TECHNICAL BRIEF

LuMPIS – A modified luminescence-based mammalian interactome mapping pull-down assay for the investigation of protein–protein interactions encoded by GC-low ORFs

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The GC content is highly variable among the genomes of different organisms. It has been shown that recombinant gene expression in mammalian cells is much more efficient when GC-rich coding sequences of a certain protein are used. In order to study protein–protein interactions in Varicella zoster virus, a GC-low herpesvirus, we have developed a novel luminescence-based maltose-binding protein pull-down interaction screening system (LuMPIS) that is able to overcome the impaired protein expression levels of GC-low ORFs in mammalian expression systems.

Most processes in mammalian cells are directed and/or regulated by protein–protein interactions (PPIs). Defining the putative interaction partners of a specific protein is one approach to elucidate its cellular function. Several methods to study PPIs in parallel, such as yeast-two-hybrid (Y2H) screening, luminescence-based mammalian interactome mapping (LUMIER), biomolecular fluorescence complementation, bioluminescence resonance energy transfer (BRET), protein complementation assay, fluorescence resonance energy transfer, or co-immunoprecipitation (co-IP) of tagged proteins have been developed in the last couple of years and are reviewed elsewhere [1–5]. In principle, each method for the systematic detection of PPIs relies on the terminal fusion of one pair of tags (*i.e.* peptides, protein domains or whole proteins) to the respective binding partners. After positive interaction of the tested proteins, the respective pairs of tags accomplish either the pull-down of a reporter protein, as for example in LUMIER or co-IP, the complementation of a reporter protein, as for example in Y2H, protein complementation assay, or the biomolecular fluorescence complementation, or the resonance energy transfer to the reporter protein, as for example in fluores-

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Abbreviations: aa, amino acid; **BRET,** bioluminescence resonance energy transfer; **co-IP,** co-immunoprecipitation; **eGFP,** enhanced green fluorescent protein; **eGFP-Luc,** eGFP-luciferase fusion protein; **HEK,** human embryonic kidney; **HSV-1,** herpes simplex virus 1; **LIR,** luminescence intensity ratio; **LUMIER,** luminescence based mammalian interactome mapping; **LuMPIS,** luminescence-based MBP pull-down Interaction screening system; **MBP,** maltose binding protein; **MIF,** migration inhibitory factor; **PCA,** protein complementation assay; **PPI,** protein–protein interaction; **Rluc,** Renilla-Luciferase; **RPS19,** ribosomal protein 19; **VZV,** Varicella zoster virus; **Y2H,** yeast-two-hybrid; **YFP,** yellow fluorescent protein

cence resonance energy transfer or BRET. Due to its simplicity, high-throughput compatibility and cost-effectiveness, the Y2H-screening technology has been established as the method of choice for the initial analysis of PPIs in genome-wide scales. We have recently performed a comprehensive Y2H-analysis of all 71 known Varicella zoster virus (VZV) ORFs. This interactome analysis revealed 173 putative PPIs within the tested proteins [6]. The Y2H technology, as any high-throughput screen, can exhibit a high rate of false-positive and -negative PPIs, making their validation in different mammalian cell-based assay systems extraordinarily important. The validation of these putative PPIs in any mammalian cell-based assay system requires as a prerequisite the abundant recombinant expression of the individual, terminally tagged, binding partners. VZV, however, comprises the lowest genomic GC content of all human pathogenic herpesviruses with an overall of 46% [7]. The GC content is, for sure, not the only factor influencing protein expression levels of a particular ORF, but it has been shown that gene expression in mammalian cells is much more efficient when GC-rich coding sequences of a transgene are used. The mechanism of how the GC content of a transgene influences its gene expression is not completely understood, but effects on the transcription efficiency, polyadenylation, nuclear export, stability (half-life) and translation rate of the transcribed mRNA's may be involved [8]. In order to validate the putative PPIs of VZV that have been identified by Y2H, we have developed a novel luminescence-based MBP pull-down interaction screening system (LuMPIS) that is able to overcome impaired protein expression levels of GC-low ORFs.

