Supporting Online Material

The spontaneous cellular uptake of exogenous messenger RNA in vivo is nucleic acid-specific, saturable and ion-dependent.

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Detailed material and methods

Nucleic acid production

mRNA

We produced capped mRNA *in vitro* by run-off transcription with T7 RNA polymerase (T7-Opti mRNA kits, CureVac, Tübingen, Germany). The coding sequence of these mRNA (either *Eschericha coli* ß-galactosidase [lacZ] cloned from Acc. U02445 or *Photinus pyralis* luciferase [luc] cloned from Acc. U47295) was flanked at the 3' end by an alpha-globin untranslated region and a poly A (n=70) tail. For experiments in mice we precipitated mRNA with lithium chloride. Then we resuspended the mRNA in water and determined the yield by spectroscopy at 260 nm. Finally, we further purified the mRNA by extraction with phenol/chloroform/isoamylalcohol and precipitation with ammonium acetate. The mRNA was then resuspended in sterile water. For experiments in humans we produced GMP-grade mRNA in the dedicated clean room facility at CureVac in agreement with the specific quality management system.

Plasmid DNA

We prepared endotoxin free pCMV-luc DNA with the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). The pDNA was ammonium acetated precipitated and finally sterile resuspended in water. We engineered the pCMV-luc plasmid by inserting a Xba I (blunted with Klenow fragment) Hind III fragment of pGL3 (Acc. U47295) into the Nsi I (blunted with Klenow fragment) Hind III digested plasmid backbone of pCMV-HB-S (Acc. A44171).

Quality control

Before utilization for the experiments presented in the manuscript, for each batch of nucleic acid we determined the concentration by spectroscopy and assessed the integrity by formaldehyde agarose gel electrophoresis (mRNA) or restriction digestion and TBE agarose (plasmid DNA) gel electrophoresis (**Fig. S 1**). In addition, we validated the functionality of each batch of nucleic acid by electroporation of the nucleic acids in BHK21 cells. Therefore, we electroporated 1-3 Mio cells in 200 µl PBS with 10 µg nucleic acid at 300 V and 150 µF in 0,4 cm cuvettes. We analyzed the transfected cells 8-24 h post electroporation using an appropriate detection method (X-gal staining or luminescence detection) for protein expression (**Fig. S 1**).

Injection buffer

We used the following buffers: 2x phosphate buffered saline (PBS: 274 mM sodium chloride, 5,4 mM potassium chloride, 20 mM disodium hydrogen phosphate, 4 mM potassium dihydrogen phosphate, pH 7,3 at 20,8°C), 2x HEPES buffered saline (HBS: 300 mM sodium chloride, 20 mM HEPES, pH 7,4 at 20,8°C) and 1x Ringer-lactate (102,7 mM sodium chloride, 5,4 mM potassium chloride, 1,8 mM calcium chloride, 28 mM sodium lactate).

For 2x PBS and 2x HBS we dissolved all components in water and adjusted the pH. Then we added diethyl pyrocarbonate (DEPC, Sigma, Schnelldorf, Germany) to a concentration of

0,1% (v/v) and incubated the buffers for >1 hour at 37°C. Finally, the buffers were autoclaved.

The 1x Ringer-lactate was either purchased from Fresenius-Kabi (Bad Homburg, Germany) or self made from 20x stock solutions of the four different salts (sodium chloride, potassium chloride, calcium chloride and sodium lactate). In some experiments we omitted one of the four different salts without compensating the lowered osmolarity. With exception of the sodium lactate racemat solution (Fluka, Schnelldorf, Germany) each of these components was treated with DEPC and autoclaved as described for 2x PBS and 2x HBS.

We tested all buffers and buffer components for ribonuclease activity by incubating 1 µg of mRNA in 1x buffer for >2 hours at 37°C. When we analyzed the mRNA by formaldehyde agarose gel electrophoresis we observed no degradation.

Mice

We performed all animal experiments in accordance with institutional and national guidelines. We purchased female BALB/c mice of 8-15 weeks in age from Charles River (Sulzfeld, Germany).

Before the intradermal injection we anaesthetized the mice and we wetted the mouse ear pinna with isopropanol. To analyze for mRNA uptake and expression, we sacrificed the mice after defined time, shaved the ears with a razor blade to remove disturbing hairs and further proceeded with them as outlined in the following paragraphs.

Injection of mRNA

For HBS and PBS we diluted the mRNA in 1x concentrated buffer. For Ringer-lactate and the single depletion variants of Ringer-lactate (without one of the four salts), we diluted the mRNA in 0,8x concentrated buffer. If not otherwise stated we used 20 µg mRNA in 100 µl injection solution per mouse ear. To remove secondary structures in the mRNA, we heated

injection solutions for 5 min to 80°C. Next, we chilled the solutions on ice for another 5 min. Finally, we dispersed the injection solution into Sub-Q (Becton Dickinson, Heidelberg, Germany) syringes. We used separate syringes for each injection.

To test the mechanisms of uptake of the mRNA, we treated in selected experiments the mice 3 hours before the mRNA injection with 300 µg amiloride (Sigma), 6 µg chloropromazine (Sigma), 80 µg cytochalasin B (Sigma) or 100 µg nystatin (Sigma) applied intra-peritonealy in a total volume of 200 µl. Drug doses where selected according to the literature [1-4].

