Delivery and optical imaging of luminescent and fluorescent reporter genes

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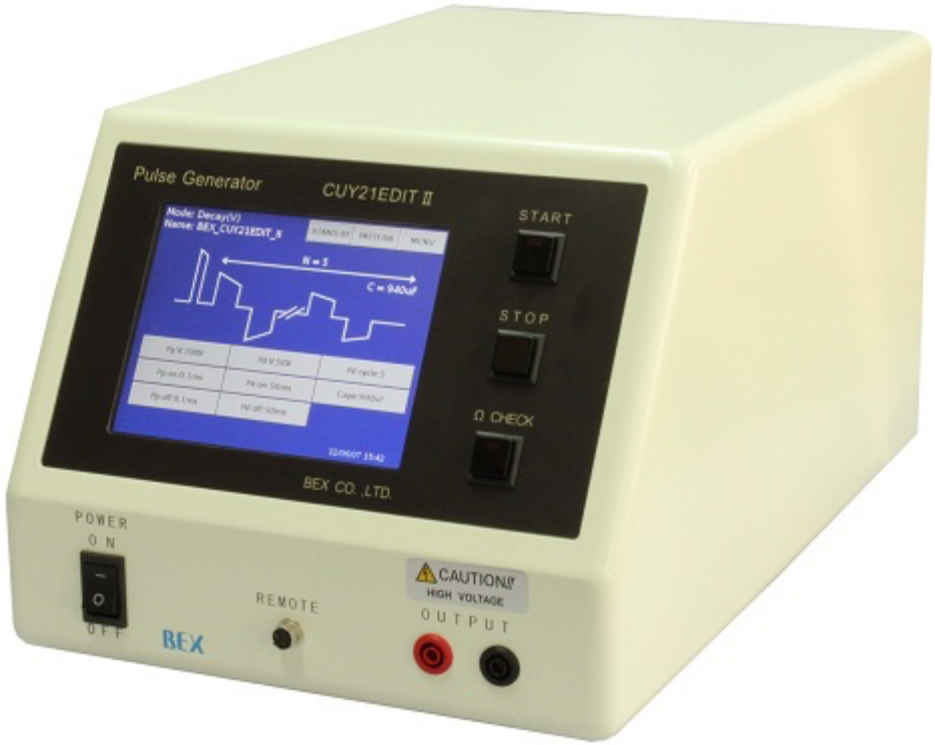
Electroporation

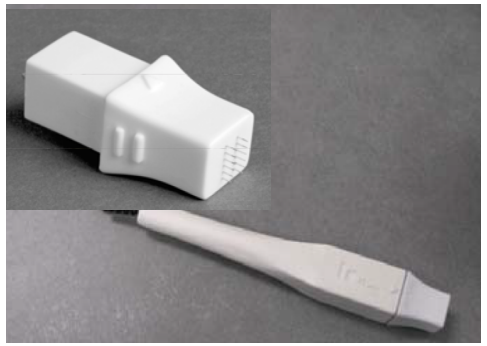
“Application of an electrical field to cells in order to increase the *permeability* of the cell membrane, allowing the introduction of chemicals, drugs, or **DNA**.”

CUY21EDIT II pulse generator

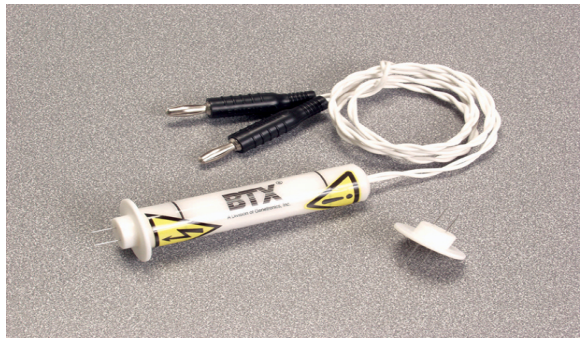
- In vivo and in vitro electroporation
- Patterns of elecroporation pulses
  - → Square
  - → Decaying
  - → Change of polarity
- First constant current electroporator
  - → The user can set desired current



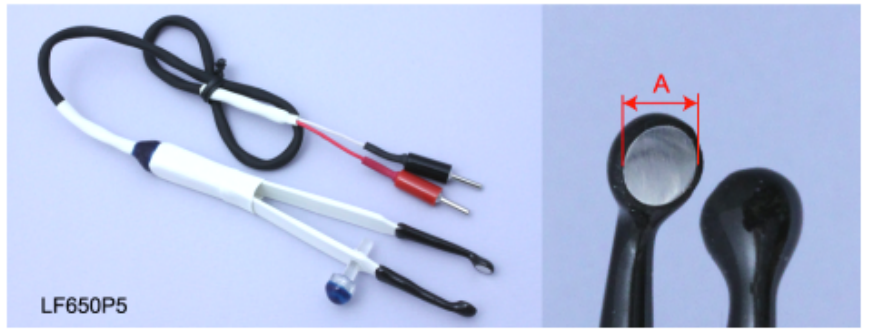
Electrodes for skin electroporation



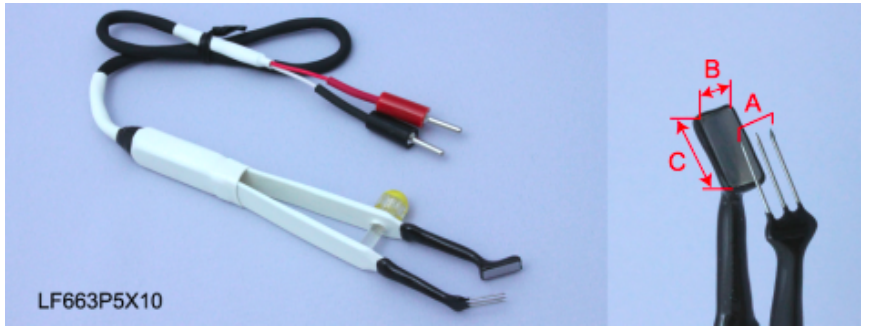
- Multineedle array electrodes, BTX



- 2-needle electrode array, BTX



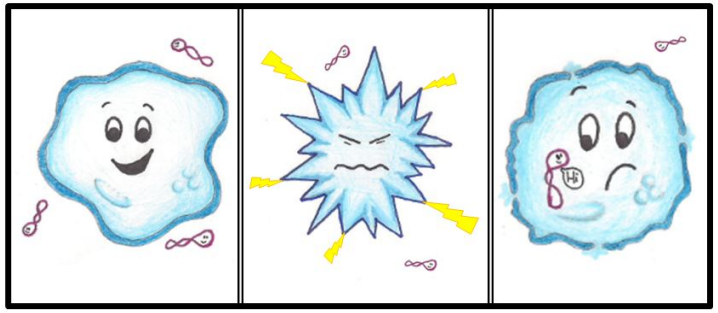
- Platinum-coated tweezers with plate electrodes, BEX



- Tweezers with fork and a plate electrode, BEX

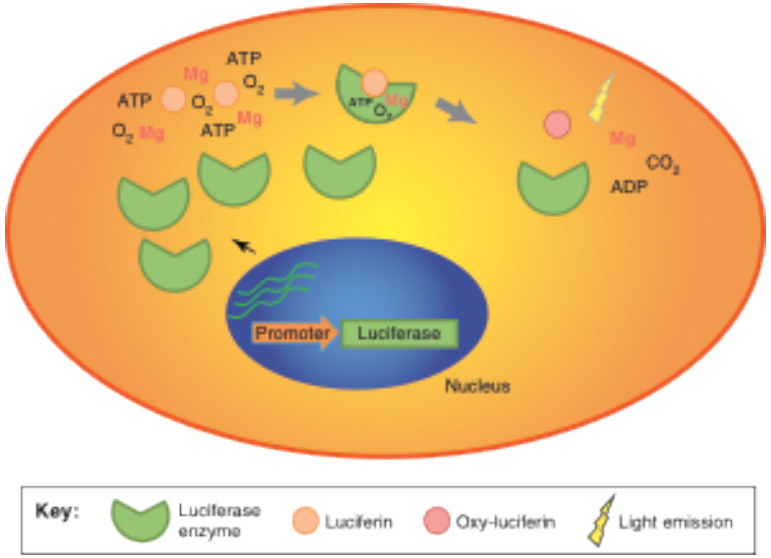
Immunization parameters

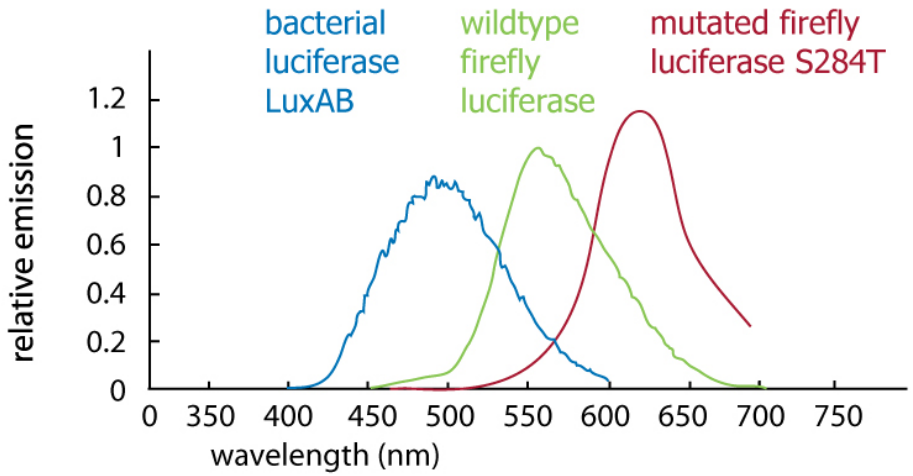
- Intradermal injection of 10 µg of reporter gene dissolved in 20 µl PBS
- Electroporation immediately after injection
  - → 1 poration pulse of 400 V (0.05 ms)
  - → 8 driving pulses of 70 V/100V
  - → All pulses had a 10 ms duration with 20 ms gaps