We have recently performed comprehensive Y2H-analyses of the ORFeomes of different human pathogenic herpesviruses. These included not only the ORFeomes of VZV and human herpesvirus 8 [6], but also murine cytomegalovirus, herpes simplex virus 1 (HSV-1) and Epstein–Barr virus (Fossum *et al.* 2009, submitted for publication). In order to validate the putative PPIs that have been identified by respective Y2H analyses in an independent, mammalian cell-based assay system, a co-IP-based strategy utilizing N-terminally HA- and Myc-tagged proteins was performed [6]. In this case, respective herpesviral ORFs were expressed transiently in human embryonic kidney (HEK) 293 cells utilizing customized, pCR3 (Invitrogen) based, N-terminally HA- and Myc-tagged, Gateway^{TM}-compatible mammalian expression vectors and FuGENE HD^{TM} (Roche) as transfection reagent. It soon became clear that the systematic mammalian expression of the majority of VZV ORFs, in contrast to that of all other systematically assayed herpesviral ORFeomes, was impaired with respect to abundant protein expression levels (unpublished observations). One striking difference between VZV and the other assayed herpesviruses is the GC content of their respective genomes, with VZV having the lowest GC content of 46% in contrast to overall higher GC contents of 54% (human herpesvirus 8), 59% (murine cytomegalovirus), 60% (Epstein–Barr virus) and 68% (HSV-1) [7].

When we performed Western blotting experiments to assay the transient mammalian expression levels of two VZV proteins, namely ORF63 and ORF25, with rather extreme (57 *versus* 41%, respectively) GC contents, it became obvious that protein expression is abundant in case of the relatively GC-rich ORF63, but impaired in case of the relatively GC-low ORF25. In case of ORF25 this effect of impaired protein expression levels could be reconstituted by using long, GC-rich protein tags as, for example, maltose-binding protein (MBP) (462 amino acid (aa)/53% GC) or enhanced green fluorescent protein (eGFP-Luc) (812 aa/51% GC), but not by using short peptide tags, as for example the eight aa Flag tag, or GC-low tags, as for example GST (223 aa/39% GC). The abundant expression levels of the rather GC-rich ORF63 have not been affected negatively by any of the four utilized N-terminal tags (Fig. 1A). In case of ORF25-abundant protein, expression levels could further be reconstituted by utilizing ORF25GC, a completely synthetic, GC-rich (60% GC) version of this gene (GENEART), demonstrating the direct effect of the GC content on recombinant protein expression levels in HEK 293 cells (Fig. 1B). When we assayed six randomly selected VZV tegument ORFs, namely ORFs 9, 10, 23, 34, 21 and 22N systematically for their protein expression levels in mammalian cells, only three of them (ORFs 9, 10 and 23) could be expressed abundantly when using a N-terminal Flag tag. In contrast, all six ORFs could be expressed in relatively even and abundant amounts, when utilizing the MBP tag, demonstrating the positive effect of a long and GC-rich tag on systematic protein expression levels in mammalian cells (Fig. 1C).

After this observation, we have decided to modify the original LUMIER technology described by Barrios-Rodiles *et al.* [4] in order to overcome the impaired protein expression levels of GC-low ORFs. In the original LUMIER protocol, prey and bait proteins are expressed with N-terminal Flag and Renilla-Luciferase (Rluc) tags, respectively. Positive PPIs can be detected after pull-down of the Flag-tagged prey protein using sepharose beads and Flag-specific monoclonal antibodies and bioluminescence detection of the bound Rluc-tagged bait protein (Fig. 2, left side). In case of our modified LuMPIS protocol, the Flag tag of the bait protein has been replaced by MBP and the Rluc tag by eGFP-Luciferase. In case of LuMPIS, positive PPIs can be detected after pull-down of the MBP-tagged bait protein using amylose beads and bioluminescence detection of the bound eGFP-Luc tagged prey protein after elution with maltose (Fig. 2, right side). These LuMPIS specific modifications offer three main advantages: (i) the use of two long, GC-rich tags increases the systematic expression levels of the overall GC-low VZV ORFs, (ii) the use of amylose beads for pull-down is much more economic compared with sepharose beads and monoclonal antibodies and (iii) the use of an eGFP-Luc tag enables the qualitative control of transfection efficiencies by fluorescence microscopy prior to starting the assay.