Luciferase activity detection *ex vivo*

To detect luciferase expression *ex vivo* we had to prepare tissue lysates. Therefore we minced the tissue under liquid nitrogen with mortar and pestle and homogenized the resulting "crumbs" with 800 µl lysis buffer (25 mM Tris HCl, 2 mM EDTA, 10% (w/v) Glycerol, 1% (w/v) Triton X-100, plus freshly added 2 mM DTT and 1 mM PMSF). We recovered the supernatant of the homogenate after centrifugation (10 min, 13.000 rpm, 4°C) in a minifuge. We stored 110 µl aliquots of this lysate at -80°C.

To measure luciferase activity we thawed an aliquot on ice and measured the light emission of 50 µl lysate for 15 s with a luminometer (LB 9507, Berthold, Bad Wildbad, Germany). The luminometer added automatically 300 µl buffer A (25 mM glycyl-glycine, 15 mM magnesium sulfate, 5 mM freshly added ATP, pH 7,8) and 100 µl buffer B (250 µM luciferin in water) to the lysate prior to the measurement.

For standardization, we used serial dilutions of recombinant luciferase protein (QuantiLum®, Promega, Madison, USA) in all measurements. According to this standard, we calculated the amount of luciferase molecules per single measurement. For each lysate we measured the luciferase activity at two different days in duplicates and calculated the mean amount of luciferase activity. The coefficient of variation (n=4) for the amount of luciferase molecules was below 10% for all lysates with luciferase activity above the detection limit. This detection

limit (indicated by thick line with number in all diagramms) was calculated from the mean of measurements with lysis buffer only plus 3 times the standard deviation of these values (n=80).

In vivo bioluminescence detection

To detect luciferase expression in living animals, we anaesthetized mice with 1.5% isoflurane at defined time points post nucleic acid injection. Subsequently we injected them intra-peritoneally with 200 µl of 20 mg/ml luciferin (Synchem, Kassel, Germany) in PBS (filtrated sterile). 5 min after luciferin injection we started to collect the bioluminescence light emission of the mice for 20 min. Therefore we placed the mice on a temperate plate (37°C) in a dark box equipped with an Orca II (Hamamatsu, Photonics, Germany) camera system. We present the light emission in pseudocolours overlaying a greyscale photographic picture of the mice at normal light. Analysis was performed by the SimplePCI (Hamamatsu) software.

ß-galactosidase activity & histology

We dissected shaved mouse ears, embedded them in Tissue-Tek® O.C.T™ compound (Sakura, Zoeterwoude, Netherlands) medium and stored them at -80°C. Out of these blocks, we collected sequentially 20 µm transfersal cryosections in 5 sets (**Fig. S**) on SuperFrost® Plus slides (Langenbrinck, Emmendingen, Germany) i.e. that the vertical distance between two sections of one set is approximately 100 µm. Next we air dried the sections and stored them at -20°C until we stained them. For a first screening in which area the transferred mRNA (coding for *Eschericha coli* ß-galactosidase) is taken up and translated, we stained one set of sections with X-gal. Therefore, we adapted the slides to room temperature and circumscribed them with an ImmEdge™ Pen (Vector, Burlingame, USA). Next, we fixed the sections for 15 min with 2% Formalin in PBS. Then we washed the slides 3 times 2 min with PBS and stained them overnight at 37°C in a humidified chamber with X-gal staining solution (1 mg/ml freshly added X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide,

1 mM magnesium chloride, 15 mM sodium chloride, 60 mM disodium hydrogen phosphate, 40 mM sodium dihydrogen phosphate). We completed the staining by washing the slides 3 times 2 min and mounted them with Hydro-Matrix® (Micro-Tech-Lab, Graz, Austria, twofold diluted in water) medium.

To get information of the tissue morphology we combined for another set of sections the X-gal staining with a hematoxylin eosin (HE) staining. Therefore, we washed the sections after the X-gal staining 3 times 2 min in PBS and additional 5 min in bidistilled water, before we performed a 2 sec staining with Mayers Hämalaun (Merck, Darmstadt, Germany). We developed the staining for 10 min under flowing tap water before we counterstained for 10 min with 0,1% Eosin Y (Sigma, Schnelldorf, Germany) in water. We stopped the staining by washing briefly in bidistilled water and started next the dehydration with increasing alcohol concentration (2 min 80% ethanol, 2 min 95% ethanol, 2 min 100% ethanol, 5 min 100% xylol). Finally, we mounted dried sections with Roti®-Histokitt (Roth, Karlsruhe, Germany) medium.

To address the question whether antigen presenting cells (APC) can take up the injected mRNA we performed a double staining for MHC class II molecules (expressed by APC) and mRNA transfer (in terms of ß-galactosidase expression). We used both, an immunohistochemical and an immunofluorescent detection of the MHC class II molecules. For both protocols we washed the sections 3 times for 2 min with PBS between all steps. For the immunohistochemical approach, we fixed the sections with 1% (w/v) formalin (Fluka) in PBS. Next, we removed lipids by 30 sec incubation in pure acetone. Immediately after, we blocked for 30 min at RT with 4% goat serum (Vector) and 50 µg/ml avidin D (Vector) in PBS. We blocked the remaining biotin binding sites with 50 µg/ml biotin (AppliChem, Darmstadt, Germany) and stained at the same time for MHC class II molecules with the monoclonal antibody 2G9 (Becton Dickinson) or the appropriate isotype control (rat IgG 2a,

R35-95, Becton Dickinson) diluted each to 1 µg/ml (all in PBS). We next incubated the sections for 30 min at RT with biotinylated goat anti-rat IgG (3 µg/ml, Vector) and 2% mouse serum (CCPro, Neustadt, Germany) in PBS. Subsequently, we added ABC complex (1:100 of reagent A and B in PBS, Vector) for 30 min at RT. The MHC class II staining was completed by detection with freshly prepared and 0,45 µm filtrated 3-Amino-9-Ethylcarbazole (AEC, Sigma) substrate solution (0,5 mg/ml AEC, 0,015% hydrogen peroxide, 50 mM sodium acetate pH 5,5). We stopped the substrate reaction by washing 2 times 5 min with water and 3 times 5 min with PBS. Subsequently, we performed an X-gal staining as described above.

