Video Article

Studying Protein Function and the Role of Altered Protein Expression by Antibody Interference and Three-dimensional Reconstructions

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Abstract

A strict management of protein expression is not only essential to every organism alive, but also an important strategy to investigate protein functions in cellular models. Therefore, recent research invented different tools to target protein expression in mammalian cell lines or even animal models, including RNA and antibody interference. While the first strategy has gathered much attention during the past two decades, peptides mediating a translocation of antibody cargos across cellular membranes and into cells, obtained much less interest. In this publication, we provide a detailed protocol how to utilize a peptide carrier named Chariot in human embryonic kidney cells as well as in primary hippocampal neurons to perform antibody interference experiments and further illustrate the application of three-dimensional reconstructions in analyzing protein function. Our findings suggest that Chariot is, probably due to its nuclear localization signal, particularly well-suited to target proteins residing in the soma and the nucleus. Remarkably, when applying Chariot to primary hippocampal cultures, the reagent turned out to be surprisingly well accepted by dissociated neurons.

Video Link

The video component of this article can be found at http://www.jove.com/video/53049/

Introduction

A tight control of protein expression is essential for every living organism to command its own development as well as to react to environmental signals. Hence, a multitude of mechanisms has been invented during evolution to precisely regulate the expression level of each protein encoded by the app. 20,000 genes existing in any eukaryotic cell at any given time of its life. Taking place at different stages of protein production, regulatory mechanisms range from the management of chromatin structure, transcription and RNA handling to the direction of posttranslational protein modifications, transport and degradation.

It is therefore not surprising that malfunctions in the underlying molecular machineries and altered protein expression levels have been associated with diverse diseases such as cancer or intellectual disabilities. Indeed, looking at the outstanding complexity of neuronal development and mammalian brain function, the sensitivity of these sophisticated systems to alterations in protein expression manifests itself in several well-known intellectual deficits including Alzheimer and Parkinson disease (AD and PD) as well as autism spectrum disorders (ASD) like the Fragile X Syndrome (FXS). The latter disease is characterized by an extensive misexpression of a variety of proteins, which is due to the loss of a single translation regulating protein, FMRP (Fragile X Mental Retardation Protein)[1-4]. Furthermore, chromosomal rearrangements affecting the Variable charge x linked protein A (VCXA), a protein, that manages mRNA stability and translation by modifying mRNA capping[5], have recently been associated with intellectual deficits, while point mutations were not identified in patients with cognitive disabilities as of now[6,7], suggesting that the observed mental impairments originate from altered VCXA expression and dysregulated expression of its target proteins. In line with these findings, a study investigating whether *de novo* copy number variations of genes are associated with ASD established that novel gene duplications and deletions are a significant risk factor for ASD[8], thus supporting the idea that elevated or diminished protein expression levels may cause intellectual deficits.

Remarkably, recent research further provided evidence that the expression level of a given protein is precisely adjusted to prevent its aggregation as a consequence of high protein amounts with almost no safety margins[9]. It has therefore been proposed that even small increments are sufficient to induce diseases such as AD and PD[9]. Although the variety of molecular machineries contributing to protein expression control suggests a complex regulation scheme in the light of these findings, a study investigating the expression level of over 5,000 mammalian genes[10] demonstrated that nature preferred a more parsimonious scheme: The cellular abundance of proteins was shown to be predominantly regulated at the level of translation[10], thus illustrating that the management of RNA availability mainly serves to fine-tune protein expression.

Studying the dose of proteins of interest (POIs) is therefore not only important to the understanding of the endogenous functions of a protein, but also to the investigation of many diseases and the development of therapies. Thus, last decades have seen the advance of several strategies using RNA interference to manipulate POI dosage. Though RNA interference is widely used to study protein function and is even being applied in clinical trials to treat cancer or ocular diseases as well as to pursue antiviral therapies in patients[11-13], some difficulties may arise which might render the strategy impossible. For example, the seed sequence, which drives the knockdown by homology is comparable short, hence promoting off-target effects. Since highly efficient sequences are rare and need to be found among thousands of options (reviewed in [14]), identifying the right sequence can be time-consuming and costly, but results may still be disappointing.

An alternative strategy is to directly target the POI by antibodies. Here, we illustrate the use of the protein carrier Chariot (manufactured by Active Motif) to reduce cellular protein availability, and the employment of three-dimensional reconstructions to study protein function following knock-down.

