In our study (Bugaj *et al, Nature Methods,* 2013*)* we showed that light inducible clustering of Rac1 and RhoA can activate their respective signaling pathways. Below are a few experimental considerations to keep in mind when implementing these tools:

1) Care must be taken to maintain transgene expression at low-to-medium levels, as suggested in the publication. Since RhoA is maintained in an inactive state by endogenous RhoGDIs, overexpression of RhoA may saturate the GDI pool and lead to a constitutive-like activity of RhoA that is insensitive to light activation. Additionally, potential basal self-association of Cry2 at high concentration levels may be sufficient to activate RhoA independently of light, again pointing to the necessity to maintain low transgene expression levels. For this reason, it is recommended to conduct studies in cells transduced with a single copy of the virus and with expression repressed using the tetracycline-regulatable ("Tet-Off") promoter encoded in the CLPIT retroviral construct. Optimal expression levels would be best determined empirically for each individual expression host.

2) Relatively weak light is sufficient for activation– typical focal activation protocols used 0.1-10% laser power from a 450 nm or 488 nm laser on a scanning confocal microscope, which would roughly correspond to whole field illumination in the low microwatt range. Ambient light has not emerged as an issue in using this construct, though cells were shielded from ambient light whenever possible as a precaution.

3) Varying activating light levels could lead to differing phenotypes. For instance, when working with Cry2-Rac1, lamellimpodial extension typical of Rac1 activation can be induced with low levels of blue light (from 0.1-1% laser power, depending on protein expression levels), but at very high activation intensities, this phenotype is not produced. We can only speculate at this point as to why this is (photosensitivity vs. Rac1 biochemistry) and we are actively elucidating the mechanisms of how clustering activates GTPases, as well as best practices for using these tools.

All of the sequences can be found here:

http://www.nature.com/nmeth/journal/v10/n3/extref/nmeth.2360-S2.xlsx

Best of luck with your experiments!

-Schaffer Lab