For the immunofluorescence detection, we used a similar staining protocol. Subsequently to the acetone step, we blocked the sections for 40 min at RT in blocking buffer (1% bovine serum albumin in PBS). We incubated the sections next with primary antibodies (2G9 or isotype control) diluted to 1 µg/ml in blocking buffer for 40 min. Next, we incubated for 40 min at RT with Alexa Fluor 546 goat anti-rat IgG (1:400, Molecular Probes, Leiden, Netherlands) in blocking buffer. Finally, we performed a Magenta-gal staining. Therefore we replaced X-gal in the staining solution with 0,1 mg/ml Magenta-gal (Peqlab, Erlangen, Germany).

We analyzed sections with a Zeiss (Oberkochen, Germany) Axioplan 2 microscope equipped with an Axiocam HRc camera and the Axiovision 4.0 software. Colors and contrast in photographic pictures were adjusted in a linear way.

Human experiments

For these experiment informed consent was obtained from the injected healthy male volunteer who is competent to foresee, understand and report the possible side effects that can be associated with such tests.

We shaved, disinfected and treated the injection sites (lower leg) with RNaseZap (Ambion, Austin, USA) solution. Next, we injected 120 µg of mRNA in 0,8X Ringer-lactate in a total

volume of 150 µl. 15 h post injection we took 2 mm diameter punch biopsies under local anesthesia. We snap froze the biopsies in liquid nitrogen and prepared them as described. Minces biopsies were resuspended in 600 µl lysis buffer.

Statistics

We compared the median values of two different groups with the non-parametric Mann-Whitney rank sum test. A p-value of <0,05 was considered as significant difference and is shown in the diagrams.

Supporting Results

To identify the cell type(s) that is (are) responsible for mRNA uptake and expression *in vivo* we developed a histological method that allows the detection of ß-galactosidase expression in combination with a cell type specific staining.

mRNA transfer can not be detected with fluorescent probes

The first and ideal option for this purpose would be the use of two different fluorescent probes, one for the ß-galactosidase activity (encoded by the exogenous injected mRNA) and the second for the cell type specific marker. Unfortunately we did not succeed in establishing a fluorescent staining method for the ß-galactosidase activity. We tested first the use of fluorescent substrates. Neither FDG [5;6] nor the X-Fluor [7] method allowed the detection of ß-galactosidase in sections. Next we tested the use of eGFP encoding mRNA. Again it was not possible to detect a specific fluorescent staining. Finally we performed immunofluorescent stainings using different antibodies directed either against *Eschericha coli* ß-galactosidase (3 different atibodies tested) or against eGFP (1 antibody tested). We detected these primary antibodies with secondary or tertiary reagents labelled with Alexa Fluor 546. While some of these antibodies were suitable for staining of ß-galactosidase- or eGFP-coding mRNA electroporated BHK21 cells, they all failed in detecting the mRNA transfer (protein expression) *in vivo*. In conclusion all the fluorescent methods we tested may work fine in background free / signal high systems (e.g. staining of transfected cells) but for the detection of mRNA transfer *in vivo* they are either not sensitive enough or the high background (due to the complexicity of a complete tissue) hinders efficient detection.

The only reliable method to detect the mRNA uptake and subsequent expression of a protein marker was the use of *Eschericha coli* ß-galactosidase endoding mRNA in combination with different indigo dyes (X-gal or Magenta-gal).

Special features of the ß-galactosidase detection system.

The selection of the indigo dye method for the visualisation of the mRNA uptake bears some obvious disadvantages. To ensure that cells are in a single cell layer, thin sections have to be performed (ideally ≤ 10 µm). Because of the morphology of the ear pinna (thin sheet of about 0,5-1 mm) and the fact that only cryosections can be used (ß-galactosidase is heat inactivated during some steps that are necessary to perform paraffin sections) it turned out to be difficult to obtain high quality ´subsequent sections. However, under certain prerequisites (ears had to be shaved, well orientated and trimmed carefully), we managed to perform series of good quality 20µm sections. We used two different dyes to detect ß-galactosidase activity: X-gal and Magenta-gal. For X-gal (positive cells turn blue-green) we have gotten better results than for Magenta-gal (positive cells turn violet). At the same time, unspecific background staining, particularly at hair follicles was seen with X-gal whereas it was virtually absent for Magenta-gal.