The luciferase reporter gene

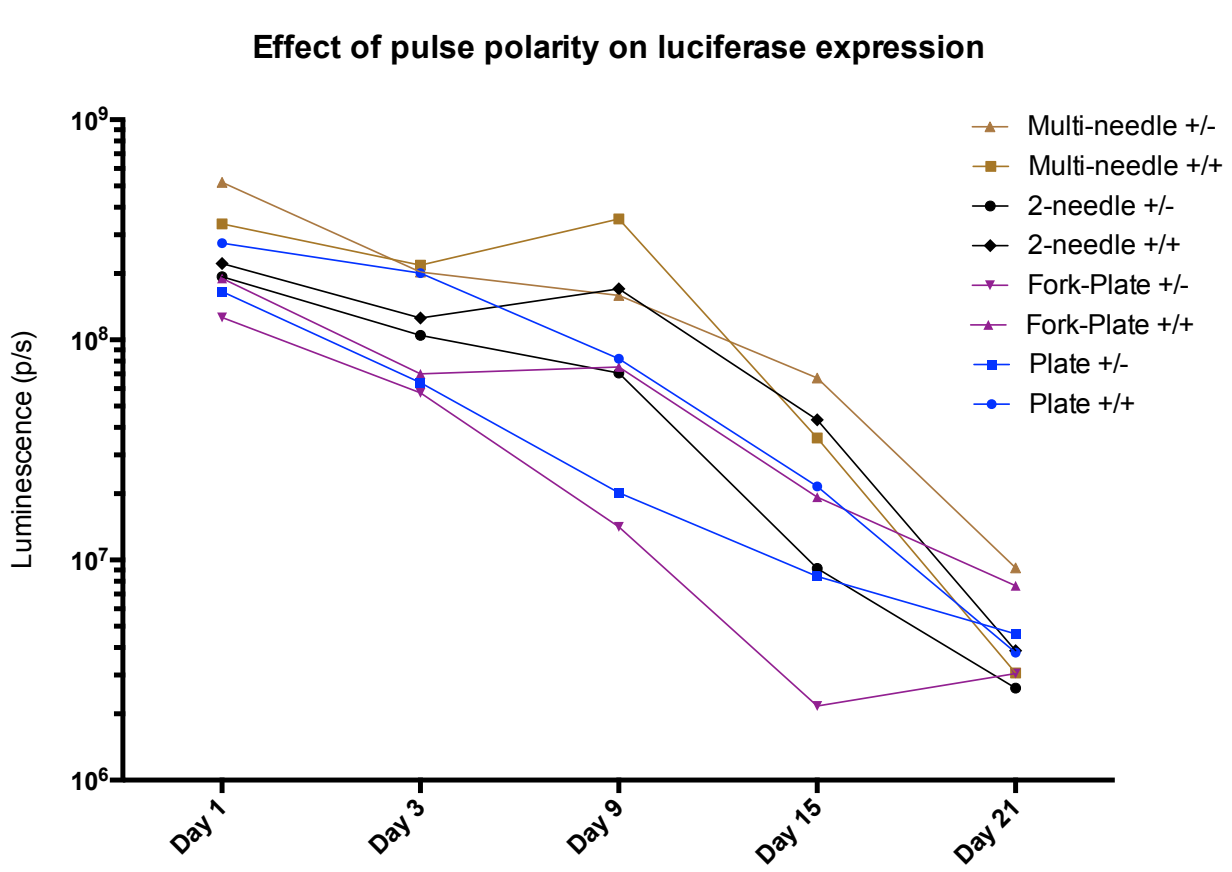
- Luciferases
  - → Firefly, Renilla/Gaussia, Bacterial
  - → Generate luminescent light (490 – 560 nm)
  - → Low immunogenicity

