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SHORT COMMUNICATION

The Biarsenical Dye Lumio™ Exhibits a Reduced Ability to Specifically Detect Tetracysteine-Containing Proteins Within Live Cells

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Abstract Investigating the localisation of proteins within live cells via fluorescence microscopy typically involves the fusion of the protein of interest to a large fluorescent protein such as green fluorescent protein (GFP). Alternate fluorescent labelling technologies such as the fluorescent biarsenical dye molecules (e.g. FlAsH, ReAsH) are preferable to the use of large fusion proteins in many respects and allow greater flexibility in terms of the location of the labelling site. We assessed the ability of the FlAsH-derived biarsenical dye molecule Lumio™ to label a range of tetracysteine containing proteins within live cells and report that although in some circumstances Lumio is capable of positively detecting such proteins, the sensitivity and specificity of labelling is significantly reduced, making the Lumio-labelling system unsuitable for the detection of a wide range of protein within live cells.

Keyword Biarsenical Dye · In-cell labelling · Lumio · FlAsH · ReAsH

Introduction

The membrane permeable biarsenical dyes FlAsH and ReAsH fluoresce upon binding to tetracysteine motifs within proteins and represent an appealing method for visualising the localisation of proteins within live cells [1]. This system has obvious advantages over the use of large fluorescent fusion proteins due to the potential issue of structural or conformational obstruction, particularly when working with relatively small proteins. Additionally, the required tetracysteine binding motif (CCXXCC) is only 6 amino acids in length and can theoretically be incorporated into proteins with only minor disruption to coding sequence. The FlAsH and ReAsH labels are currently marketed by Invitrogen under the Lumio trademark.

To assess the potential of the Lumio-labelling technology for visualising a range of different proteins within cells, we selected five proteins which differ in both their cellular localisation and their expression levels. p64 (c-*myc*) is the protein provided with the Lumio-labelling system as a positive control and localises predominantly within the nucleus and nucleolus of cells due to its nuclear targeting and DNA binding properties [2]. Apoptin or viral protein 3 (VP3) is a 13.6 kDa protein of the chicken anaemia virus which is also predominantly nuclear within transfected cells due to the action of a bipartite nuclear localisation sequence [3], although it has been observed to form filamentous structures within the cytoplasm at early stages post-transfection [4]. Non-structural protein 5 (NS5) is an RNA polymerase from the Dengue-2 virus and the full length protein (aa 1–900, 104 kDa) localises to the nucleus of transfected cells [5] due to the action of dual nuclear localisation signals within the interdomain region of the protein [6]. In contrast, the N-terminal region of NS5 (aa 1–368, 41.5 kDa) lacks the crucial nucleophilic region (aa 369–405; Pryor et al., [7]) and is found primarily within the cytoplasm of transfected cells (Rawlinson et al., submitted for publication). The matrix (MA) protein of the human

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immunodeficiency virus-1 (HIV-1) is a 17-kDa structural protein which is contained within the cytoplasm of transfected cells [8] and typically exhibits lower than average levels of protein expression.

Although biarsenical reagents theoretically bind to any CCXXCC motif, the insertion of the helix-breaking amino acids proline and glycine within the tetracysteine sequence (CCPGCC) is thought to create a hairpin structure which increases the affinity and specificity of the biarsenical dyes. We were concerned that the insertion of this structure-altering motif into the protein coding region may affect the structural and thus functional properties of the proteins, so we chose to utilise the pnLumio vectors for Lumio-labelling experiments. These Gateway™ compatible vectors contain the tetracysteine recognition site as an N-terminal fusion to the protein of interest and have the additional advantage of containing a V5 site which allows detection of the protein via immunostaining.

Materials and methods

Plasmid construction

Transfection constructs were created using the Gateway™ cloning technology (Invitrogen). DNA fragments encoding MA residues 2–132, NS5 residues 1–900 (full length, FL) and 1–368, and VP3 residues 2–121 were generated via PCR incorporating attB1 and attB2 sites into the N- and C-termini, respectively. This enabled the initial incorporation of the fragment into the pDONR207 vector (Invitrogen) via a BP reaction, and subsequent recombination into the pnLumioDEST vector (Invitrogen) via an LR reaction according to the manufacturer's recommendations. The positive control vector pcDNA™6.2/nLumio™-GW/p64 (encoding the p64 protein) was also obtained from Invitrogen. PCR products were engineered to contain a stop codon after the final aa and where appropriate, the N-terminal methionine codon was omitted from constructs to prevent internal priming. The integrity of all constructs was determined by DNA sequencing.