The active motif of Chariot, itself a 2.8 kDa peptide, is used to shuttle peptides, proteins and antibodies across membranes of mammalian cells[15]. The peptide associates with POIs by forming non-covalently coupled macromolecular complexes utilizing hydrophobic interactions, whereupon Chariot-POI complexes are internalized into cells in an endosome-independent manner. Importantly, Chariot was indicated to neither affect the intracellular localization of shuttled proteins, nor to exert cytotoxic effects or to affect the biological activity of its cargo[15].

Protocol

1. Stock Solutions

1. Resuspend the lyophilized active motif powder in sterile H_2O to a final concentration of 2 µg/µl. Tap carefully for mixing.
2. Prepare small aliquots (*e.g.,* 12 µl each, 2 µl are required per reaction) and store them at -20 °C.

2. Preparation of Cells

1. Seed mammalian cells such as HEK293 cells or primary neurons on a 24 well plate in 500 µl growth medium including antibiotics.
   NOTE: When using neurons, seed the cells at low density and use only the two middle rows of the 24 well plate. Each well will thus have an empty counterpart to harbor the medium during incubation (step 4.2). When handling neurons, always make sure that the cells are not kept outside the incubator for more than a few minutes.
2. Culture the cells at appropriate conditions (humidified, 5% CO_2 and 37 °C) until the cells are app. 50% confluent.
   NOTE: When using neurons, culture the cells until the desired developmental stage is reached and change app. 25-50% of the medium twice a week. Medium: Neurobasal medium (containing 1x B27, 5 mM L-glutamine and 1x Penicillin and Streptomycin; compare with ref 16).

3. Chariot Complex Formation

1. Per reaction, dilute 0.1-2 µg of the POI or the corresponding antibody as well as the control protein, or control antibody, respectively, in 50 µl of PBS.
   NOTE: The technique also works with macromolecular complexes consisting of pre-bound primary and secondary antibodies (cp. 'Representative Results', **Figure 1**). Mix and spin.
2. For each sample, dilute 2 µl Chariot stock solution in 50 µl of PBS using separate tubes in order to avoid self-association of Chariot. Mix and spin.
3. Transfer the diluted POI or antibody (step 3.1) to the Chariot dilution by pipetting. Mix and spin.
4. Incubate the mixture for 30 min at room temperature (Chariot-POI complexes will form).

4. Transfection of Cells

1. Dilute the Chariot complexes (step 3.4) in 100 µl pre-warmed growth medium (37 °C, no additives). Remove the medium and wash the cells once using pre-warmed 1x PBS.
   NOTE: When using neurons, do not discard the medium, it will be reused. Keep it at 37 °C. For washing, use PBS containing 0.5 mM $MgCl_2$ and $CaCl_2$.
   Suggestion: keep the medium in empty wells while incubating the plate (step 4.5).
2. Apply the Chariot complex solution (step 4.1) to the cells and rock the cells gently to ensure an even distribution of the solution. Grow the cells under standard conditions (step 2.2) for 1 hr. Add serum to a final concentration of 10%. When using neurons, add the medium from step 4.1. Grow the cells for 1 to 2 more hours.
   NOTE: The optimal incubation time depends on the size and properties of the cargo and may need to be adjusted empirically. The following suggestions may serve as a guideline: For peptides, 1 hr is usually sufficient, for proteins 1-2 hr are recommended, for antibodies 2 hr, and for the transfection of neurons with antibodies 4 hr.
3. Process cells for analysis as usual. Please note: the technique is compatible with experiments using fixation protocols as well as live imaging.

5. Imaging

1. Using a laser scanning microscope, take z-stacks of cells and/or cell compartments of interest using approximately 0.25-0.5 µm distance. The precise layer distance depends on the size of the structure to be reconstructed and needs to be determined individually.

6. Reconstruction

1. In the following steps, use the Esc bottom to switch between Select and Navigate, Cntr to select multiple objects by clicking on them and shift key to cut objects (cp. step 6.10).
2. Open the lsm-file in Imaris.
3. Using the Display Adjustment for each channel, contrast the picture until the brightest structures reach saturation. Please note that this adjustment will not influence the surface construction, it only serves to assist the experimenter in thresholding the image.
4. Click on the Add new Surfaces icon. A wizard will open.
5. Select: Segment only a Region of Interest. Proceed to the next step.
6. Adjust the margins of the selection box to fit your object of interest. Please bear in mind that the image has 3 dimensions and that the selection needs to be adjusted at the z plane as well. If the rectangular shape of the selection box should not match the object of interest properly, turn the object and/or enlarge the box until all required structures are embedded. Undesired objects may be removed later on (cp. step 6.10). Proceed.
7. Select the appropriate channel. The Surface Area Detail Level or sphere diameter should be set to match the structures being reconstructed. Depending on the signal characteristics, either Absolute Intensity or Local Contrast can be used. Generally, Local Contrast works better for diffuse signals. In any case, the settings need to be adjusted separately for each signal or structure of interest, respectively.
8. Using the Thresholding tool, reconstruct the structure of interest.
9. Complete the reconstruction by clicking on the green arrow bottom.
10. Adjust the object further by using the pencil tool to cut and remove surfaces as required. It is only possible to cut in north-south direction, therefore, tilt the image in order to cut the desired object.
11. Obtain measurements of volume, area and intensities for each surface by selecting Statistics, Detailed Statistics, and Average Values.
12. (Optional step) To observe color-coded statistics (cp. **Figure 2E** and **F**), select the color tab of the corresponding surface and mark 'Statistic Coded' instead of 'Base'. Several options will be displayed.