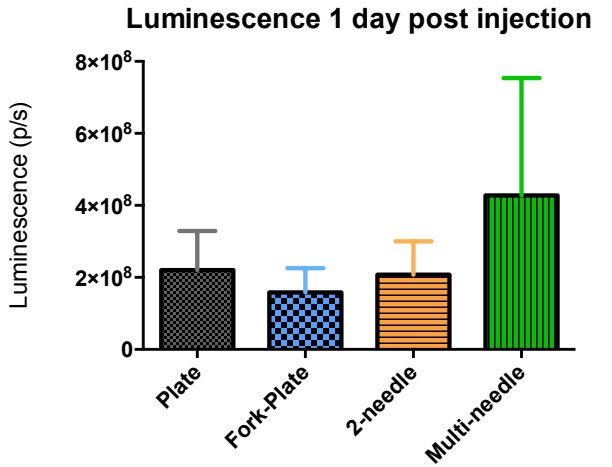


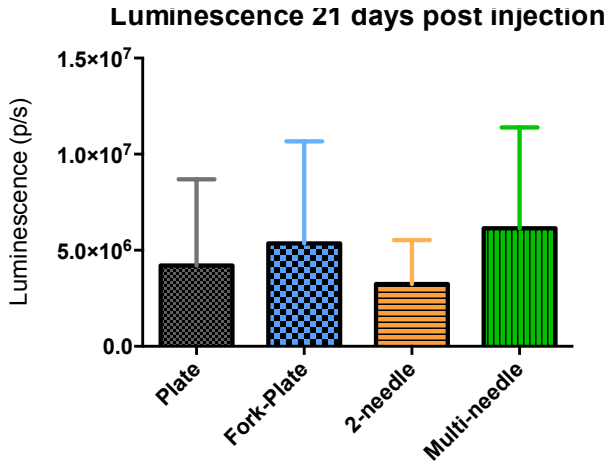


Modified from Keyaerts, M. (2012) *Trends Mol. Med.*

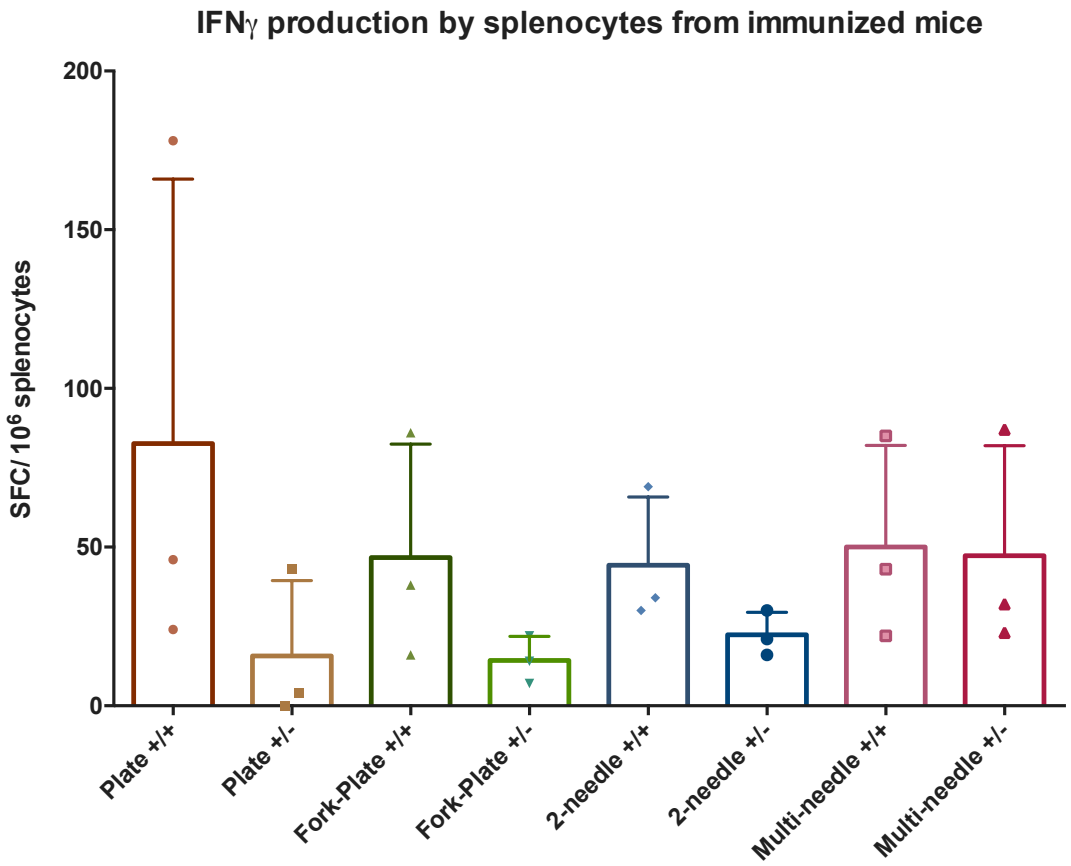
Luciferase in vivo transfection







Anti-luciferase response



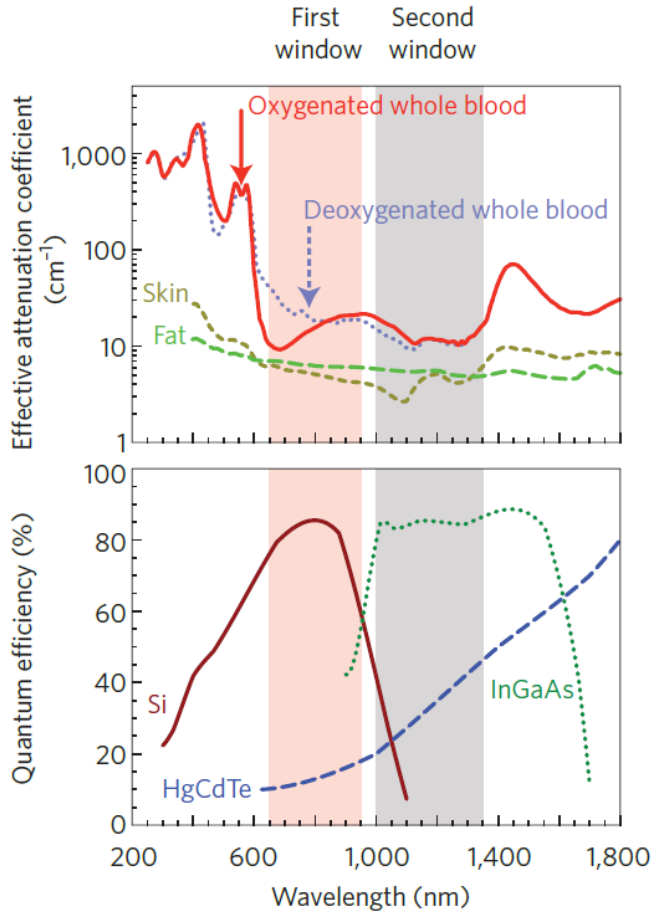
Issues with luminescence imaging

1. Poor deep-tissue detection due to spectral properties
2. Luciferase detection requires substrate (D-luciferin) presence

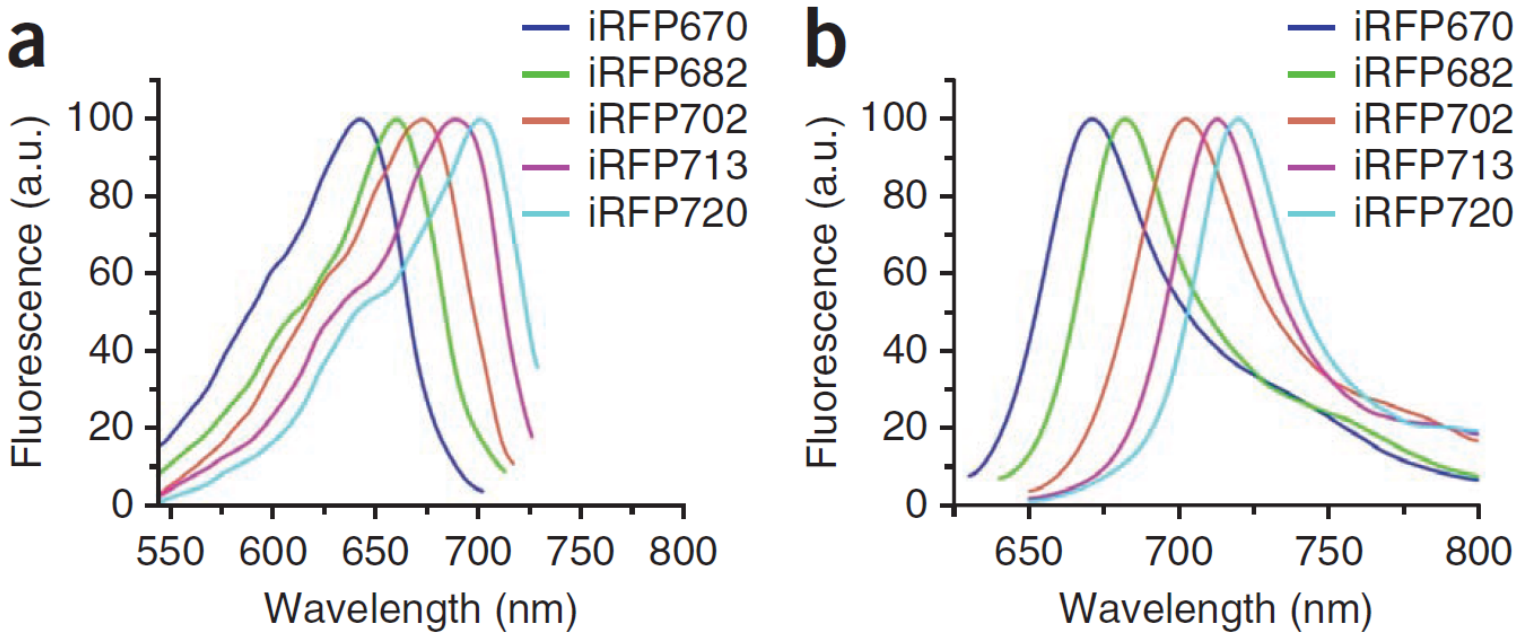
Near-infrared reporters | iRFP670

→ Near-infrared optical window
- Well-defined window for imaging 650-950 nm
- High tissue penetration due to low scatter and absorbance
- Reduced autofluorescence

→ iRFP670
- Uses bacterial phytochrome photoreceptors (BphP) as a template
- Has the most red-shifted absorption spectrum among the phytochromes



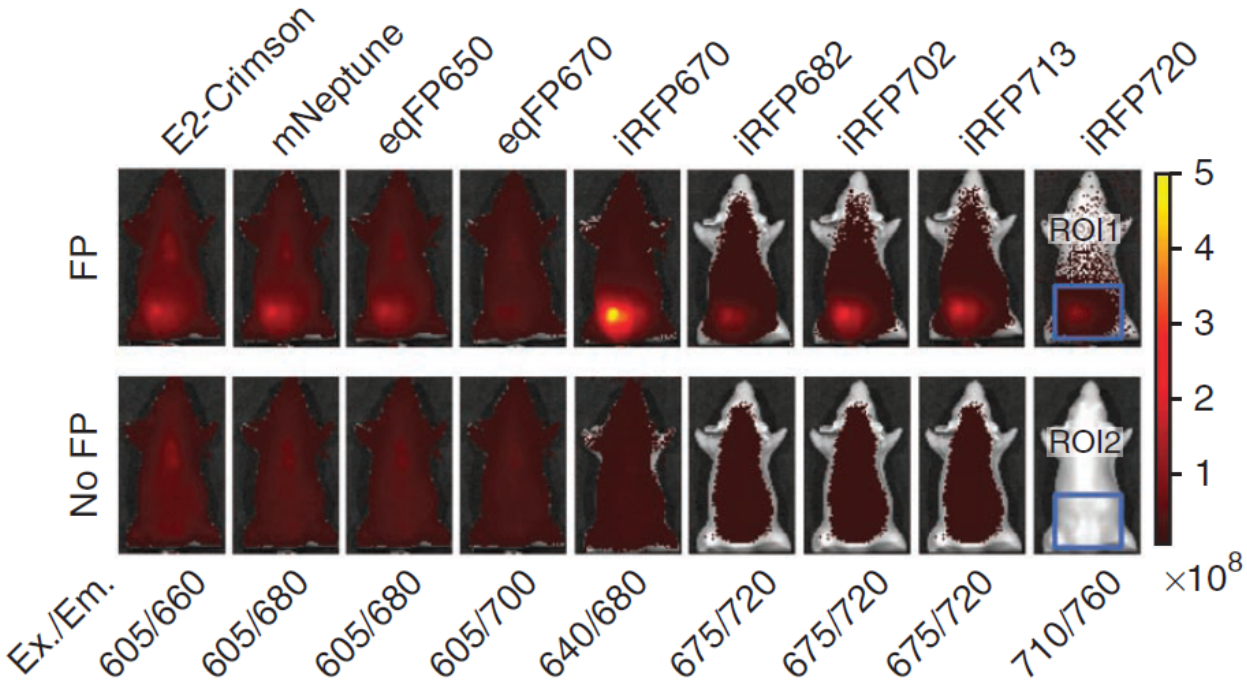
Fluorescence in tissue

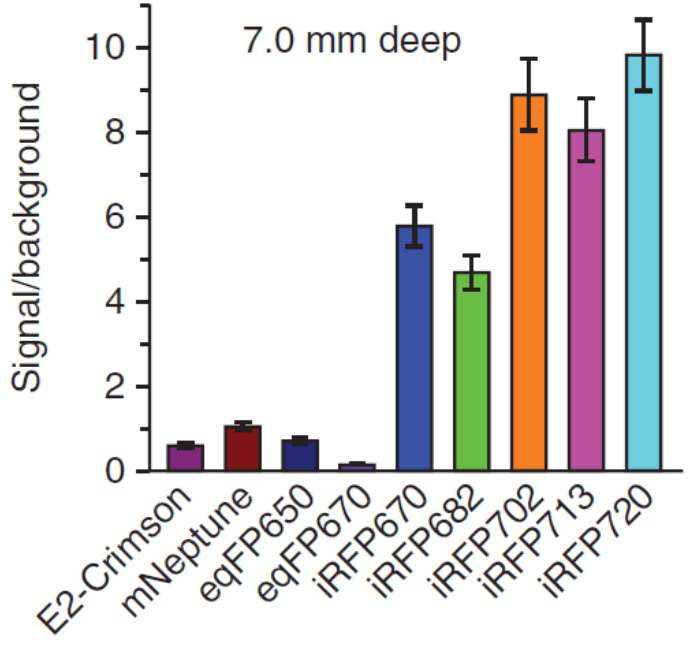


Excitation (a) and emission (b) of BphP-based fluorescent proteins.