Protocol requirements for a combined indigo- and immunostaining

The combination of ß-galactosidase detection (indigo dye) and cell type specific marker staining (specific antibody) required some more adaptations. Particularly critical was the use of the fixative and the order of the combined staining (the indigo staining includes a 14 h long incubation step at 37°C). Best results for antibody staining (against MHC II molecules) were observed with an acetone fixation and the antibody staining in the first place. In contrary, best results for ß-galactosidase activity were obtained for a fixation with a mixture of formaldehyde and glutardialdehyde and the indigo staining in the first place. Considering the pros and cons of the different combinations we selected the following one: Fixation with formaldehyde but without glutardialdehyde and the antibody staining in the first place. Glutardialdehyde had to be omitted because it increased dramatically the autofluorescence of the tissue while it had only little if any effect on the quality of the indigo staining. The

fixation with formaldehyde was used for several reasons. First the morphology of the tissue was better preserved than for acetone. Second a sharp and strong indigo staining required a fixation with formaldehyde and third the quality of the anti MHC II antibody staining was still acceptable with formaldehyde. We however used a short incubation in pure acetone to remove lipids and fat. This allowed better quality mounting (less/no air bubbles) with the water soluble medium we used. Finally we had only good quality antibody staining if we did this antibody staining before the indigo staining.

Dye compatibility in combined indigo and immunostainings

Combination of two different stainings requires not only compatibility of the different steps in the two protocols but also compatibility of the probes used for detection. In principle an immunostaining is possible with precipitating dyes (enzymatic probe) or with fluorescent dyes (labelled probe). To combine the indigo staining with a precipitating dye we used X-gal and AEC. Double positive cells appear then black (**Fig. S**) and are difficult to discriminate from intensively stained single positive cells. Therefore we preferred the combination of Magenta-gal with the fluorescent dye Alexa Fluor 546. We used Magenta-gal instead of X-gal for two reasons. First the intensity of the Magenta gal staining was consistently weaker (even if the dye was given in saturated amounts) thus preventing (too) strong staining. Second the colour of Magenta-gal positive cells fits better with the excitation/emission wavelengths of the Alexa Fluor 546 fluorescent dye. Both factors should minimize quenching of the Alexa Fluor 546 fluorescent signal. Indeed this dye combination allows detection of both signals (**Fig. S**), at least when the indigo staining is not too strong (this was consistently the case for ß-galactosidase-expressing positive cells in sections of mRNA-injected ears).

Supporting figures

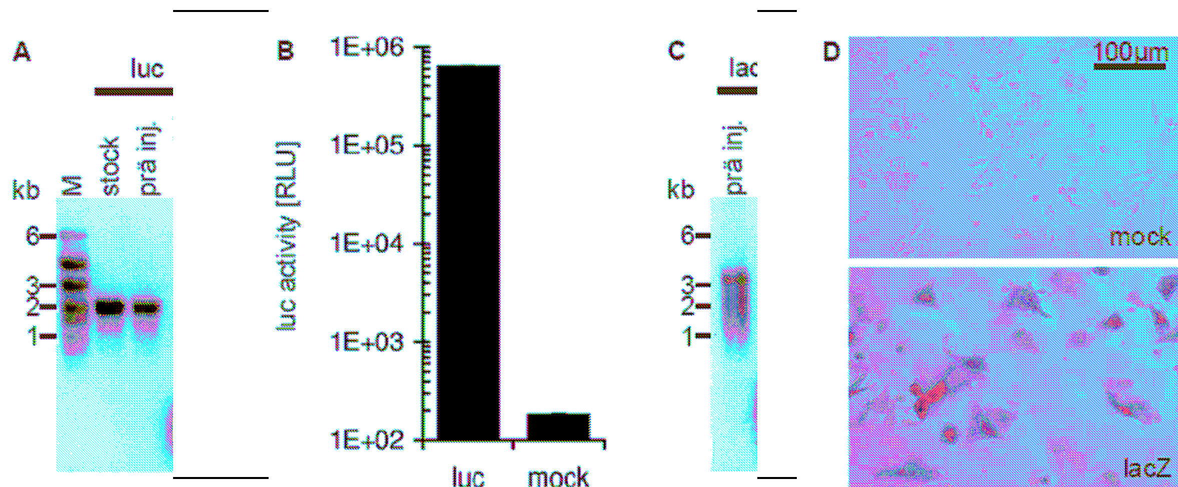

Fig. S 1. Integrity and translation capacity of the injected mRNA. We investigated the integrity of the injected mRNA by formaldehyde agarose (1,2% w/v) gel electrophoresis. Therefore we analysed 1 µg of mRNA coding either for *Photinus pyralis* luciferase (luc, 1.9 kb, **A**) or for *Eschericha coli* ß-galactosidase (lac, 3.5 kb, **C**). We observed no difference in integrity when we compared the mRNA before (stock) its dilution in the appropriate injection buffer and after its dilution in the injection buffer (prä inj.). We analyzed the capability of the mRNA for translation by transfection. Therefore we electroporated BHK21 cells with 10 µg of mRNA or as negative control with irrelevant or without mRNA (mock). Subsequently, cells were either lysed and analyzed for luciferase expression with a luminometer (**B**) or stained with X-gal and analyzed for lacZ expression with a light microscope (**D**).