Representative Results

In the following paragraphs, exemplary results illustrating a functional knockdown of a POI (Simiate, for further details please see [16,17]) using Chariot reagent and antibody interference are presented. The findings demonstrate that diminished expression of Simiate impairs transcriptional activity, and, in a dosage dependent manner, induces apoptosis, culminating in mortality rates of over 99% if higher antibody amounts (> 1 µg antibody) are applied (these discoveries were published previously in [16]). Here, we now present the techniques and protocols employed in detail and furthermore demonstrate how Chariot reagent can be used to transfect dissociated hippocampal neurons in primary cultures.

In order to investigate whether Chariot peptides allow for an efficient shuttling of antibodies and, in particular, if these antibodies remain functional during the procedure, we expressed FLAG-Simiate for 24 hours in HEK293 cells, and subsequently transfected the cells utilizing Chariot coupled rbαSimiate-gtαrbAlexa568 macromolecules (**Figure 1**). The FLAG-Simiate construct is well expressed by HEK293 cells and localizes to the somata as well as the nuclei of these cells (**Figure 1A**). Here, FLAG-Simiate clusters significantly (**Figure 1B**), thereby mirroring the speckled distribution of endogenous Simiate in the nucleus.

The antibody complexes shuttled across the cellular membrane appear in assemblages (**Figure 1A**, in red, red arrows), but the released antibodies detect FLAG-Simiate specifically as shown by a profound colocalization of FLAG-Simiate and the rbαSimiate-gtαrbAlexa568 macromolecules (**Figure 1B**, in yellow, yellow arrow). As of note, the experiment not only demonstrates that the antibodies remain functional during a Chariot-mediated membrane passage, but that they are also able to enter the nucleus. Since their size excludes antibodies from embarking the nucleus via diffusion, it is likely that the nuclear localization signal present in Chariot itself facilitates the translocation of rbαSimiate-gtαrbAlexa568 macromolecules into the nucleus. Indeed, antibody assemblages are also observed at or in, respectively, the nuclear compartment (**Figure 1B**, red arrows).

Using rbαSimiate-Chariot complexes to functionally deplete endogenous Simiate in HEK293 cells (**Figure 2**), we found that we were able to target up to 80% of the rbαSimiate binding sites, using 2.0 µg of antibodies[16]. Interestingly, while shuttling gtαrbAlexa568 antibodies alone into HEK 293 cells had no obvious effect on the appearance of nuclear speckles (**Figure 2A-C**), loss of functional Simiate radiused nuclear speckles (**Figure 2D-F**, cp. [16]) as illustrated by three-dimensional reconstructions (**Figure 2B,C** vs. **E,F**) and, in a dose dependent manner, induced apoptosis (**Figure 3**, cp. [16]).

Given that neuronal cells are very sensitive to toxic effects, we also applied the technique in primary hippocampal cultures (**Figure 4**). Using TUNEL assay and MAP2 staining to analyze cell integrity, we found no effect on the viability of neurons when comparing mock or untreated and Chariot treated cells (app. 20-25% TUNEL positive cells after 1+4 hours of treatment, cp. 4.4-4.6). Hence, we tested Chariot mediated antibody shuttling in neuronal cultures. Following the same application scheme, gtαgpAlexa568 antibody assemblages were seen to varying extends in about 83% of the cells, where they occurred in somata as well as nuclei (**Figure 5A**). These observations are further supported by a three-dimensional analysis (**Figure 5B**) and closely reassemble the picture seen in HEK293 cells, together thus illustrating that the Chariot technique can be used successfully to transfect dissociated primary hippocampal neurons as well.