Shcherbakova, D. M., & Verkhusha, V. V. (2013). *Nature Methods*.

iRFP670: a working compromise between signal and noise



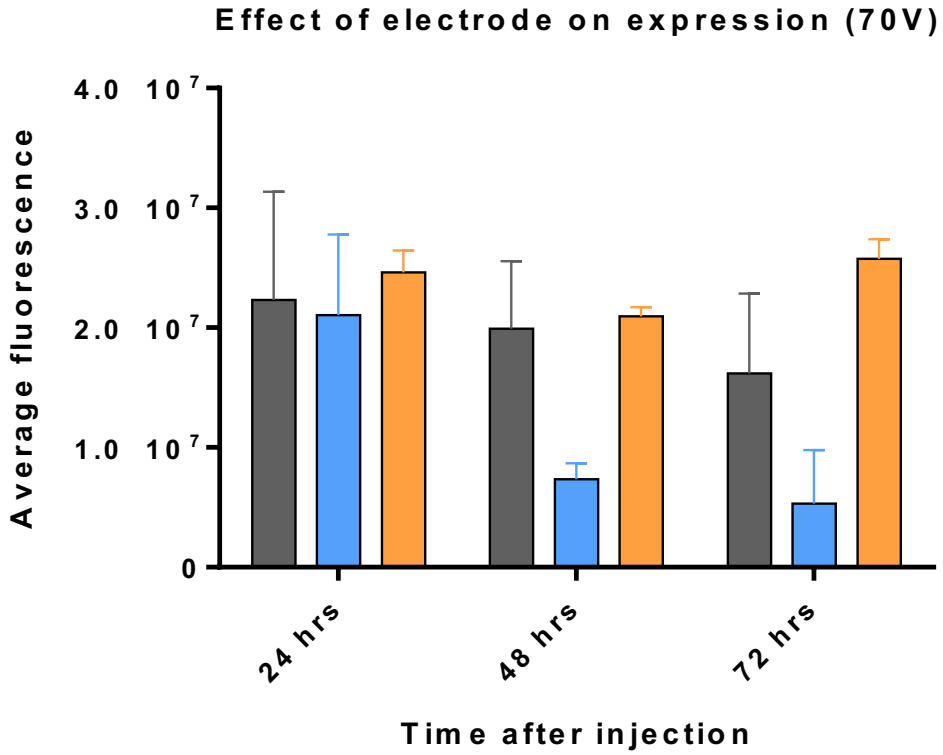


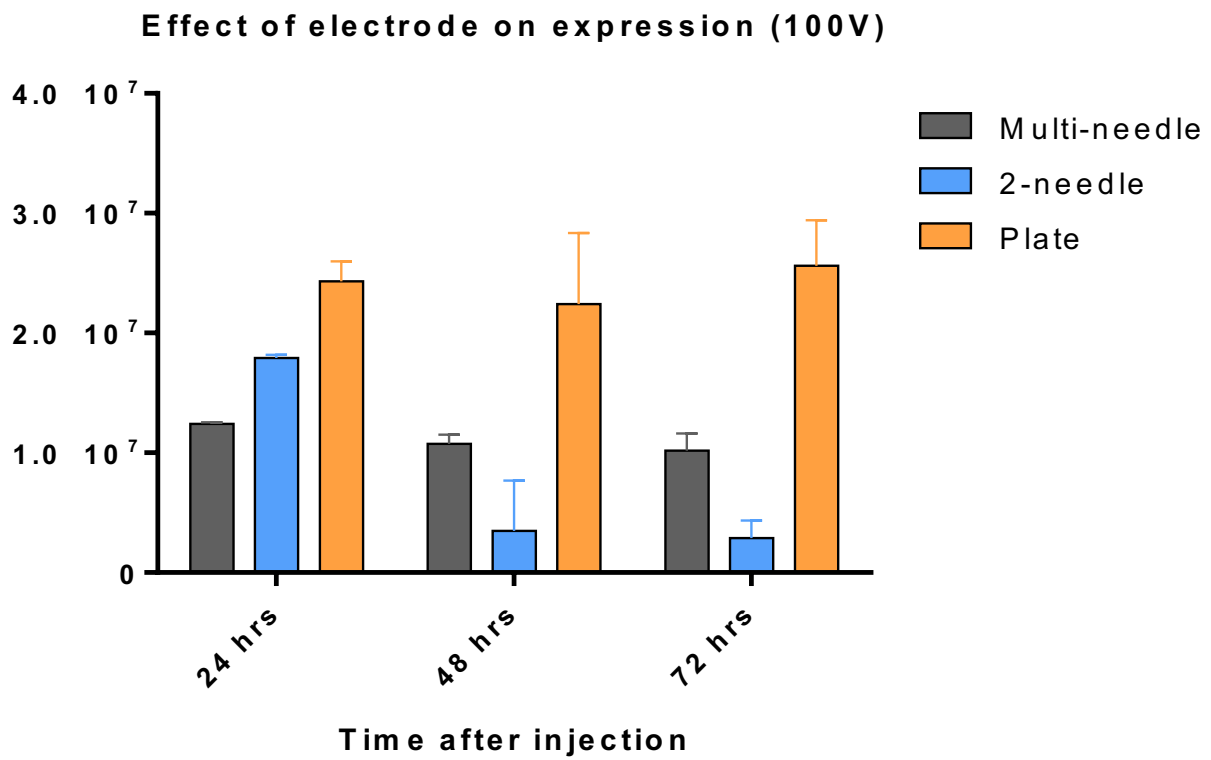
Shcherbakova, D. M., & Verkhusha, V. V. (2013). *Nature Methods*.

Experimental plan (marmoset skin explants)

1. Administer ID injections of 10µg iRFP670
2. Electroporate injection site
3. Excise injection site and culture in growth medium for 72 hours
4. Monitor fluorescence levels in skin explants
5. Collect & analyze crawl out cells from the skin explants

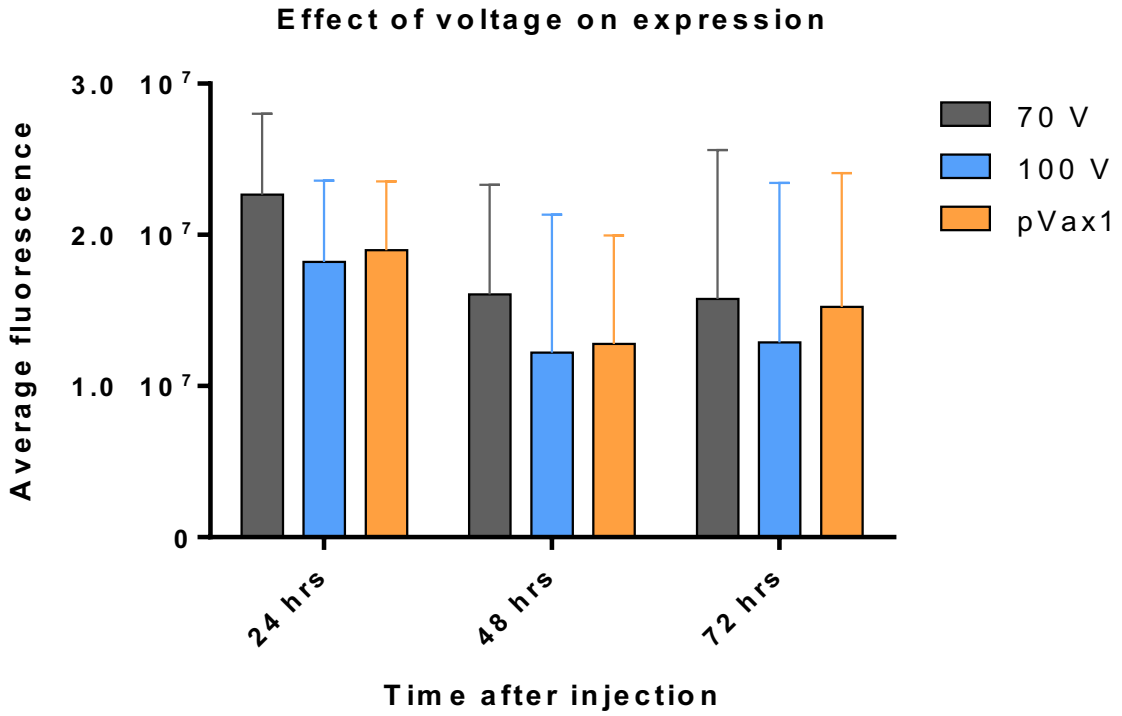
Transfection efficiency is voltage-dependent





