY FORMULATION CANDIDATE MATERIALS

NOTES on Gel formulation requirements/approach:

Nematodes require oxygen between 6-18%
Use M9 medium to mix with gel agents for nematode survival (see below)
Some evidence that nematodes more susceptible to viral uptake at pH ~5.1
Need to NOT destroy virus with Gel
Various approaches: mutant strains/enzymatic (pre&with spray)/low pH shock(pre-spray)/ DMSO shock(pre-spray)
For visible light (excite in green ~500nm/detect in red ~650nm) - don't need UV or FUV (~180-300nm) wavelengths
No autofluorescence - need to minimize background signal
Maintain wet for ~2 days
Prevent invasive bacteria that may compete w/ nematodes for viral uptake
Expect droplets of ~300 microns - use sprayer system for large droplets to not shear/kill nematodes

(C. Elegans physiological saline): 6 g Na2HPO4, 3 g KH2PO4, 5 g NaCl, 0.25 g of MgSO47H2O per liter (autoclaved medium to prevent bacteria).
Incubate nematodes in M9 with 0.0025U chitinase/ul and 0.83U collagenase/ul for 3 hours at 26 degC.

Inventory-based Materials		pH	opacity	Comments
Gelling Agents				
Xanthan Gum	**x**	varies	**transparent**	Anionic polysaccharide, hydrocolloidal, **salt-stable gel**, typ conc of <0.5%, **X-link with cations**, water-soluble, **emulsifier, temp & pH-stable gels - acid/neutral w/ trivalent cations**/basic w/ divalent cations, **pseudoplastic**
USE ONLY IF NEEDED TO INCREASE VISCOSITY				

> Synthetic materials				
Fumed Silica	**x**	acid-neu	varies	
USE AT ~1-2% CONCENTRATION				
***CHECK PARTICLE SIZE & SURFACE CHARGE/HYDROPHOBICITY**				
***USE COMBINED W/ PAAs**				

(reduced viscosity under shear w/ instantaneous regain of viscosity), **used with nematodes** at 0.3% conc + an emulsifier/surfactant (0.3% Rimulgan or ***Tween 80***) to spray on plants. Note: Rimulgan biodegradable, from natural oils (68% castor oil, 25% ionic oleic acid, 5% calcium in alcohol).

>Syloid Silicas W900: Amorphous silica gel, 10-20 microns diam., matting film applications. <--pH ~9.2 XXXXXX >**CAB-OSIL M5 & H5**: Untreated Fumed silica, **pH ~4 (4% aqueous)**, **submicron (~14nm, 200nm aggregate) particles**, medium soluble in water & organics, thickening agent applications, **thixotrophic** (reduced viscosity when under shear).
> Aerosils:
*160: 14nm, H2Ophilic, 150 m2/g BET area, pH 3.7-4.7, <1.5% H2O dry loss
*200: 12nm, H2Ophilic, 180 m2/g

				BET area, pH 3.7-4.7, <1.5% H2O dry loss *300: 7nm, H2Ophilic, 300 m2/g BET area, pH 3.7-4.7, <1.5% H2O dry loss *380: 7nm, H2Ophilic, 380 m2/g BET area, pH 3.7-4.7, <2% H2O dry loss
Polyacrylic Acids (PAAs) *** USE COMBINED W/ SILICA**	**x**	**acidic**	**transparent**	MW ~21K (ave), 1-6% (by Vol) in **spray fire-fighting gels**, **anionic superabsorbent**, **stable at low pH**, salts reduce gel structure.
*Thermo-Gel 200L	x	**acidic**	???	20-30% PAA, 20-30% petrol hydrocarbon, 1-5% surfactant
Poly(isobutylene-co-maleic acid) *** SUBSTITUTE FOR PAAs IF NEEDED**	**x**	**acidic**	**transparent**	similar to PAAs, **superabsorbent used in soils f**or water retention, **T-stable gels**, water soluble. Superabsorbent fiber. Amount of aqueous fluid absorbed decreases w/ increasing salt. Stable up to 250°C.
Poly(...propane-sulfonic acid) *** SUBSTITUTE FOR PAAs IF NEEDED**	**x**	**acidic**	**transparent**	similar to PAAs, **superabsorbent** used for water retention, **T- & pH(2-14)-stable gels**, used w/ hydroxyethyl cellulose, used w/ glycerins/glycols in lotions, often blended w/ polyacrylamides.

Humectants/Emulsifiers				
Glycerol				
Polyethylene Glycols (PEGs/PEOs) ***LOOK AT LITERATURE RE VIRAL ENTRY & INFECTION**	**x**	neutral	**transparent**	>PEG dioleate: MW ~914, nonionic plasticizer/humectant, soluble in ethanol/organics - water dispersible. >Dipropylene glycol: **nontoxic** **plasticizer/humectant/polymer initiator**, hydrocolloid/gel former, used in animal feed. >Polypropylene Glycol 400 or 2000: info???
Sorbitol ***ONLY IF NEEDED TO RETAIN WATER**	**x**	???	???	sugar alcohol (reduced glucose via aldehyde -> alcohol, humectant/plasticizer.
Surfactants				
Tween 80	**x**	???	???	**Nonionic surfactant/emulsifier**, MW ~1310, water miscible, critical micellar conc ~0.006-0.012 mM, oil/alcohol soluble. (Polyoxyethylene (20) sorbitan monooleate)
Other Additives				
Citric Acid				Used for citrate buffer (pH 4-7)
Potassium/Sodium Citrate				Used for citrate buffer (pH 4-7)

Antimicrobials: QACs: Variquat		To prevent bacteria (compete w/ nematodes for viral uptake)

NOMENCLATURE

United States Department of Agriculture (USDA)
Rift Valley Fever Virus (RVFV)
Vaccinia Virus (VV)
Vescicular Stomatitis Virus (VSV)
National Institute of Allergy and Infectious Disease (NIAID)
Biosafety in Microbiological and Biomedical Laboratories (BMBL)
Biosafety Containment Level 2 (BSL2)
Risk Group 2 (RG2)
American Type Culture Collection (ATCC)
Green fluorescent protein (GFP)
Red Fluorescent protein (RFP)

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