Lumio-labelling and immunofluorescence

HeLa cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% foetal calf serum, L-glutamine, penicillin and streptomycin. Cells were transfected with 2 μg pnLumio constructs using Lipofectamine 2000™ (Invitrogen) according to manufacturer's instructions. Cells were either labelled with Lumio reagent and imaged live, or fixed and immunostained with antibodies to the V5 epitope, 16–24 h post-transfection. For Lumio-labelling, cells were stained with 0.6 μM of either Lumio-Green or Lumio-Red reagent in Hank's balanced salt solution (HBSS) containing 5 μM β-mercaptoethanol (βME) for 1 h at room temperature. Cells were washed once in HBSS and incubated in HBSS containing 100 μM βME for 30 min prior to imaging in phenol-free DMEM using confocal laser scanning microscopy (CLSM) and utilising a ×40 water-emersion lens. Cells transfected in parallel were fixed with 4% paraformaldehyde and immunofluorescence utilised to visualise proteins via their V5 tag using an anti-V5 antibody (Invitrogen, 1:500) and an Alexa-488 conjugated secondary antibody (Molecular Probes, 1:1,000). Images were acquired of unmounted immunostained cells using CLSM as described above.

Results and discussion

Initial attempts to use the Lumio dyes to label proteins within cells were performed in the absence of dithiol inhibitors, but the level of non-specific fluorescence was so significant that positive staining cells could not be discerned above background (data not shown). Indeed, it has been shown that fluorescent biarsenical dyes bind not only to engineered tetracysteine motifs but also to endogenous cysteine-rich proteins as well [9]. The addition of dithiol reagents such as 1,2-ethanedithiol (EDT) has been reported to reduce the level of non-specific staining within cells [1]. In our hands, the inclusion of 10 μM EDT to the Lumio-labelling reaction did not significantly improve the specificity of staining and the high level of background made it virtually impossible to detect positive staining cells (data not shown).

Whilst attempting to optimise these experiments, we were encouraged by the findings of Langhorst et al. [10] who report that the non-specific staining of the Lumio reagents can be significantly reduced by the inclusion of low concentrations of thiol-containing reagents in the labelling mix in combination with a high concentration thiol wash following labelling. Additionally, they report that βME can be used as the thiol-containing reagent in place of EDT, which is highly toxic and problematic to use experimentally. We examined whether these additional measures would allow for the sensitive yet specific detection of the abovementioned proteins within live cells.

Following labelling with Lumio-Green reagent, specific staining could be observed within a small number of cells expressing the p64, VP3 and, to a lesser extent, the NS5 (FL) Lumio-tagged proteins (Fig. 1a). Within cells transfected to express NS5(1–368) and MA, however, it was near impossible to detect a definitively positive fluorescent signal above the background level of fluorescence exhibited by all cells (Fig. 1a contains representative images of NS5 (1–368) and MA expressing cells which were deemed to