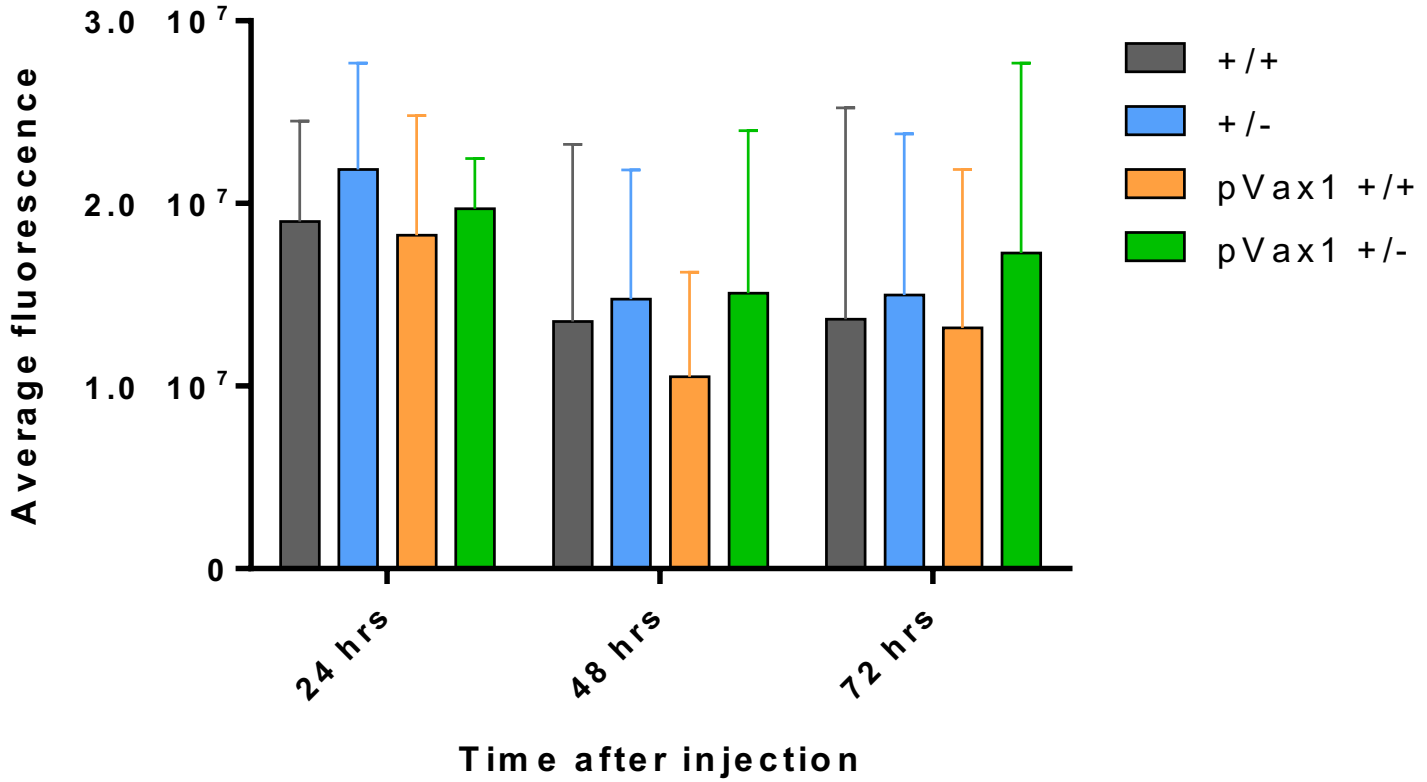
70V is optimal for multineedle EP and 100V rusults in slightly better transfection using a plate electrode.

Transfection in vivo (explants)



70V EP results in slightly higher iRFP670 expression as compared to 100V.

Effect of pulse polarity on iRFP670 expression



Alternating polarity pulses result in slightly higher fluorescence.

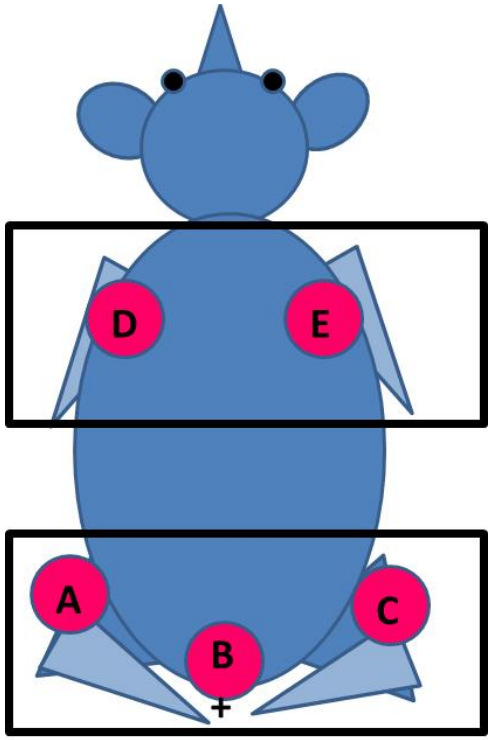
Conclusion

It did not work!

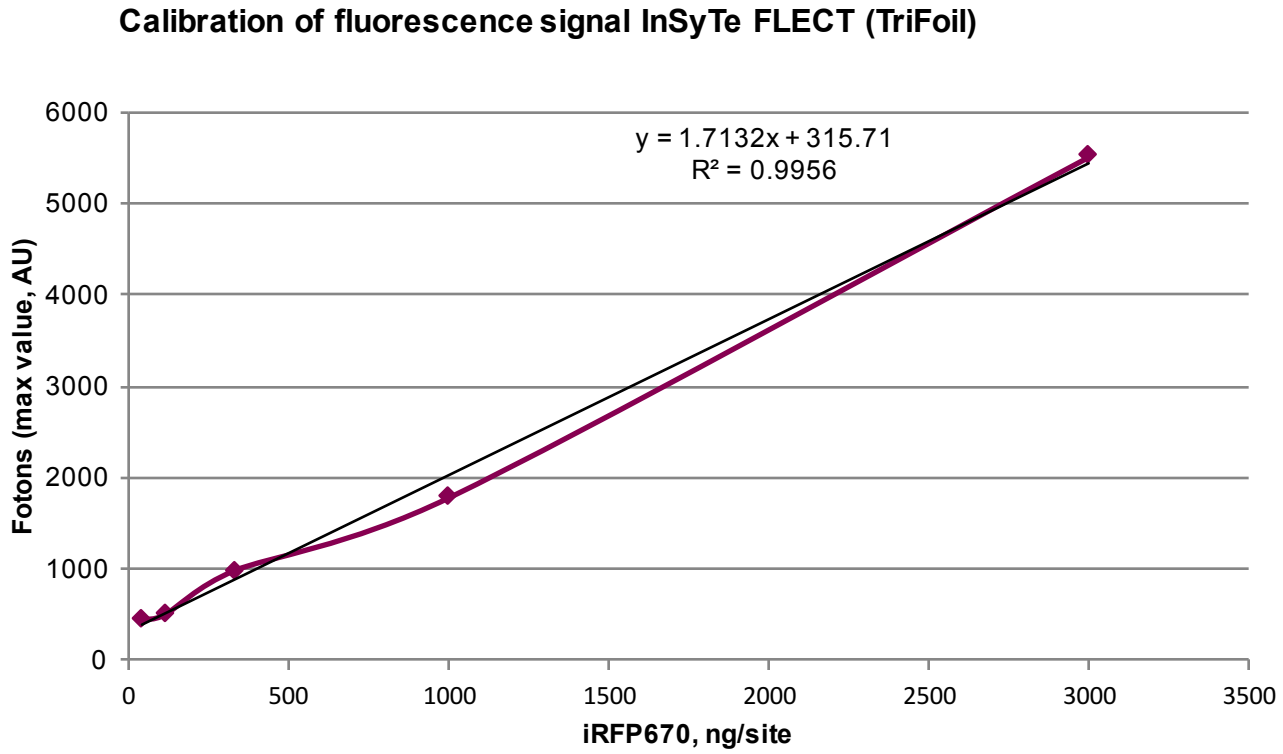
10 µg is too little to provide reliable detection in explants*

* With the IVIS

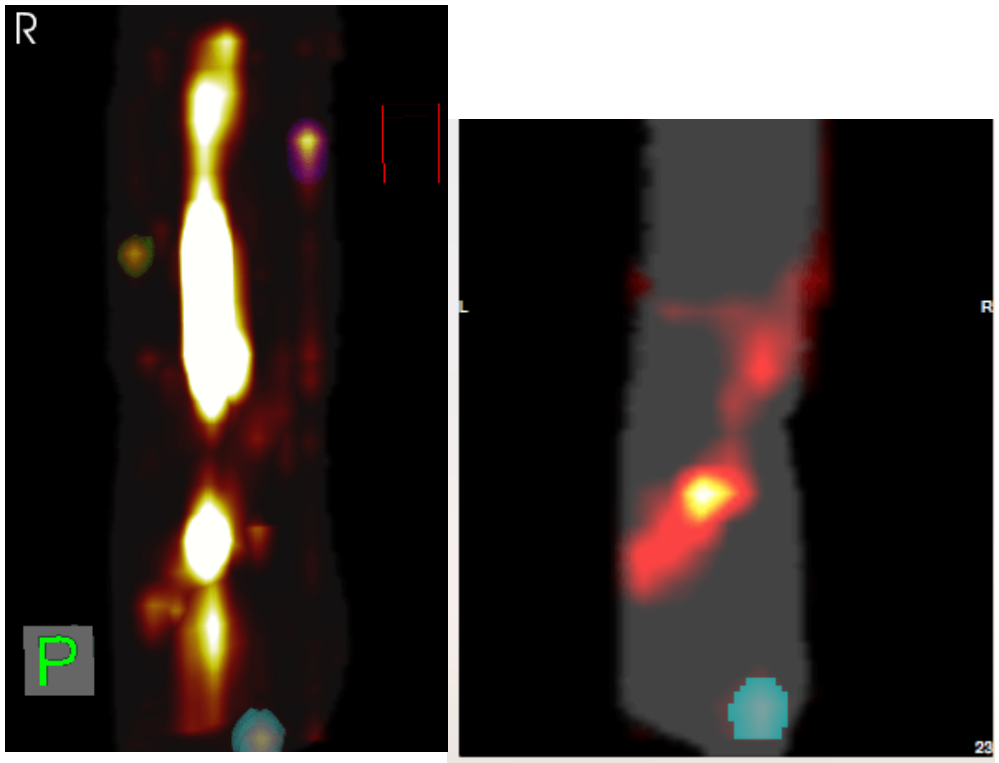
iRFP670 protein detection (TriFoil)