For a typical LuMPIS experiment, semiconfluent HEK 293 cells grown in 12-well plates were transfected with the respective prey and bait vectors using FuGENE HD^{TM}
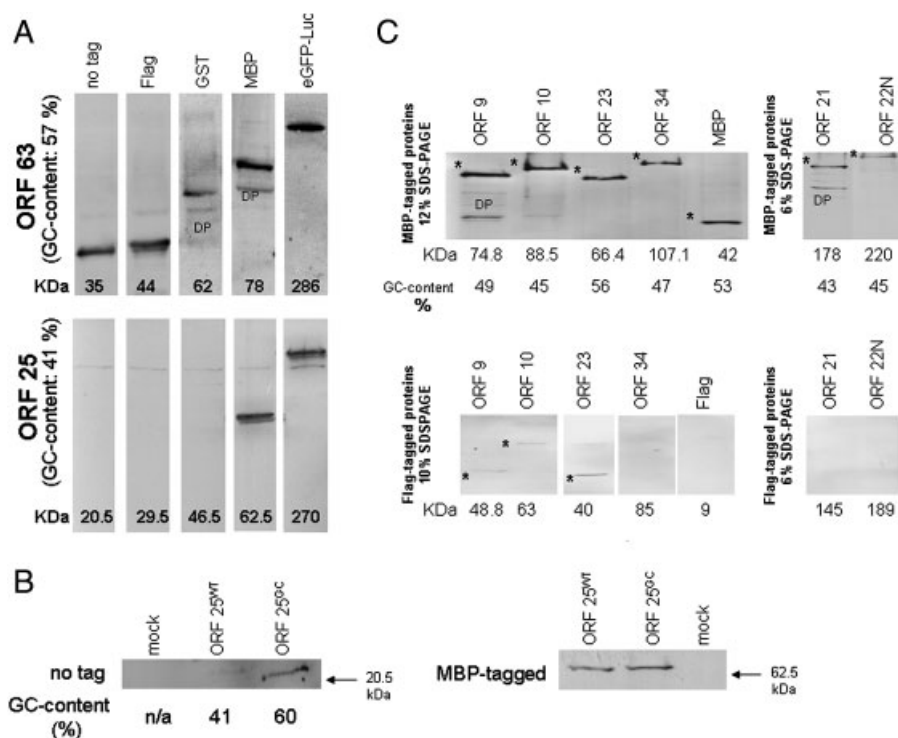
Figure 1. Protein expression levels of different tagged and untagged VZV ORFs in HEK 293 cells. For all transient protein expression experiments customized, pCR3 (Invitrogen) based, N-terminally tagged, Gateway[TM]-compatible mammalian expression vectors were used. FuGENE HD[TM] (Roche) was used as transfection reagent according to the manufacturer's instructions. (A) Western blot experiments to assay the protein expression levels of VZV ORFs 63 (upper panel) and 25 (lower panel). Protein expression was visualized by ORF25 and ORF63 specific polyclonal rabbit antisera (a generous gift from Paul Kinchington). Protein expression is abundant in case of the relatively GC-rich (57%) ORF63, but impaired in case of the relatively GC-low (41%) ORF25. In case of ORF25 impaired protein expression levels could be reconstituted by using long, GC-rich protein tags. (B) Western blot experiments to assay the protein expression levels of wild-type VZV ORF25 (ORF25[WT]) and a completely synthesized, GC-enriched (60%) version of this protein (ORF25[GC]) as well as their MBP-tagged variants. ORF25 protein expression was visualized by an ORF25 specific polyclonal rabbit antiserum. Abundant ORF25 protein expression levels could be reconstituted by utilizing the GC-enriched version of the protein. (C) Western blot experiments to assay the protein expression levels of six randomly selected VZV tegument ORFs (ORFs 9, 10, 23, 34, 21 and 22N) when utilizing N-terminally Flag tagged (upper panel) or N-terminally MBP tagged (lower panel) proteins. Tagged protein expression was visualized by Flag and MBP-specific mouse monoclonal antibodies (Sigma-Aldrich and NEB, respectively). All six ORFs could be expressed in relatively even and abundant amounts, when utilizing the N-terminal MBP tag, demonstrating the positive effect of a long and GC-rich tag on systematic protein expression levels in mammalian cells.