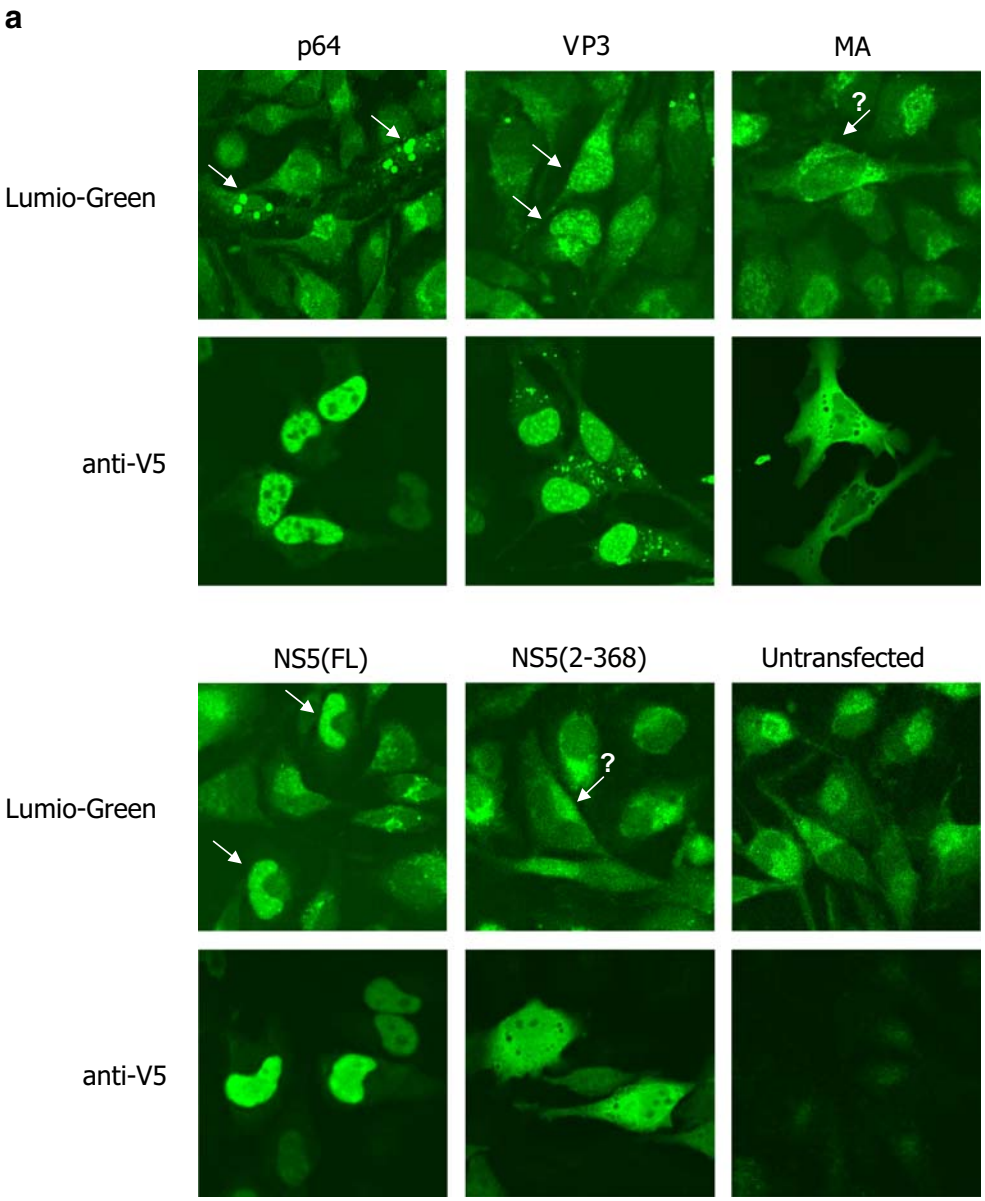

Fig. 1 **a** Cells transfected to express pnLumio constructs encoding p64, VP3, NS5(FL), NS5(1–368) or MA, together with non-transfected cells, were either labelled with Lumio-Green (*upper panels*) or fixed and immunostained with antibodies to V5 (*lower panels*) as described in the "Materials and methods" section. Example CLSM images are shown for each construct as indicated with *arrows* indicating cells containing regions of positive staining. *Question marks* indicate cells in which the presence of specific Lumio-labelled regions was unclear. **b** The percentage of cells containing regions of positive staining following either Lumio-Green or anti-V5 immunostaining was determined from CLSM images such as those in **a** by counting the number of cells containing a specific fluorescence signal clearly discernable above background. Graphs depict the average percentage of positive staining cells (±standard error, SEM) following Lumio-Green (*white bars*) or anti-V5 labelling (*grey bars*) calculated from 8 randomly selected fields of view (×40 magnification) containing at least 30 cells per field of view. Results presented are from one individual experiment representative of at least three separate experiments. **c** The signal to noise ratio of both Lumio-Green and anti-V5 labelled cells was determined using the equation $F_{\text{specific}}-F_{\text{bg}}/F_{\text{non-spec}}-F_{\text{bg}}$ where F_{specific} represents the fluorescence of specifically labelled regions, $F_{\text{non spec}}$ the fluorescence of cells exhibiting only non-specific fluorescence and F_{bg} the background fluorescence of the intercellular regions. The average signal to noise ratio (±SEM, $n\geq 20$ for Lumio-labelling and $n\geq 100$ for immunofluorescence) of cells labelled with Lumio-Green (*white bars*) or anti-V5 antibody (*grey bars*) is shown from one individual experiment representative of three separate experiments