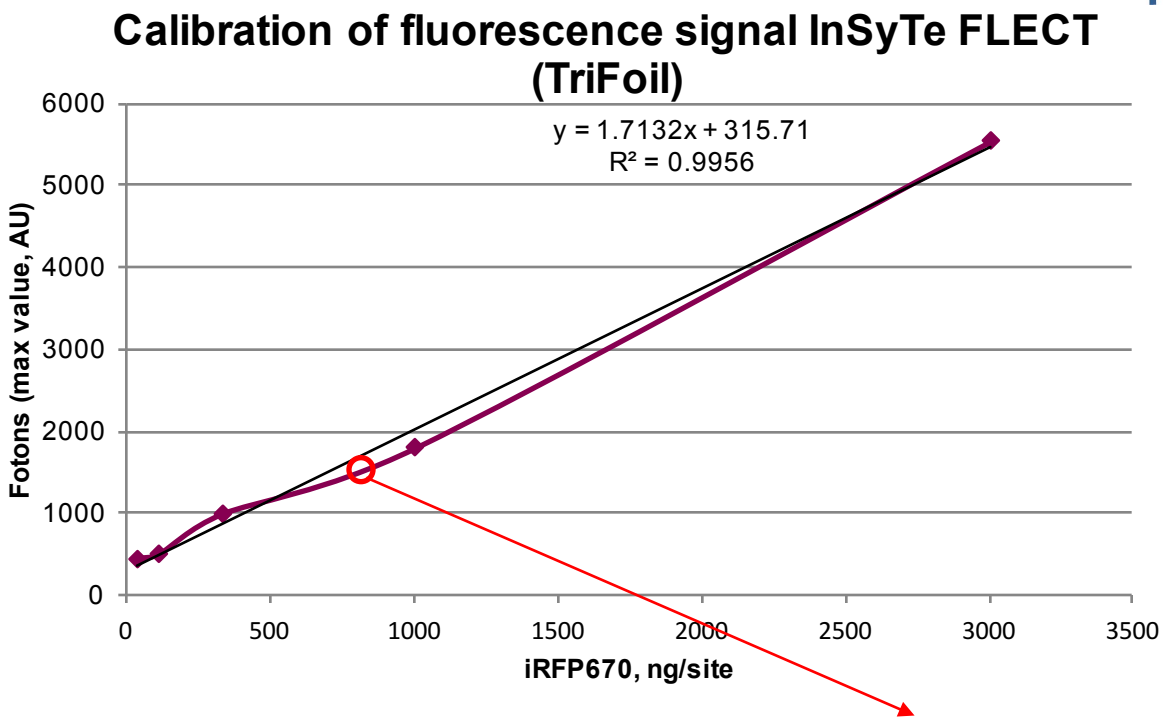


A - 3 ug
B - 1 ug
C - 330 ng
D - 110 ng
E - 33 ng



Results: iRFP670 plasmid injection

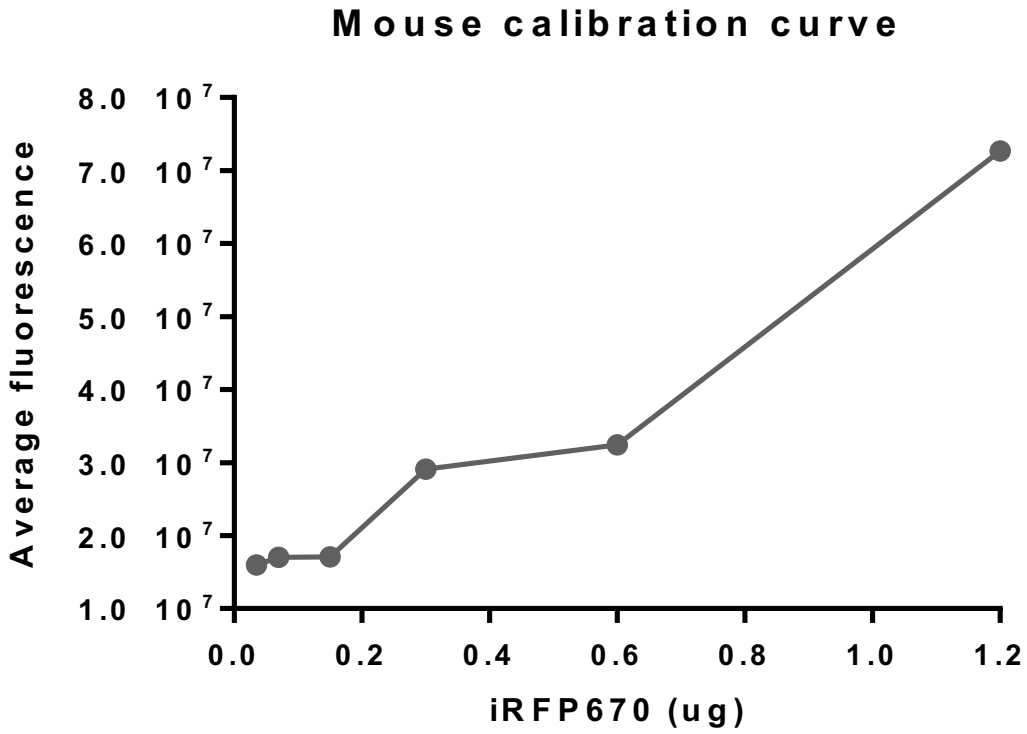


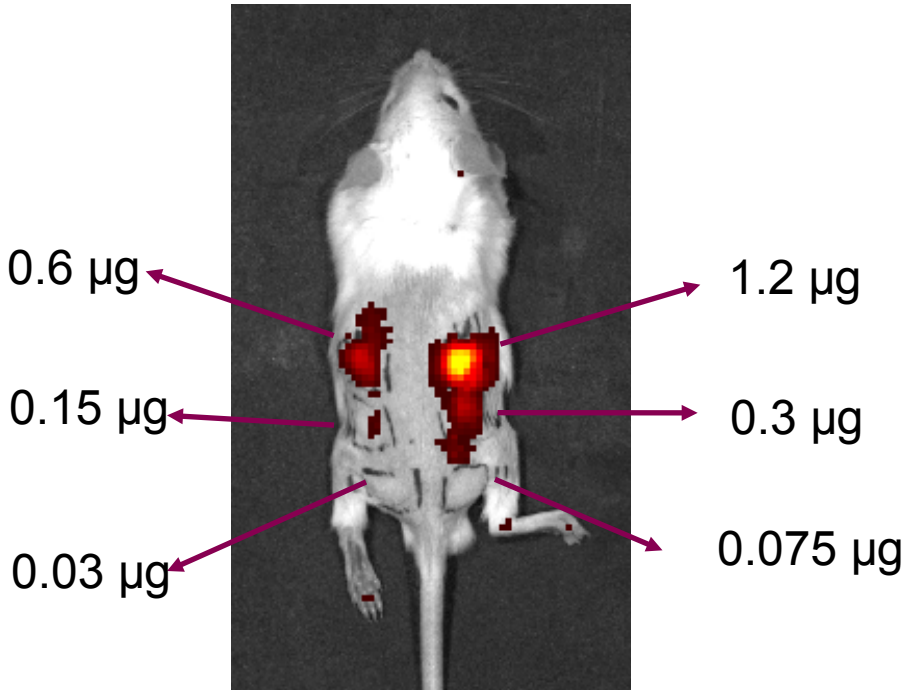


~820 ng

Location	Voxels	Volume mm³	Conc
Right hip	559	35.7	1543.3

iRFP670 protein detection (IVIS)