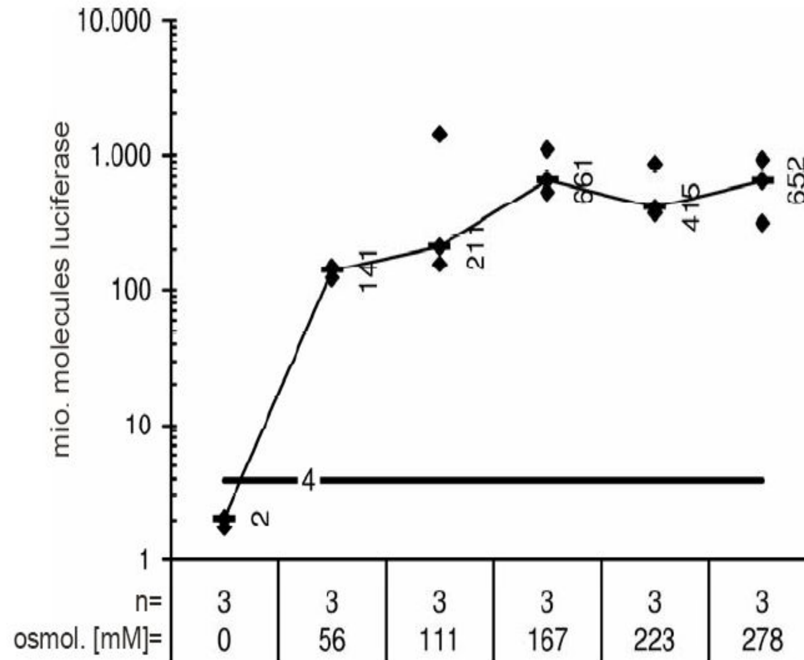

Fig. S 2. Influence of the ion concentration on the mRNA transfer *in vivo*. We used different concentrations of Ringer-lactate to prepare injection solutions (100 µl) with same amount of *Photinus pyralis* luciferase encoding mRNA (20 µg) but different osmolarity (osmol.). We used these solutions to inject BALB/c mice into the ear pinna. 15 h later we sacrificed the mice and prepared ear lysates. We show the calculated total amount of generated molecules luciferase for each ear, the median of the different groups (bar with number), the size of each group (n) and the detection limit of the assay (thick line with number).

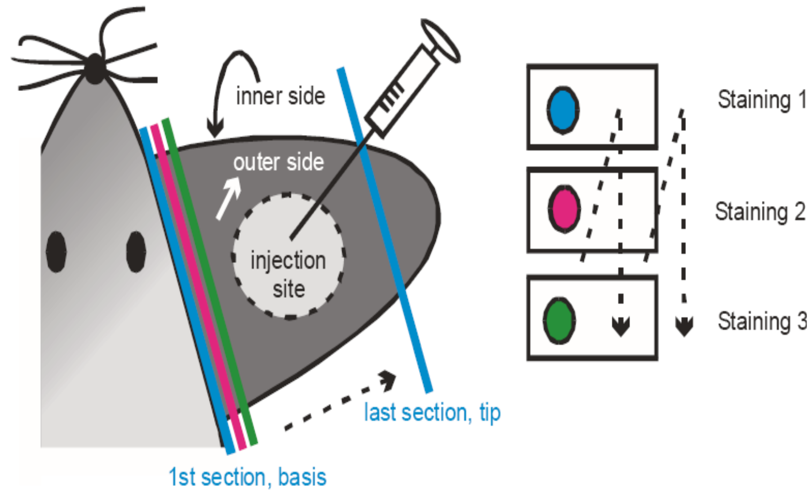

Fig. S 3. Method for the characterisation of the cell type(s) that take-up exogenous mRNA. The schema shows a mouse from the top. We injected the mice with mRNA into the ear pinna. We made consecutive transversal sections of the ear (represented by lines different colors). Sections were collected in different sets (represented by dots in different colors), air dried and stored at -20°C until different stainings were performed.

