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TECHNIQUES AND RESOURCES

RESEARCH REPORT

Transfection of *Capsaspora owczarzaki*, a close unicellular relative of animals

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ABSTRACT

How animals emerged from their unicellular ancestor remains a major evolutionary question. New genome data from the closest unicellular relatives of animals have provided important insights into the evolution of animal multicellularity. We know that the unicellular ancestor of animals had an unexpectedly complex genetic repertoire, including many genes that are key to animal development and multicellularity. Thus, assessing the function of these genes among unicellular relatives of animals is key to understanding how they were co-opted at the onset of the Metazoa. However, such analyses have been hampered by the lack of genetic tools. Progress has been made in choanoflagellates and teretosporeans, two of the three lineages closely related to animals, whereas no tools are yet available for functional analysis in the third lineage: the filastereans. Importantly, filastereans have a striking repertoire of genes involved in transcriptional regulation and other developmental processes. Here, we describe a reliable transfection method for the filasterean *Capsaspora owczarzaki*. We also provide a set of constructs for visualising subcellular structures in live cells. These tools convert *Capsaspora* into a unique experimentally tractable organism to use to investigate the origin and evolution of animal multicellularity.

KEY WORDS: Transfection, *Capsaspora owczarzaki*, Holozoa, Multicellularity, Origin of Metazoa

INTRODUCTION

The transition to animal multicellularity from a single-celled ancestor is one of the most intriguing events in the history of life (King, 2004; Ruiz-Trillo et al., 2007; Rokas, 2008; Knoll, 2011; Richter and King, 2013; Cavalier-Smith, 2017; Sebé-Pedrós et al., 2017). Analysis of the genomes of extant unicellular relatives of animals, hereafter unicellular holozoans, recently showed that the unicellular ancestor of animals was genetically more complex than previously thought (Sebé-Pedrós et al., 2017). Strikingly, genes thought to be animal specific are now known to be present in unicellular holozoans. These include genes involved in cell adhesion, such as those encoding integrins and cadherins, cell-to-cell communication, such as those encoding tyrosine kinases, and transcriptional regulation, such as the developmental transcription factor *Brachyury* (Sebé-Pedrós et al., 2010, 2011; Sebé-Pedrós et al., 2013a; Nichols et al., 2012; Suga et al., 2012). These findings imply that the co-option of ancestral genes into new functions was an important mechanism for the transition to animal multicellularity. However, understanding how these genes were co-opted will only be possible through functional analyses in extant unicellular relatives of animals.

There are three known lineages of unicellular holozoans: choanoflagellates, teretosporeans (ichthyosporeans and corallochytreans) and filastereans (Torruella et al., 2015; Grau-Bové et al., 2017). These three lineages show very different developmental modes, such as the clonal colony formation of choanoflagellates (Fairclough et al., 2013), the coenocytic growth of teretosporeans (Marshall et al., 2008; Suga and Ruiz-Trillo, 2013) and the aggregative behaviour present in filastereans (Sebé-Pedrós et al., 2013b). To develop a comprehensive view of the transition to multicellularity, we need to understand these three different modes of development. So far, a forward genetics approach has been developed in choanoflagellates, leading to the discovery of *rosetteless*, a gene related to colony formation in *Salpingoeca rosetta* (Levin et al., 2014). Efforts are also underway to develop transfection in choanoflagellates. Transfection has already been developed in the ichthyosporean *Creolimax fragrantissima*, where it allowed the description of synchronous nuclear division during coenocytic development (Suga and Ruiz-Trillo, 2013). To date, however, there are still no genetic tools reported in filastereans.