(Roche) as transfection reagent. Transfection efficiency was qualitatively checked for green fluorescence (eGFP signal) using an inverse fluorescence microscope (Zeiss) before starting the experiment. Transfected HEK 293 cells were grown for 48 h and then lysed in 500 µL of lysis buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 0.05% Tween 20, 5 µg/mL Leupeptin, 5 µg/mL DNAse I, 2.5 mg/mL BSA, pH 7.5) by sonication (five pulses of 15 s) at 4°C in a Bandelin Sonorex RK 100 device. Lysates were cleared by centrifugation at $13\,000 \times g$ at 4°C for 10 min and then diluted 1:10 in washing buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA). MBP-tagged bait proteins were captured by incubating 150 µL cleared lysate with 100 µL of pre-equilibrated 50% slurry amylose beads (New England Biolabs) for 2 h by rotation at 4°C. After washing the amylose beads four times with 200 µL washing

buffer, the captured MBP-tagged bait proteins were eluted in two subsequent steps by incubating the beads with 75 µL 10 mM maltose for 15 min by rotation at 4°C. Eluates were pooled and the co-eluted eGFP-Luc-tagged prey proteins were detected by measuring luciferase activity in 50 µL eluate after addition of 50 µL luciferase assay reagent (Promega) using an Optima FLUOstar Luminometer system (BMG LABTech). Results are finally documented as luminescence intensity ratio (LIR). To calculate LIR, the luciferase activity in the eluates was normalized toward the activity in their corresponding lysates in order to attenuate the influence of possible transfection variations. The negative control (*i.e.* eGFP-Luc-tagged prey expression vector co-transfected with the empty MBP vector) was performed for each prey protein. LIR equals the ratio of the normalized luciferase activity of each pair of eGFP-Luc-tagged prey and
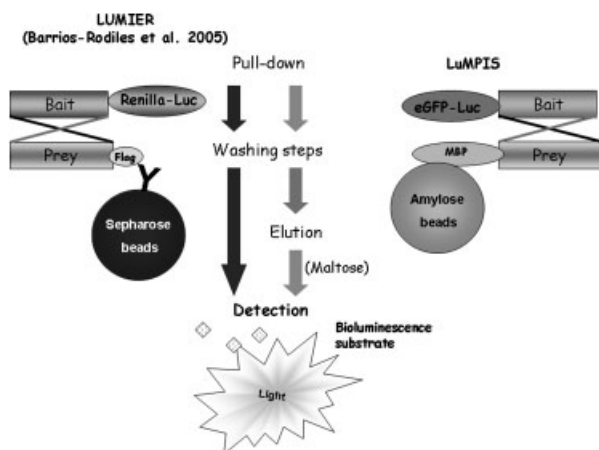
Figure 2. Schematic diagram of the LuMPIS-specific modifications of the original LUMIER technology described by Barrios-Rodiles et al. [4]. In the original LUMIER protocol, bait and prey proteins are expressed with N-terminal Flag and Rluc tags, respectively. Positive PPIs can be detected after pull-down of the Flag-tagged bait protein using sepharose beads and Flag-specific monoclonal antibodies and bioluminescence detection of the bound Rluc-tagged bait protein. In case of LuMPIS, the Flag tag of the bait protein has been replaced by MBP and the Rluc tag by eGFP-Luciferase. Positive PPIs can be detected after pull-down of the MBP-tagged bait protein using amylose beads and bioluminescence detection of the bound eGFP-Luc-tagged prey protein after elution with maltose.