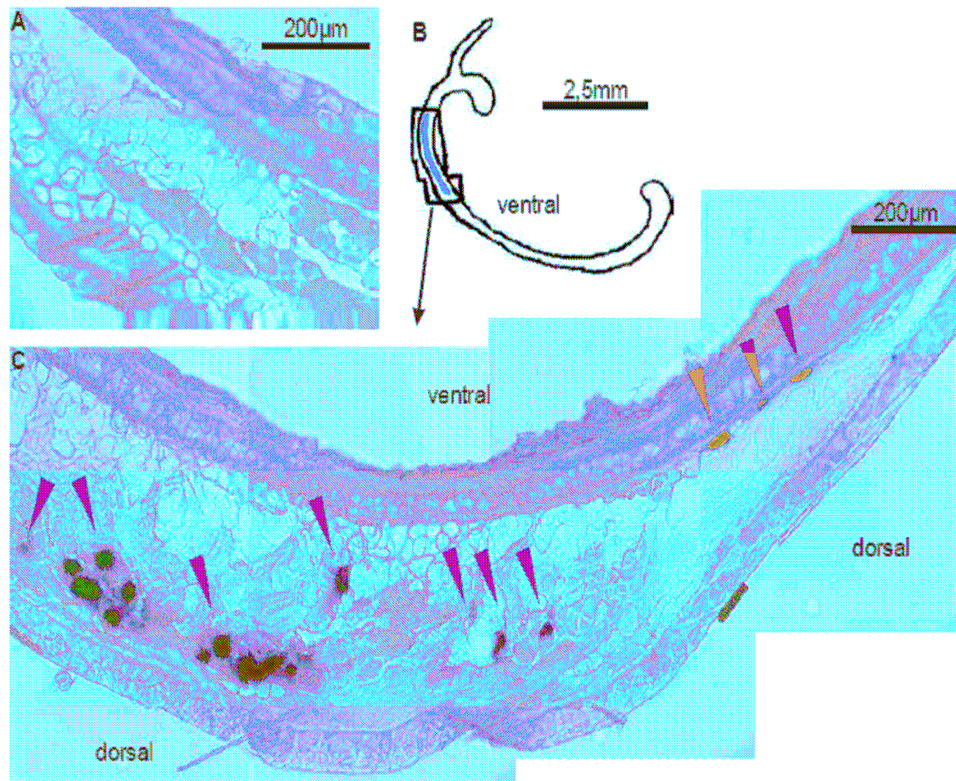

Fig. S 4. mRNA uptake *in vivo* on the cellular level. We injected 5 µg *Eschericha coli* ß-galactosidase encoding mRNA into a mouse ear pinna. 15 h post injection we embedded the ear in TissueTek O.C.T medium and performed 60 µm cryosections. Sections were stained over night with X-gal solution. (**A**) Cryosection of an mRNA transfer negative ear. No lacZ positive cells (blue) are detectable. (**B**) Overview and (**C**) detail of an mRNA transfer positive ear cross section. LacZ positive cells appear dark blue and are indicated by pink arrows.

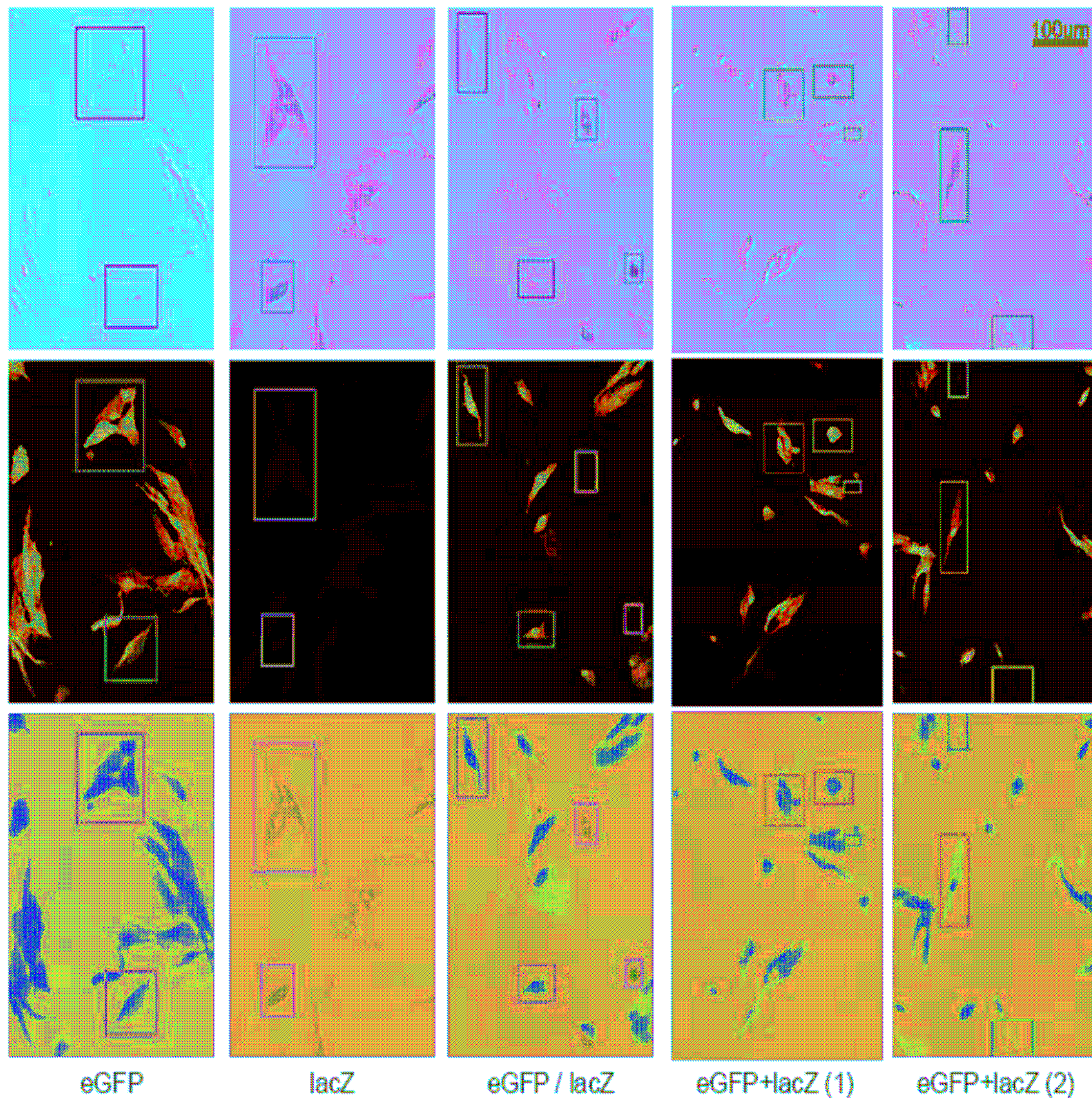

Fig. S 6. Compatibility Alexa Fluor 546 signal with the colour of Magenta-gal positive cells. To asses whether detection of Alexa Fluor 546 is possible in Magenta-gal positive cells we transfected BHK cells with combinations of eGFP or lacZ mRNA. We stained the cells with an anti eGFP antibody with Alexa Fluor 546 detection and subsequently with Magenta-gal solution. We detected Magenta-gal staining positive cells (expressing lacZ) by bright field light microscopy (upper row) and Alexa Fluor 546 staining positive cells (expressing eGFP) by fluorescence microscopy (middle row). We used the overlay of both channels (lower row) to get a precise impression of the location of the cells with respect to each other, although the

Alexa Fluor 546 signal (green) hides in this presentation the light microscopic picture. We analyzed the following combinations of transfected cells: single transfections with eGFP or lacZ mRNA only, a mixture of single transfected cells (eGFP/lacZ) and finally double transfected cells (eGFP+lacZ).