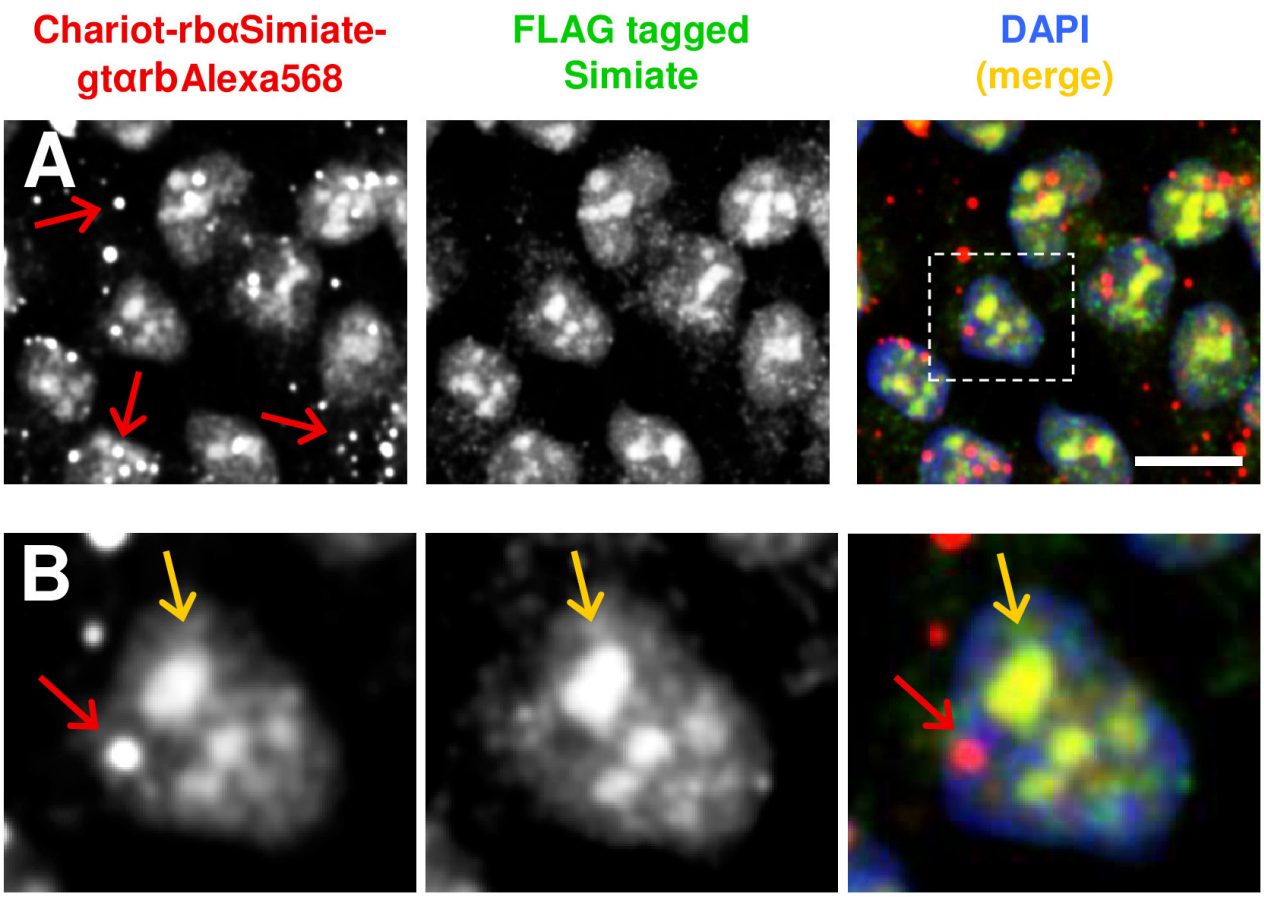

Figure 1. FLAG-Simiate is detected by Simiate specific antibodies shuttled into HEK293 cells using Chariot reagent. For antibody interference experiments, rbSimiate-gtrbAlexa568 antibodies were preassembled prior to Chariot-antibody complex formation. A) HEK293 cells expressing FLAG-tagged Simiate. Simiate antibody assemblages enter the soma as well as the nucleus following application, where they colocalize specifically with FLAG-Simiate. Round dots: Chariot-rbSimiate-gtrbAlexa568 assemblages (red arrows). B) A high power magnification of the boxed region in (A) illustrating the colocalization of FLAG-Simiate and Chariot mediated Simiate signal (yellow arrows). Please note the presence of a Chariot antibody assemblage in the nucleus (red arrow). Scale bar: 10 µm.