MBP-tagged bait against its corresponding averaged negative control:

$$\mathrm{LIR} = \frac{\mathrm{Eluate}^{\mathrm{eGFP-Luc-tagged\ prey\ and\ MBP-tagged\ bait}} : \mathrm{Lysate}^{\mathrm{eGFP-Luc-tagged\ prey\ and\ MBP-tagged\ bait}}}{\mathrm{Eluate}^{\mathrm{Negative\ control}} : \mathrm{Lysate}^{\mathrm{Negative\ control}}}$$

As in the original LUMIER publication [4] the cutoff was set conservatively at LIR > 3 for a positive interaction.

In order to validate our novel LuMPIS PPI screening technology in general, we have investigated the interaction of three well-studied (pairs of) proteins with different (i.e. relatively strong, intermediate and low) binding affinities. In detail: human Jun and Fos, human migration inhibitory factor (MIF) and human ribosomal protein S19 (RPS19), and the homodimerizing protein BVRF2 of Epstein–Barr virus. Jun and Fos form a heterodimeric transcription factor which plays a fundamental role in the physiology and pathology of mammalian cells [9]. Jun and Fos strongly interact via a ZIP region with an affinity of 110 ± 12 nM [10], making them ideal control proteins for the development of novel PPI assays [11]. As shown in Fig. 3A, our LuMPIS technology allows the detection of the Fos–Jun interaction, whereas the interaction between dFos, a mutant whose ZIP motif has been deleted [11], and Jun did not differ significantly from the negative control. In case of Fig. 3A respective PPIs have been detected by using Jun as prey and Fos variants as bait. This interaction could also be detected in the opposite direction, when using Fos variants as prey and Jun as bait (data not shown). RPS19,

which is released by apoptotic cells, directly interacts with MIF ($K_D = 1.3\,\mu$M) acting as an extracellular negative regulator of this factor [12]. This interaction could also be confirmed with our assay by using MIF as prey and RPS19 as bait (Fig. 3B). We also detected the homodimerization of BVRF2 ($K_D = 20\,\mu$M), which has the essential function of disrupting the scaffold protein after the assembly of the capsid to make space for the viral DNA [13]. Notably, our LuMPIS technology is able to qualitatively detect all three tested interactions. Since the LIR values, however, do not correlate with the affinity of the tested PPI, our system is considered to be not quantitative (Figs. 3A–C).

To further validate our LuMPIS technology in particular for the screening of GC-low VZV ORFs that have been shown to possess impaired protein expression levels in mammalian cells (Fig. 1C), we focused on PPIs of the VZV tegument protein ORF21 (43% GC), which had been detected by Y2H screening technology recently [6]. The tegument is a unique, yet poorly understood, structural and functional element of the family of Herpesviridae playing important roles in key tasks of the herpesviral replication cycle, such as entry, maturation and egress [14]. Little is known about the VZV tegument protein ORF21. Its orthologues in other herpesviruses, such as cytomegalovirus, pseudorabies virus or HSV-1, have been shown to interact with the large tegument protein corresponding to VZV ORF22 [15–17]. In VZV this interaction could so far only be detected by Y2H analysis [6], but has not been validated in any other mammalian cell-based PPI assay system. As shown in Fig. 1C, VZV ORF21 could not be expressed abundantly by using a small N-terminal Flag tag, but only when the long, GC-rich MBP tag was used. When we performed LuMPIS assays to detect the interaction of ORF21 as bait against nine other VZV ORFs as prey (Fig. 3D), we could detect an interaction between ORF21 and the large tegument protein ORF22, validating our previous Y2H data. Since the completely sequenced cDNA of the 8232 bp long ORF22 was not available, it is to notice that we performed our LuMPIS experiments with a 4119-bp long, N-terminal fragment of ORF22 (ORF22N).