possibly contain positively-stained cells). A significant degree of background fluorescence was observed within all cells, especially within the cytoplasm, which made the unambiguous identification of the cytoplasmically localised NS5(1–368) and MA proteins almost impossible. Indeed, regions of positive staining within the VP3 and NS5(FL)-expressing cells were identified more often than not by the fact that the pattern of fluorescence was different (i.e.

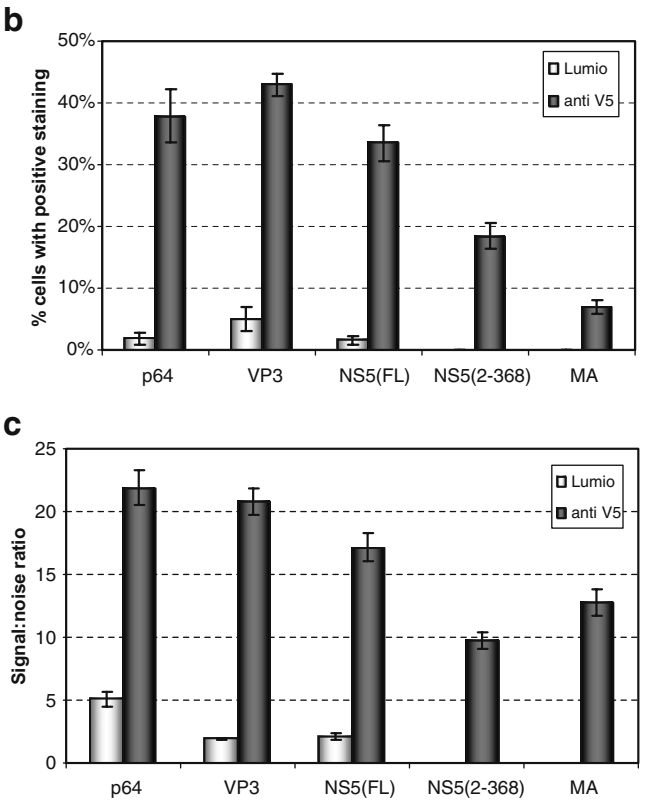

Fig. 1 (continued)

nuclear) rather than due to a striking difference in the intensity of fluorescence between signal and background. The concentration of p64 protein within the small nucleoli within unfixed cells made these regions easier to identify. The Lumio-Red reagent was found to be inferior to Lumio-Green due to a reduced number of positive staining cells and significantly higher background fluorescence (data not shown).

To confirm that these cells were indeed expressing significant levels of these proteins and to compare the efficiency of Lumio-labelling to more traditional techniques, cells which were transfected in parallel were fixed and proteins visualised via immunofluorescence using antibodies to the V5 epitope. These experiments revealed that the number of cells expressing Lumio-tagged protein was significantly higher than that indicated by Lumio-labelling. Figure 1b highlights the fact that the proportion of cells staining positive for p64, VP3 and NS5(FL) protein following immunofluorescent staining was approximately 38%, 43% and 34% respectively, whilst the Lumio-Green reagent was only able to detect protein within 1–5% of these cells (see Table 1). Of greatest concern was the observation that NS5(1–368) and MA protein was in fact present within transfected cells (in almost 20% of NS5(1–368) transfected cells) whereas the Lumio-Green reagent was unable to detect protein in either of these samples.