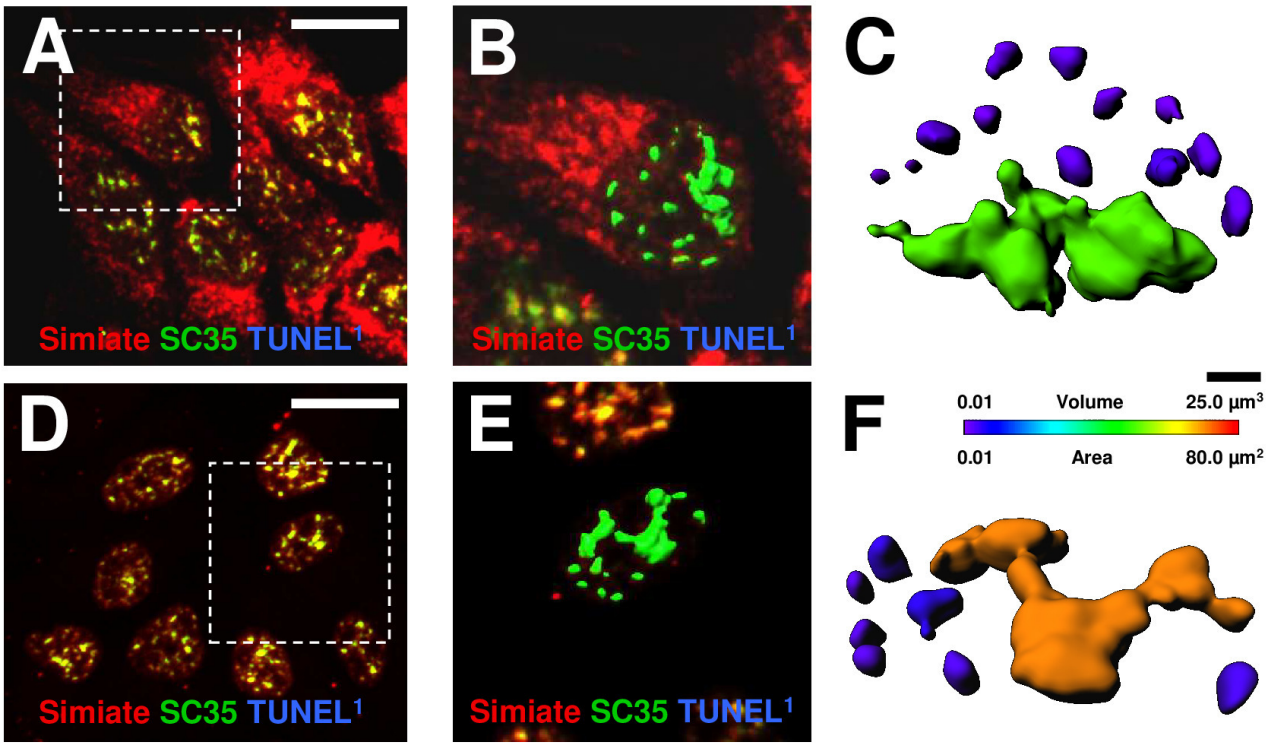

Figure 2. Simiate-specific antibodies shuttled into HEK293 cells interfere with cellular functions of endogenous Simiate. A,B) HEK293 cells treated with Chariot-gtrbAlexa568 (1 µg) serve as control. In red, endogenous Simiate is shown in false color, while Chariot-gtrbAlexa568 assemblages are not displayed. Nuclear speckles were labelled using the marker protein SC35 (in green), whereas apoptotic cells were identified by TUNEL assay (in blue). B) Reconstruction of nuclear speckles from the boxed region in A. C) Reconstruction of the same region illustrating speckle volumes and surface areas in a color coded manner. D,E) HEK293 cells following Chariot-rbSimiate treatment (0.5 µg). Endogenous Simiate as well as Chariot-rbSimiate-gtrbAlexa568 assemblages are shown in red, while nuclear speckles and apoptotic cells are displayed according to A and B. E) Nuclear speckles reconstructed from the boxed region in D. F) The same region is shown as outlined for C. Please note the radiused appearance and aggregation of speckles (all reconstructions: Imaris software). Scale bar in C,F: 2 µm. All other scale bars: 10 µm. 1) For three-dimensional reconstruction, only non-apoptotic cells were used.