Since a semi-quantitative analysis of the ORF21-ORF22N interaction was not possible by using our LuMPIS technology, we performed a BRET saturation curve in order to estimate its relative binding affinity. BRET saturation analysis has been performed as described earlier [18–20]. Briefly, COS-7 cells were co-transfected with a total of $2\,\mu$g DNA at increasing acceptor to donor ratios (BRET saturation) in three independent experiments. Luciferase signals were acquainted after 24 h upon addition of Rluc substrate to the living cells. Light emission was collected over 10 s at 475 nm (Rluc signal) and 535 nm (BRET signal). The BRET ratio was calculated using

$$\frac{\mathrm{BRET_{probe}} - \mathrm{Rluc_{probe}} \times cf}{\mathrm{Rluc_{probe}}}$$

where cf is a correction factor $= \frac{\mathrm{BRET_{control}}}{\mathrm{Rluc_{control}}}$ with the negative control being the co-transfection of donor fusion proteins with yellow fluorescent protein in the absence of the protein of interest. PPI parameters were calculated by $Y = B_{max} \times \frac{X}{K_D + X}$,
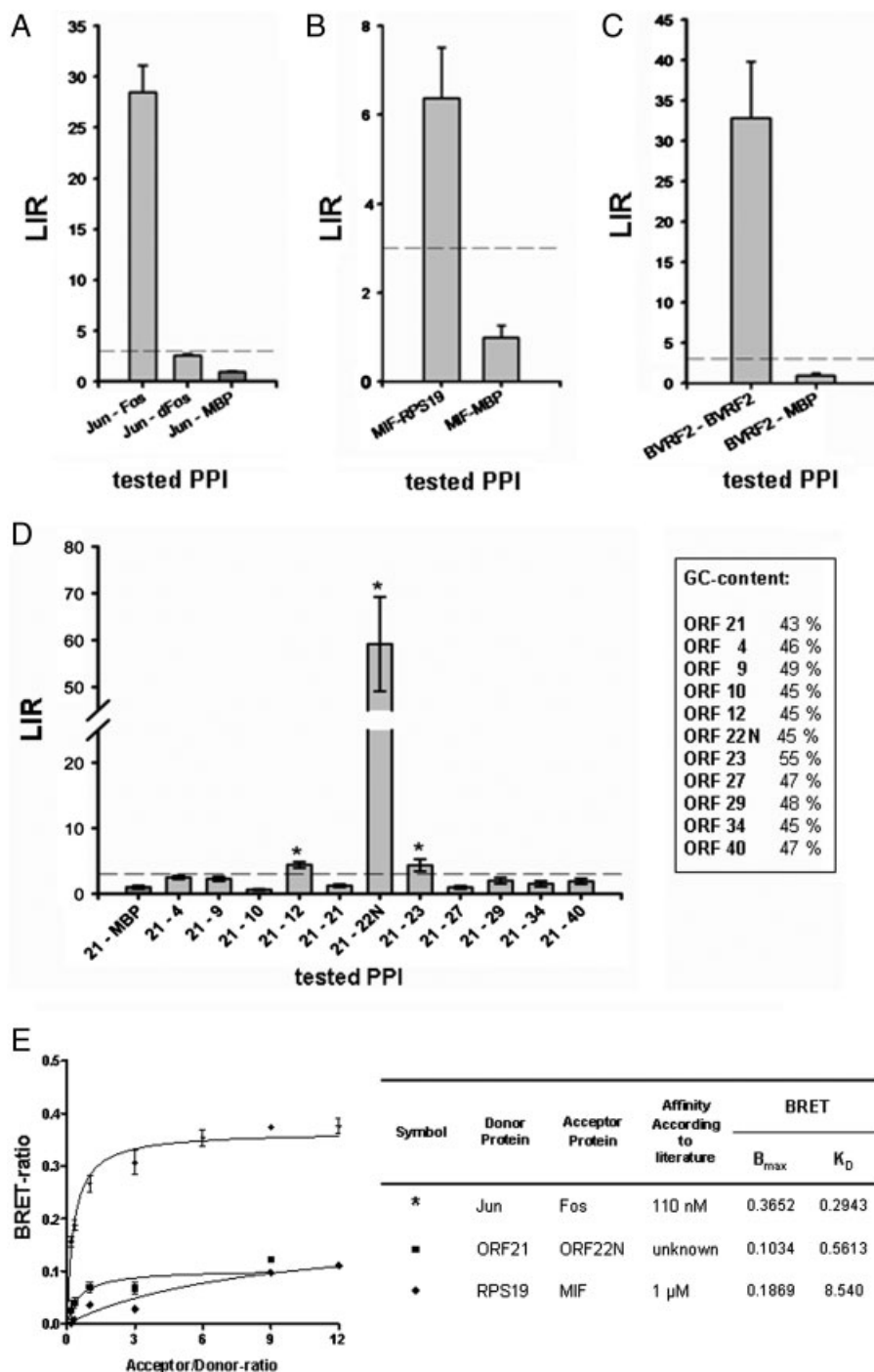
Figure 3. Validation of the LuMPIS PPI screening technology. (A–C) The LuMPIS technology allows the detection of three well-established strong, intermediate and weak PPIs between Fos and Jun (A), MIF and RPS19 (B) and the homodimerizing BVRF2 (C), respectively. (D) The LuMPIS technology allows the detection of a protein interaction between the GC-low VZV ORFs 21 and 22. Furthermore, two other interactions between ORF21 and ORFs 12 and 23 could be observed. This assay has been performed by using ORF21 as prey and respective other VZV proteins as bait. All experiments have been performed three times in duplicates. Error bars indicate standard error of the mean. Asterisks indicate positive PPIs with LIR values above the cutoff. (E) The relative affinity of known protein interactions and our pair of interest (ORF21 and ORF22N) were analyzed by BRET. Cells were transiently co-transfected with the respective donor (Rluc) and acceptor (yellow fluorescent protein) fusion proteins in an increasing acceptor to donor ratio. The BRET ratio, defined as the acceptor luminescence signal over the donor luminescence signal, was determined 24 h after transfection. Non-linear regression analyses were performed to assess the affinity of oligomer formation (K_D) and the maximal BRET ratio (B_{max}). Three independent experiments were performed and values are given as means. Error bars represent standard error of the mean.