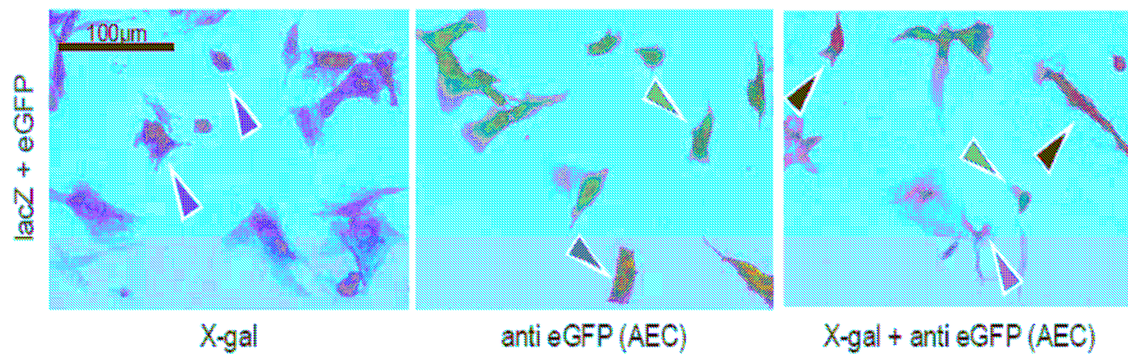

Fig. S 5. Compatibility of X-gal dye and AEC dye positive cells. To asses whether the X-gal precipitate is compatible with the detection of AEC positive cells we cotransfected BHK cells with eGFP and lacZ mRNA. The cells were stained with an anti eGFP immunostaining with AEC (red positive cells express eGFP), with X-gal solution (green-blue positive cells express lacZ) or a combination of both. We analyzed the stained cells by bright field microscopy. Double positive cells appear blackish (black arrows). Discrimination of single and double positive cells (green an red arrow) becomes difficult when the single staining is strong (and therefore rather dark).