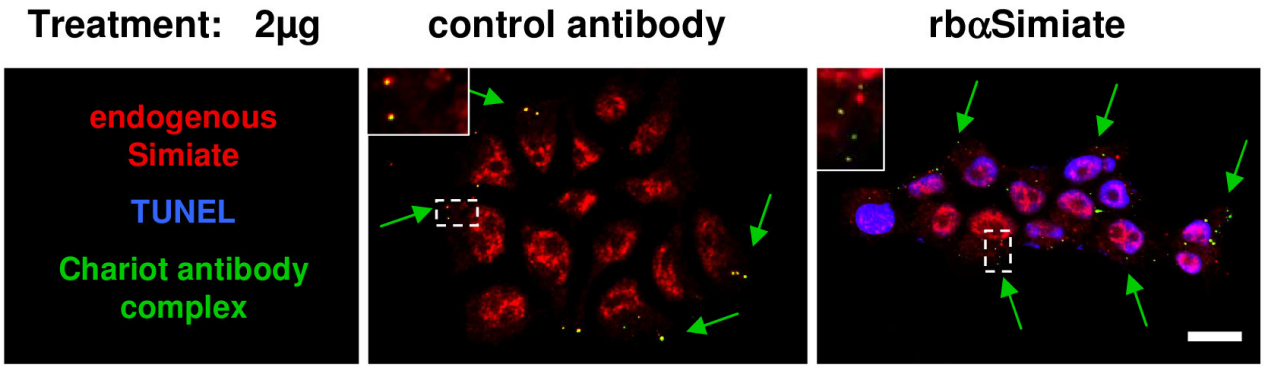

Figure 3. High doses of Chariot-rbSimiate-gtrbAlexa568 induce massive cell death. HEK293 cells were treated with 2 µg of Chariot-gtrbAlexa568 or Chariot-rbSimiate-gtrbAlexa568, respectively (both complexes shown in green and indicated by arrows as well as magnifications). TUNEL labelling (in blue) was applied to identify apoptotic cells. Scale bar: 10 µm.

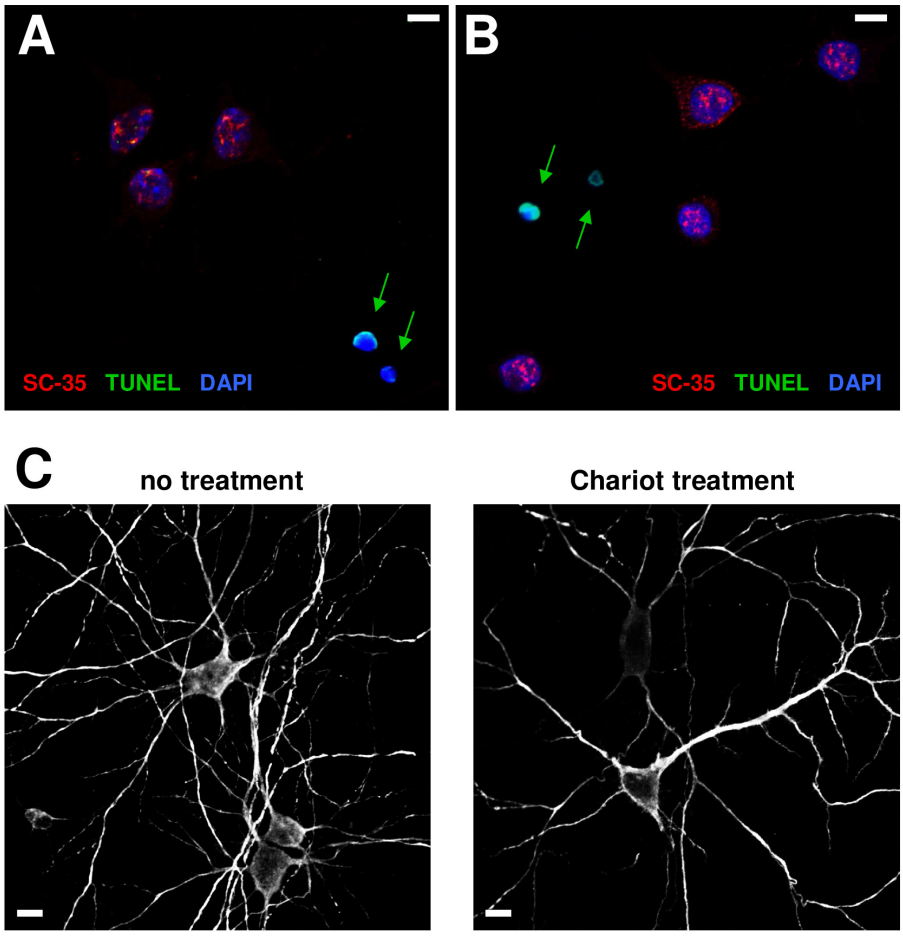

Figure 4. Chariot is not toxic to primary hippocampal neurons. A-B) DIV 7 neurons were either mock treated (medium, A) or subjected to Chariot reagent (B) as outlined in the protocol for antibody shuttling (1+4 hours, cp. 4.4-4.6). While SC-35, a marker protein for nuclear speckles, served to label the transcription and splicing machinery, TUNEL staining was applied to identify apoptotic cells (green arrows). C) Untreated and Chariot treated DIV 16/17 neurons. MAP2 staining was applied to visualize the dendritic trees. Scale bars: 10 µm.