Table 1 Average percentage of cells (±SEM) exhibiting positive staining following Lumio-Green and anti-V5 labelling

Protein	Lumio-Green	Anti-V5
p64	1.87 % (±1.03)	37.88 % (±4.25)
VP3	5.10 % (±1.93)	42.92 % (±1.76)
NS5(FL)	1.53 % (±0.67)	33.51 % (±2.90)
NS5(2–368)	ND	18.44 % (±2.07)
MA	ND	7.02 % (±1.05)

Calculated from CLSM images as described in the legend to Fig. 1b.

Additionally, the signal to noise ratio (i.e. the difference in fluorescence between a 'positive' staining region and that of background fluorescence) appeared to be far inferior in the Lumio-Green labelled cells (Fig 1a). Quantitation of the fluorescent images confirmed that within cells labelled via immunofluorescence, the regions of 'positive' signal were on average 10 to 20 times higher than that of 'background' fluorescence (Fig. 1c and Table 2). In contrast, the signal to noise ratio of Lumio labelled cells was far less at only 2–5 times higher. Such a low ratio makes it difficult to distinguish regions of genuine fluorescent staining, particularly with proteins such as NS5(1–368) and MA which localise to the cytoplasm. We note that the p64 protein exhibits a more uniformly nuclear distribution within fixed cells as compared to the nucleolar localisation observed within live cells, and presume this is a consequence of fixation. These results suggest that the sensitivity of the Lumio-labelling system is approximately 10 times less than that of traditional labelling techniques such as immunofluorescence and that the signal to noise ratio of labelling is also significantly reduced, making it difficult to unambiguously distinguish positive staining regions. Additionally, these results highlight a major shortfall with the Lumio-labelling system in that it appears unsuitable for the detection of cytoplasmically localised proteins and/or those which exhibit low levels of protein expression.

Others have reported similar issues regarding the lack of specificity with in-cell labelling of tetracysteine containing proteins [9]. It has been reported that optimisation of the amino acids surrounding the labelling motif results in significant improvement to the of the biarsenical dyes [11].

Table 2 Average signal to noise ratio (±SEM) of Lumio-Green and anti-V5 labelling

Protein	Lumio-Green	Anti-V5
p64	5.07 (±0.60)	21.87 (±1.39)
VP3	1.92 (±0.12)	20.83 (±1.05)
NS5(FL)	2.09 (±0.25)	17.13 (±1.10)
NS5(2–368)	ND	9.73 (±0.70)
MA	ND	12.80 (±1.03)

Calculated from CLSM images as described in the legend to Fig. 1c.

Having two such binding motifs in tandem also appears advantageous. Whilst these modifications may improve the efficiency of labelling, the increased size of these optimised binding motifs (up to 24 aa) precludes their inclusion within proteins and limits their application to N- or C-terminal localisations. Additionally, the larger the fusion tag the more likely it is to obstruct the properties and functions of the protein of interest and the less suitable it becomes for use with small proteins and peptide sequences. The non-specific fluorescence of the biarsenical dyes within cells is significant and necessitates the need to include reagents which minimise this fluorescence. We found the presence of βME in the labelling mix in combination with a high concentration wash was useful in reducing this background fluorescence. However, this reagent is relatively toxic to cells and its use had an obvious impact on cell viability, as evident by the altered morphology of cells and the significant rate of apoptosis within Lumio-labelled cells. The biarsenical dyes themselves can have detrimental effects on cellular functioning and have been reported to accumulate within active mitochondria and cause mitochondrial swelling [10]. As mentioned above, the signal to noise ratio of the Lumio dyes is relatively low, and regions of positive staining were often distinguished primarily by their altered staining pattern. This is not ideal, particularly when the cellular localisation of the protein of interest is unknown or when localisation may be altered by varying cellular conditions. Further optimisation of the labelling motif and/or the biarsenical dyes themselves appears to be required if the specificity of labelling is to be improved.

Conclusion

Whilst fluorescent biarsenical dyes such as Lumio-Green can be used to detect the cellular localisation of certain highly expressed, nuclear localising proteins, they appear unable to convincingly detect proteins which are localised within the cytoplasm or which exhibit a low level of protein expression. Their greatly reduced sensitivity and specificity, combined with the cellular toxicity of the labelling conditions and even the dyes themselves, makes the Lumio-labelling reagents somewhat unsuitable for the detection of a broad range of proteins within live cells.

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