where B_{max} is the maximal BRET ratio and K_D is the acceptor to donor ratio required to reach half-maximal BRET, indicating the relative affinity of PPI partners. The Jun–Fos and the MIF–RPS19 PPI pairs were also used as controls (Fig. 3E). Taken together, the BRET saturation analysis of ORF21-ORF22N in combination with the performed Jun–Fos and MIF–RPS19 controls indicate a relatively strong binding affinity with a (BRET defined) K_D of 0.5613.

Furthermore, two other interactions between ORF21 and ORFs 12 and 23 could be observed in three independent LuMPIS experiments. These two PPIs of ORF21 have so far only been detected by Y2H analyses: Uetz *et al.* demonstrated the interaction of ORF21 with the small capsid protein ORF23 in a VZV specific, genome-wide Y2H screen [6] and Lee *et al.* demonstrated the interaction of the respective orthologues of ORF21 and the tegument protein ORF12 in a Y2H screen for

interactions between structural proteins in HSV-1 [21]. Both interactions have so far not been validated in any other mammalian cell-based PPI assay system. These PPIs could not be studied by BRET due to the very low expression levels of the corresponding donor and acceptor proteins (which were checked microscopically), which resulted in low BRET signals.

In conclusion, our LuMPIS method provides an economic and easy-to-handle mammalian cell-based assay system for the detection of PPIs. Due to the utilization of long, GC-rich tags (*i.e.* MBP and eGFP-Luc) and the inherent properties of MBP as fusion partner (solubility enhancement and promotion of proper folding), this system is able to reconstitute poor protein expression levels of relatively GC-low ORFs, a prerequisite of any PPI assay system. By using LuMPIS we could demonstrate three well-characterized PPI pairs of strong, intermediate and low affinities (Jun–Fos, MIF–RPS19 and BVRF2–BVRF2, respectively). Furthermore, we were able to demonstrate for the first time the interaction between the GC-low tegument proteins ORF21 and the ORF22 of VZV in a mammalian cell-based PPI assay system.

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