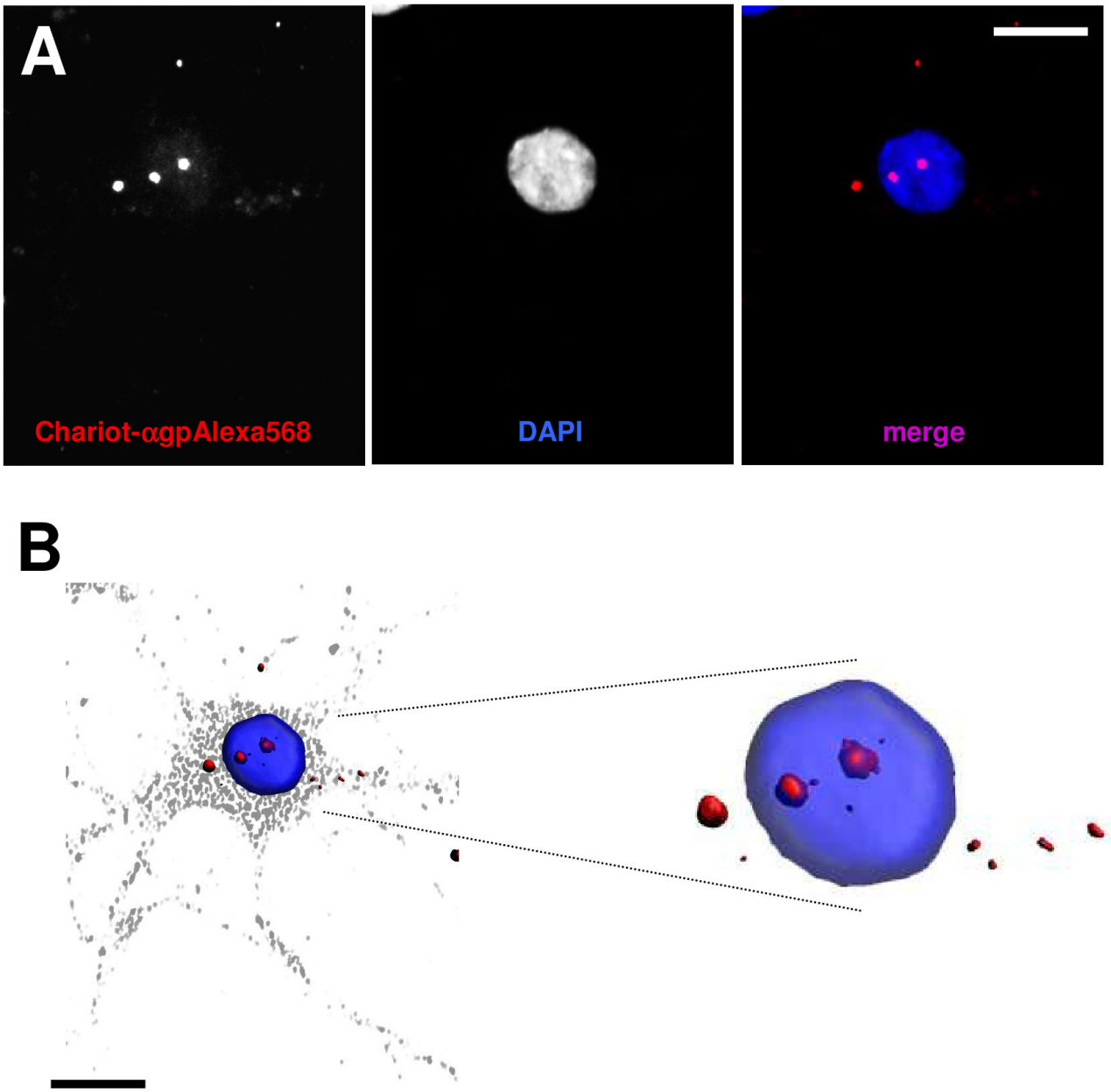

Figure 5. Chariot mediated transfection is compatible with neuronal cultures. A) A representative primary hippocampal neuron from div 5. The picture shows z-projections of stacks taken using a laser scanning microscope (Laser Scanning Microscope 710, Zeiss). Scale bar: 10 µm. Please note the occurrence of Chariot-gtgpAlexa568 complexes (red) in the nucleus and the soma of the cell. B) Three-dimensional reconstruction (Imaris software) of the nucleus as well as gtgpAlexa568 assemblages shown in A. Soma and dendrites of the neuron are indicated by grey dots, which are distilled from labelling of endogenous Simiate. Scale bar: 10 µm. Antibody amount transfected: 0.25 µg.