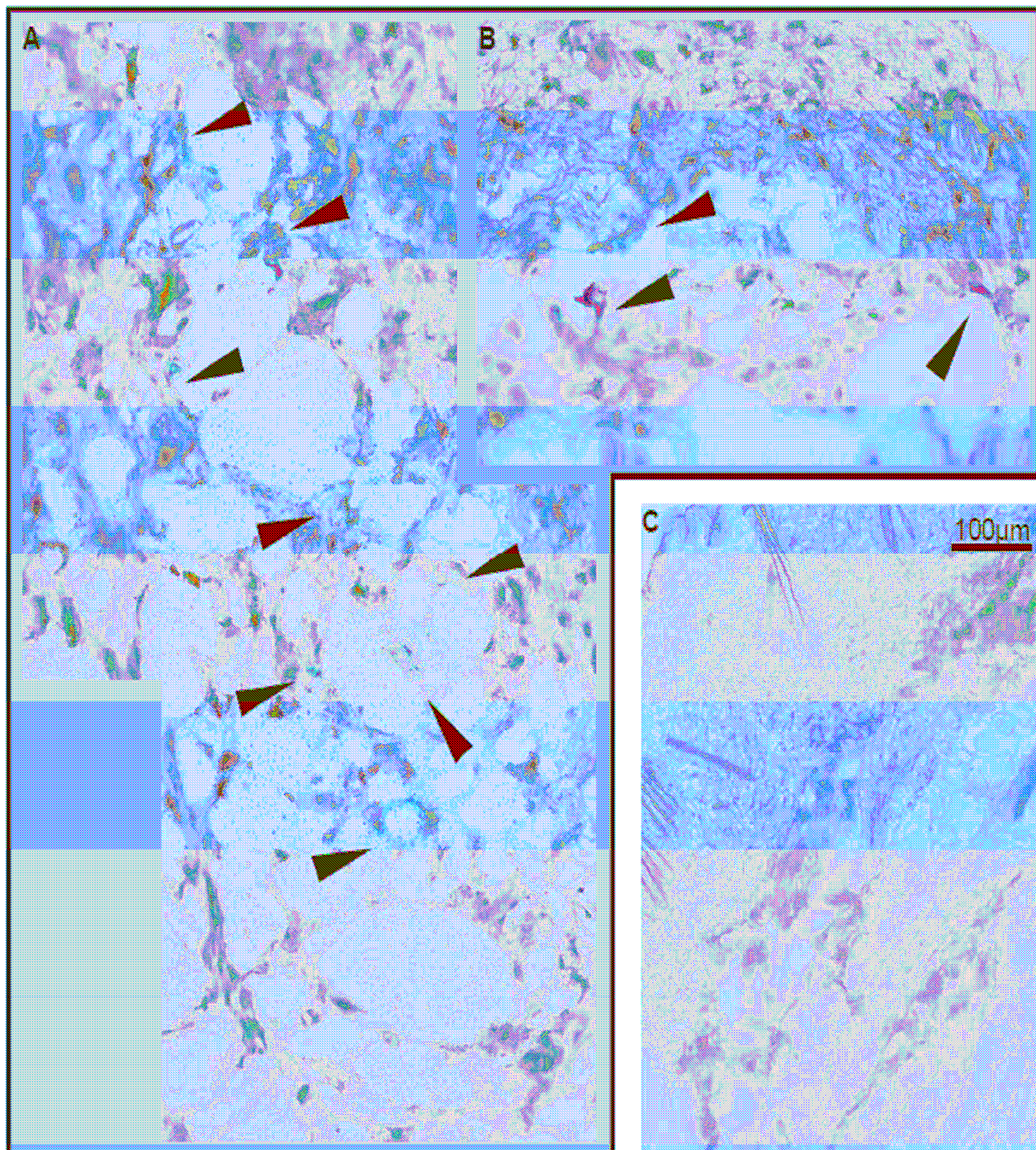

Fig. S 7. Co-staining of MHC class II and ß-galactosidase expressing cells using X-Gal. We injected 20 µg ß-galactosidase encoding mRNA in a total of 100 µl Ringer-lactate buffered solution. 14 hours after injection, we removed the ears from sacrificed mice and made transversal cryosections. We stained the sections first with an anti MHC class II (A-B) or the appropriate isotype control antibody (C) (detected by AEC staining) and second with X-gal (for ß-galactosidase expression). Cells positive for mRNA Transfer appear green-blue, cells

positive for MHC II red and double positive cells would appear black. mRNA transfer positive cells (irrespective of MHC II expression) are indicated by arrows.

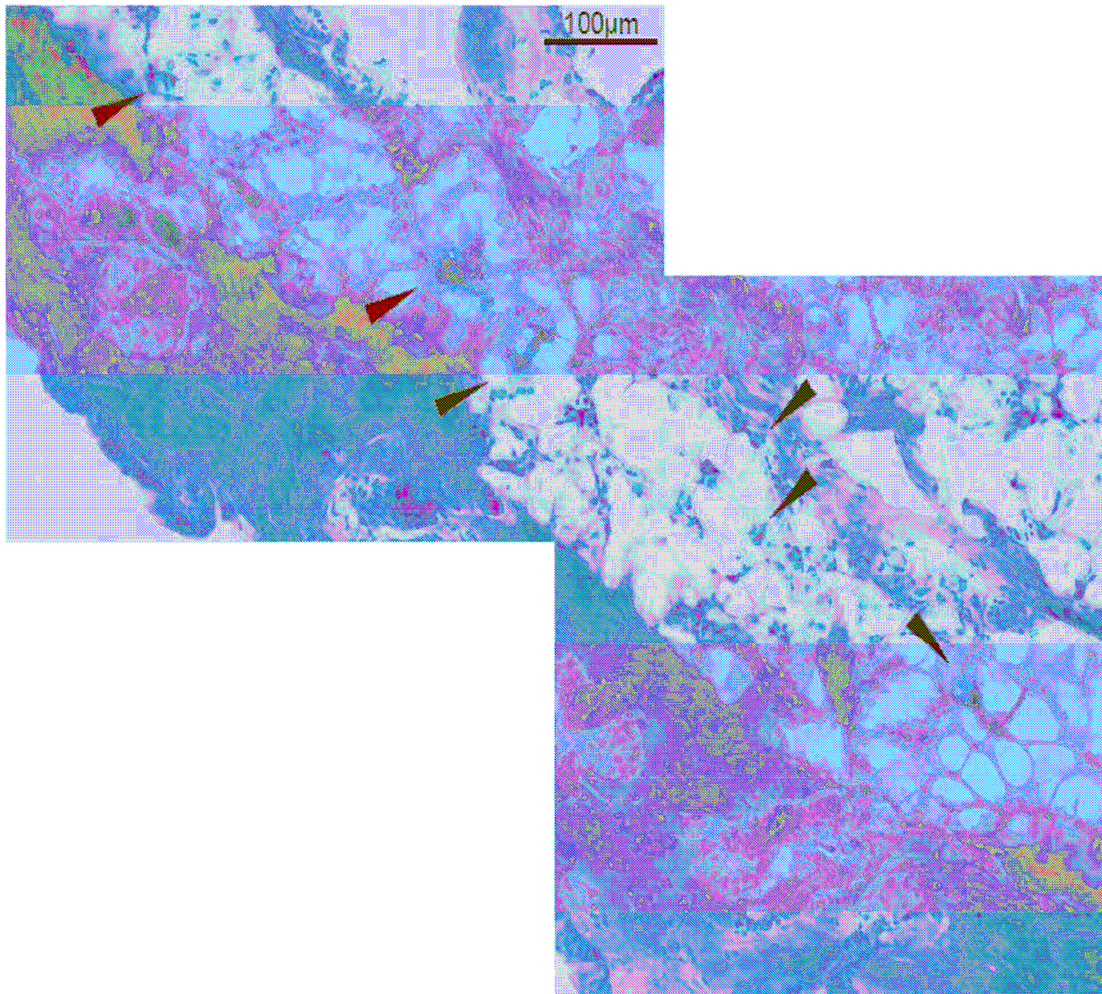

Fig. S8. mRNA uptake and ear pinna morphology. We injected 20 µg ß-galactosidase encoding mRNA in a total of 100 µl Ringer-lactate buffered solution. 14 hours after injection, we removed the ears from sacrificed mice and made transversal cryosections. We stained the sections first with X-gal (for ß-galactosidase expression) and second with hematoxylin and eosin. Cells positive for mRNA transfer are indicated by arrows and are found in a narrow layer of "parenchymatic" cells.

Supporting References

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