Recent analysis of the genome, transcriptome, proteome and phosphoproteome of the filasterean amoeba *Capsaspora owczarzaki* (Fig. S1), hereafter *Capsaspora*, provided important insights into the origins of animal multicellularity and the nature of their unicellular ancestor (Suga et al., 2013; Sebé-Pedrós et al., 2016a,b). The *Capsaspora* genome encodes an unexpected set of transcription factors known to be involved in animal development that were previously thought to be metazoan specific, such as NFκB, Runx and T-box (Sebé-Pedrós et al., 2011; Suga et al., 2013; de Mendoza et al., 2013). Similar to animals, these transcription factors are differentially regulated at the transcriptional level and are also differentially phosphorylated during the *Capsaspora* life cycle (Sebé-Pedrós et al., 2016a,b). *Capsaspora* also contains the most complete set of proteins linked to cell–extracellular matrix adhesion (the Integrin adhesome) among unicellular holozoans (Sebé-Pedrós et al., 2010; Suga et al., 2013). This highlights *Capsaspora* as the closest relative of animals in which such genes can be studied.

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Here, we present the first protocol for transfecting the filasterean *Capsaspora* with plasmid DNA. The protocol is based on the classical calcium phosphate precipitation method (Graham and van der Eb, 1973), which we coupled with a glycerol shock to increase transfection efficiency. We also constructed a set of expression vectors containing an endogenous promoter and fluorescent reporters that allow labelling of multiple subcellular structures *in vivo*. Altogether, this work provides the key step necessary to perform functional assays requiring foreign nucleic acid delivery, including overexpression, RNA interference and genome editing, rendering *Capsaspora* experimentally tractable towards addressing the transition to animal multicellularity.

RESULTS AND DISCUSSION

Capsaspora transfection using calcium phosphate precipitation

There are several protocols available for the transient transfection of plasmid DNA for eukaryotic cells. For *Capsaspora*, we initially tried electroporation because it has been successfully used to transiently transfect the ichthyosporean *C. fragrantissima* (Suga and Ruiz-Trillo, 2013). However, we obtained no more than 20 positive cells out of thousands of cells. We additionally tried lipid-based transfection (Felgner et al., 1987) and magnetofection (Buerli et al., 2007; Ensenauer et al., 2011), which have been reported to work in eukaryotic cells that are difficult to transfect. Nevertheless, both approaches resulted in few, if any, positive cells. Finally, we tested the classical calcium phosphate precipitation-based transfection method (Graham and van der Eb, 1973), which has been reported to successfully transfect *Dictyostelium discoideum* (Nellen et al., 1984; Gaudet et al., 2007), an amoebozoan without a cell wall. Given that we initially obtained ∼100 times more cells that were positive cells with the calcium phosphate precipitation protocol than with the other methods, we focused on this protocol to further improve its efficiency.

As a first step to increase the efficiency of transfection, we investigated which life stage to transfect. Under culture conditions, *Capsaspora* presents three distinct life stages: adherent, cystic and aggregative (Sebé-Pedrós et al., 2013b). We tried using cells in the adherent stage because the culture is at its exponential growth phase at this stage (Fig. 1A) (Sebé-Pedrós et al., 2013b). We observed that transfecting *Capsaspora* adherent cells at 90-95% confluence from a fresh culture resulted in higher transfection efficiency.

Next, we addressed crystal formation during DNA precipitation. We sought the smallest size of crystals possible, because smaller crystals have been associated with higher transfection efficiency (Jordan et al., 1996; Jordan and Wurm, 2004). We achieved this by keeping the same ratio of DNA, calcium and phosphate as previously described for *D. discoideum* (Gaudet et al., 2007), and setting an incubation time of 10 min at 37°C (Fig. 1B-4). Additionally, we used a transfection medium containing minimal growth components but lacking phosphate (Fig. 1B-3,6), to maintain the optimal concentration of calcium phosphate for DNA precipitation. This medium also contains buffering agents at pH 7.1 to avoid pH fluctuations that might affect the solubility of any precipitates.

Finally, to increase the number of transfected cells, we coupled the protocol with a glycerol shock, because the latter has shown to increase the transfection efficiency in other systems (Grosjean et al., 2006; Gaudet et al., 2007; Guo et al., 2017). We performed the shock using 10% glycerol in 1× HBS for 1 min (Fig. 1B-7) and immediately added growth medium to avoid compromising cell viability (Fig. 1B-8, see Supplementary Materials and Methods for further details).