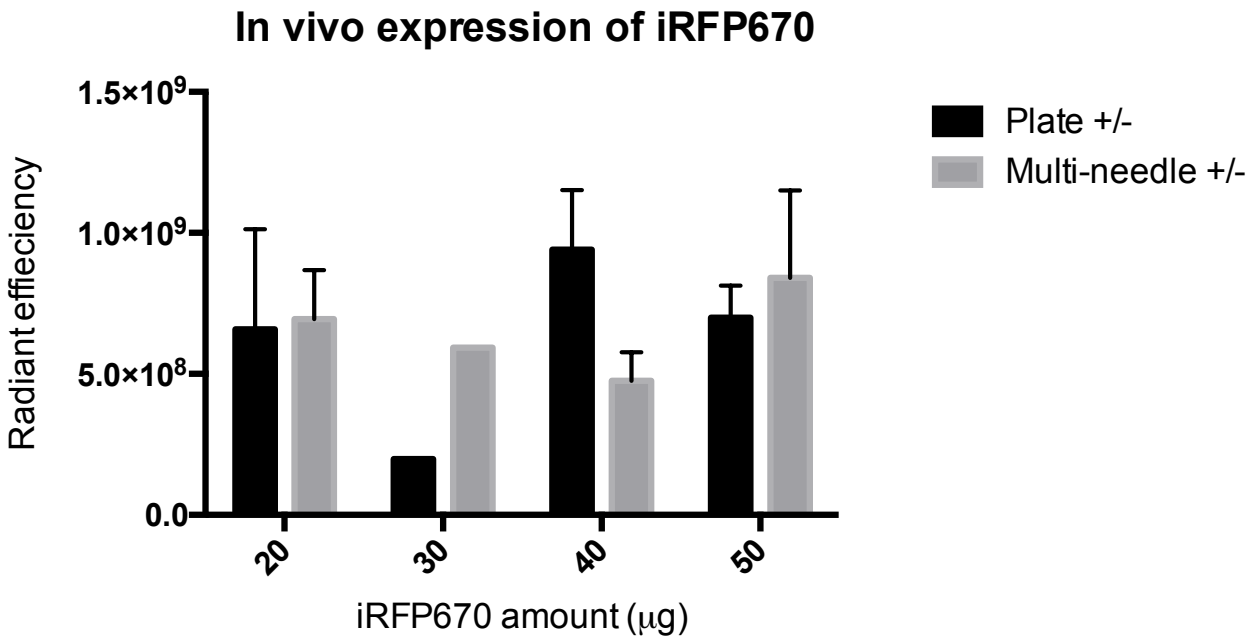


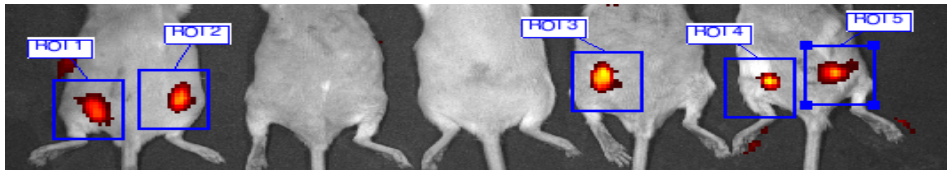


Optimization of iRFP670 delivery

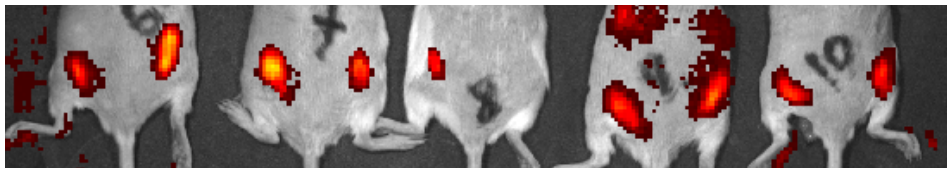
- 4 important parameters:
  - Electrodes
    - Multineedle
    - 2-needle
    - Plate
    - Plate-fork
  - Voltage
  - Polarity
  - Dose

iRFP670 expression depends on quality of electroporation





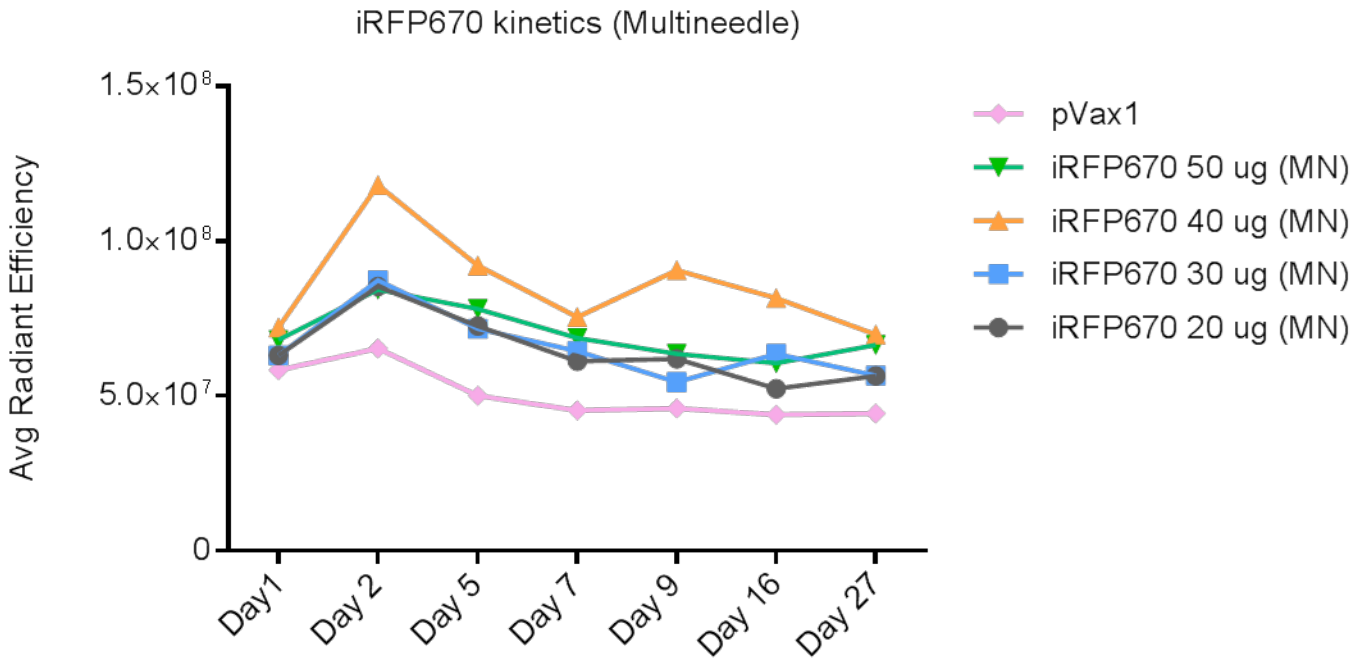
20 MN VECTOR 30 MN 40 MN

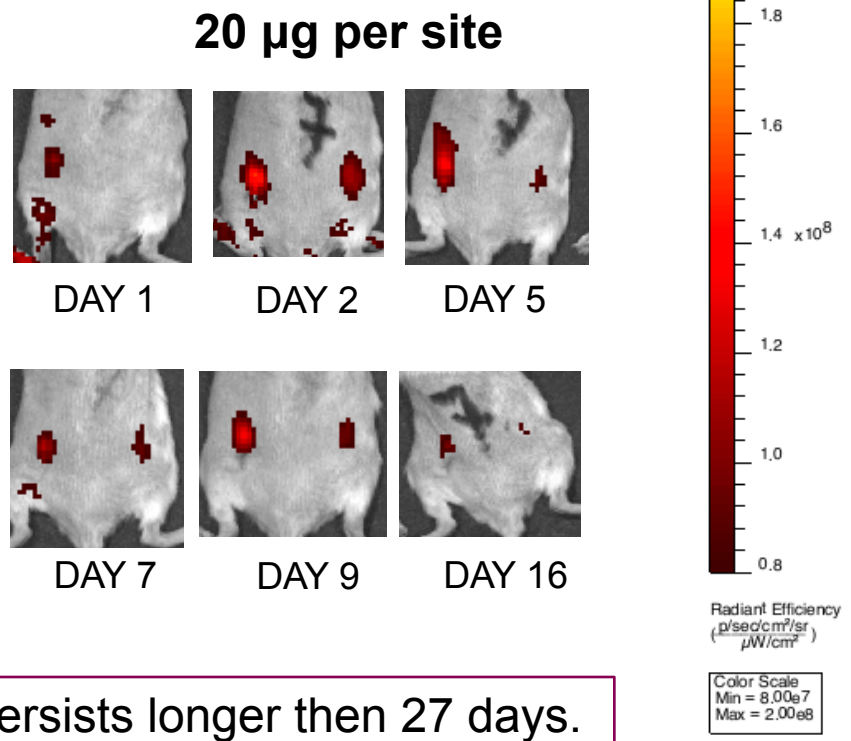


50 MN 20 PL 30 PL 40 PL 50 PL

Saturation point 40 µg or less. 20 µg is already providing sufficient fluorescence for reliable detection. 20 µg of iRFP670 plasmid injection translates into ~3 µg protein 5 days after injection.