Discussion

Here, we present a protocol to study the significance of protein expression levels in controlling cellular functions in a dose driven manner. The protocol described allows for a fine-tuned manipulation of protein expression in various mammalian cell types, including hippocampal neurons, hence facilitating detailed studies of protein function on the cellular level.

Although RNA interference represents a well known strategy to down-regulate POIs, it has its disadvantages (cp. [14]). Not only the identification of a highly specific and efficient sequence can be costly and time-consuming, but also transcript biogenesis inside the cell may cause unpredictable outcomes since the accumulation of toxic RNA constructs, an overload of the nuclear export machinery as well as a saturation of the endogenous RNAi processing systems may result in off-target effects or inefficiency. Also, high amounts of exogenous RNAi vectors may induce unspecific effects as well, and perturb cell homeostasis.

Antibodies, on the other hand, require no further processing by endogenous machineries once they have entered the cell, and are effective immediately, thus supporting not only time-saving experimentation, but also investigations of immediate cellular processes. Though they may have unspecific off-target effects, too, the increasing number of well-characterized and highly specific antibodies available makes this alternative worth a thought. Since the delivery of macromolecular complexes into cells represents a major obstacle, different transfection techniques have been developed within the recent years, including the peptides R8 and azoR8[18] as well as microsphere[19] mediated shuttling. While R8 and azoR8 were indicated to behave similarly to Chariot[18], microspheres were found to require 24 hours for uptake[19], which is considerably longer than Chariot-mediated membrane passages and may cause difficulties when studying faster and dose dependent cellular responses.

Remarkably, the Chariot technique turned out to be applicable to primary hippocampal cultures, which are very sensitive to treatments and difficult to transfect. So far, Chariot carriers have been used to analyze protein function in primary osteoblast cultures[20] as well as in the neuroblastoma-glioma cell line NG108-15[21], chick dorsal root ganglia cultures[21], or in PC12 cells[22], which originate from the neural crest, but no reports of any application in cell cultures derived from the central nervous system are available yet. Since traditional transfection reagents such as Lipofectamine are inefficient in these cells and viral transfections intricate, Chariot might represent an interesting alternative.

Notably, Chariot itself was observed to localize to the nucleus[15], probably due to its nuclear localization signal PKKKRKV (cp. [23]). In line with these observations, we found that Chariot mediated transfection of rbαSimiate-gtαrbAlexa568 macromolecules resulted in a clear labeling of nuclear compartments such as nuclear speckles. Indeed, control antibodies such as gtαgpAlexa568 were also detected in the nucleus of both, HEK293 and primary hippocampal cells. Since antibodies, in particular in assemblages, are far too big to embark the nucleus by diffusion and since Simiate itself does not contain any known nuclear localization signal, but rather enters the nucleus via diffusion, it is likely that the Chariot signal is involved in the nuclear localization of antibodies. Alternatively, a transport in larger protein assemblages could also be possible, though this would not explain the presence of gtαgpAlexa568 in the nucleus of cultured hippocampal neurons from rats, as there is no target for this antibody in these cells. Further, since Chariot was found to not interfere with the natural localization of shuttled peptides and proteins[15], these observations suggest that antibodies behave differently.

Indeed, when shuttling antibody complexes such as Chariot-rbαSimiate-gtαrbAlexa568 (**Figure 1**) or Chariot-gtαgpAlexa568 (**Figure 5**) across cellular membranes, they appear in clusters, which only dissolve, when appropriate target proteins such as endogenous Simiate or FLAG-Simiate are present (only in **Figure 1**). Using Chariot antibody complexes in osteoblasts, Selim and colleges observed the same effect[20]. Given that Chariot was demonstrated not to interfere with the localization of peptides and proteins[15], these observations indicate that Chariot assembled antibodies disassemble only if there is a molecular mechanism such as an antibody-target interaction, that overcomes the hydrophobic interactions between Chariot and its antibody cargo and, thus, encourages disassembly. Because antibodies are heavier than peptides or most other proteins and have, due to their shape, a large surface, they may associate with higher numbers of Chariot molecules and/or interact more firm with Chariot peptides, which would thus promote a more Chariot-driven behavior of antibodies. A lack of a disassembly mechanism would therefore result in a clustered appearance and a nuclear localization of the antibody cargo (**Figure 5**).

Taken together, the data shows that Chariot is particularly well suited to address the function of proteins residing in the nucleus and that it is applicable in a variety of mammalian cell types including primary hippocampal neurons.

Disclosures

The authors have nothing to disclose.

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