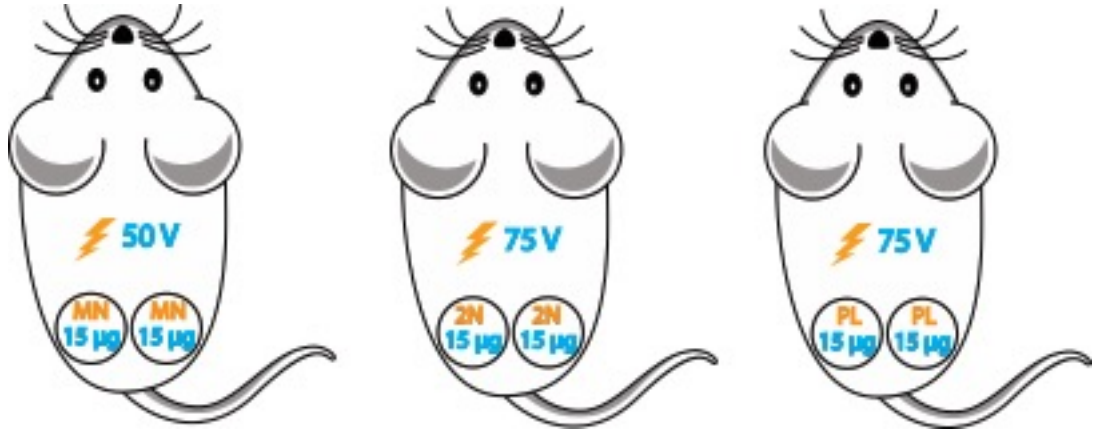
Longitudinal monitoring of fluorescence



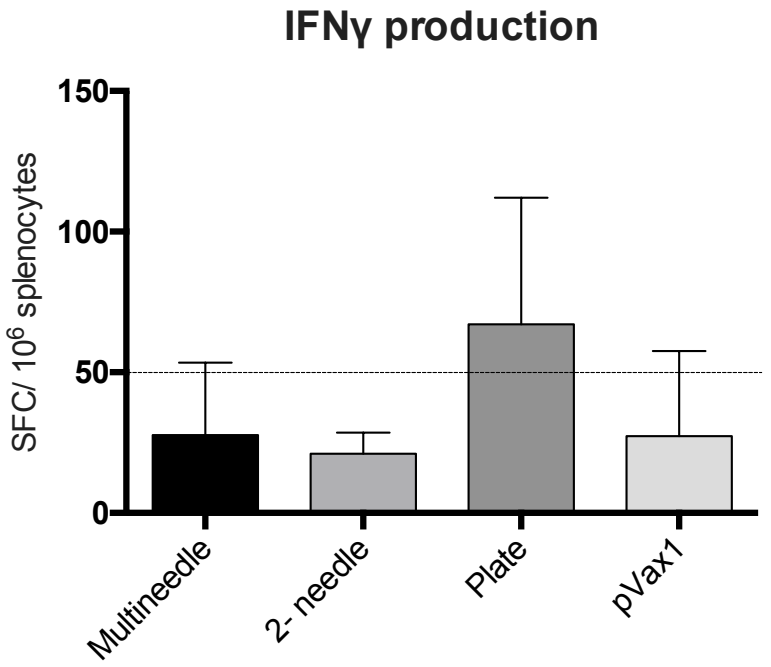


The data confirms saturation at not more than 40 µg. Expression persists longer then 27 days.

Assessment of iRFP670 immunogenicity

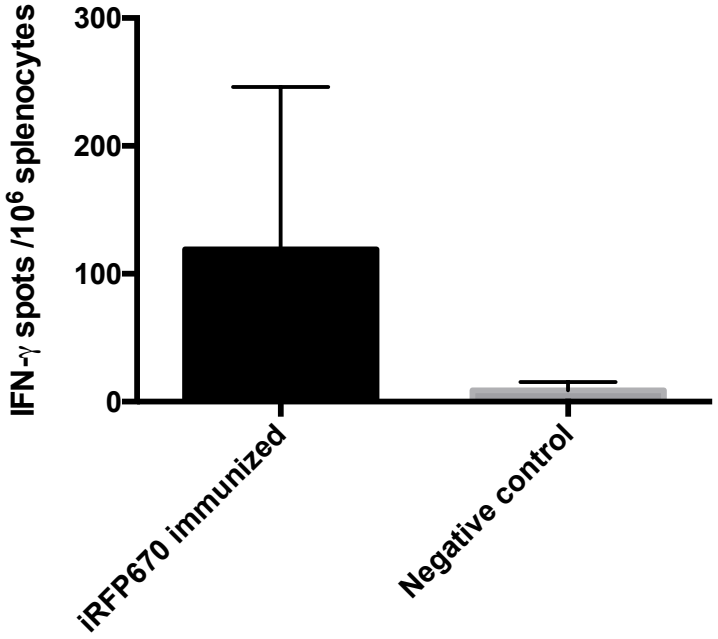


- Mice were injected ID + EP
- 21 days later spleens were harvested
- Splenocytes were stimulated in vitro with iRFP670 protein



The protein does not induce a significant cellular/humoral immune response after 21 days!

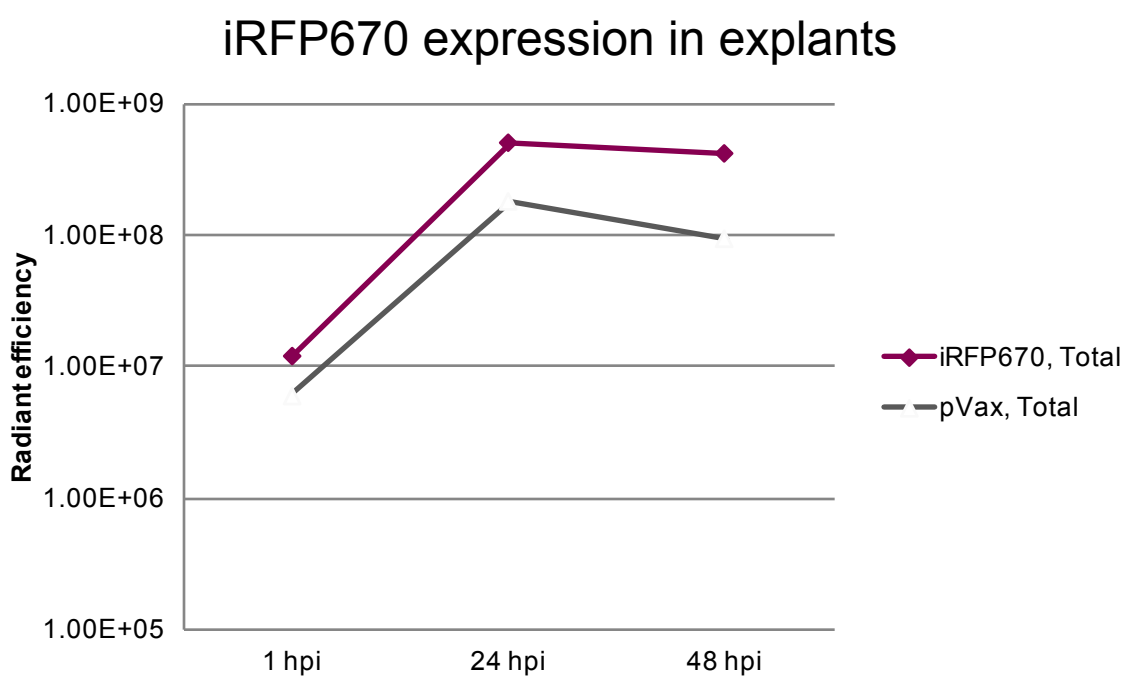
Assessment of iRFP670 immunogenicity

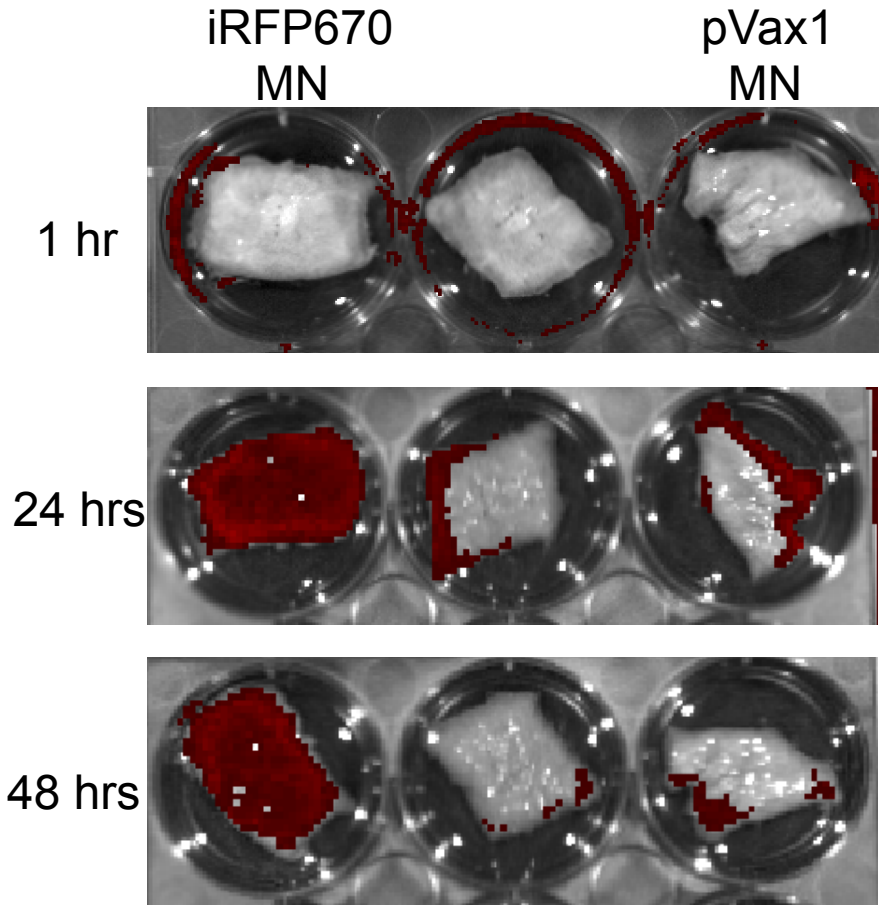


- Mice were injected ID + EP
- 21 days later spleens were harvested
- *In silico* prediction of epitopes performed
- Specific peptides were synthesized and used for assessment of responses by ELISpot

Peptide stimulation confirmed the low immunogenicity of the reporter!

Optimization in human skin (50 µg iRFP670)





Detectable difference in fluorescence between vector and iRFP670 inoculated explants (MN electrode, Derma Vax)

Conclusions and current work

- iRFP670 is a promising candidate for both *in vivo* and *ex vivo* imaging of transfected tissue
- Evaluated of reporter expression (long- and short term experiments)
  a. Corroborate expression in crawl-out cells (done in mice)
  b. Study the type of cell populations among crawl-outs

a. Immunogenicity and toxicity are low *in vivo*
b. Investigate the relationship between expression in explants and cell inflammation

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