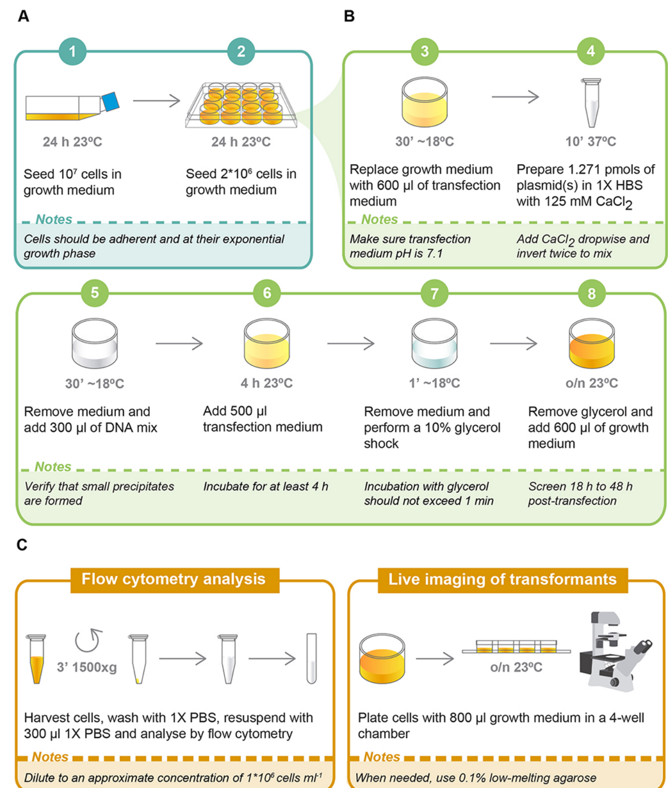

Fig. 1. Protocol for transfection of plasmid DNA in *Capsaspora*. (A) Preparation of cells (1-2). (B) Calcium phosphate precipitation (3-8). (C) Screening of transformants. o/n, overnight.

Analysis of transfected *Capsaspora* cells by flow cytometry

To evaluate DNA incorporation by *Capsaspora*, we constructed two expression vectors containing either Venus (pONSY-Venus) or mCherry (pONSY-mCherry) fluorescent proteins as cytosolic markers (Fig. 2A). These vectors contain the promoter and terminator regions of the endogenous *elongation factor 1-α* (*EF1-α*) gene (CAOG_07807) from *Capsaspora* (see Materials and Methods). We confirmed the successful expression of both fluorescent proteins by fluorescence microscopy (Fig. 2A′-A″) and flow cytometry. We performed an immunofluorescence assay on sorted cells and confirmed that the fluorescent population identified was expressing Venus (Fig. S2).

Next, we analysed the transfection efficiency by quantifying the number of positive cells by flow cytometry (Fig. 3A,B). We performed single transfection experiments using either pONSY-Venus or pONSY-mCherry in seven independent experiments (each experiment performed with a different batch of cells) with at least six technical replicates each. In both cases, the positive populations were defined using a negative control (Fig. S3). *Capsaspora* transfection efficiency was 1.132%±0.529 (mean±s.d.) with a 95% confidence interval of (0.983-1.281%) from a total of 4.9 million cells (Fig. 3C). In these experiments, individual transfection efficiencies ranged from 0.347%±0.193 to 2.083%±0.248 (Table S1). Importantly, these transfection efficiencies are sufficient to screen for positive cells and perform further manipulations, because they correspond to thousands of positive cells per well. Additionally, we compared transfection rates between *Capsaspora* cells transfected with either pONSY-Venus or pONSY-mCherry (Fig. 3D, experiments 7a and 7b, respectively, in Table S1), but no significant difference was observed (P=0.5625, Wilcoxon Signed Rank Test).

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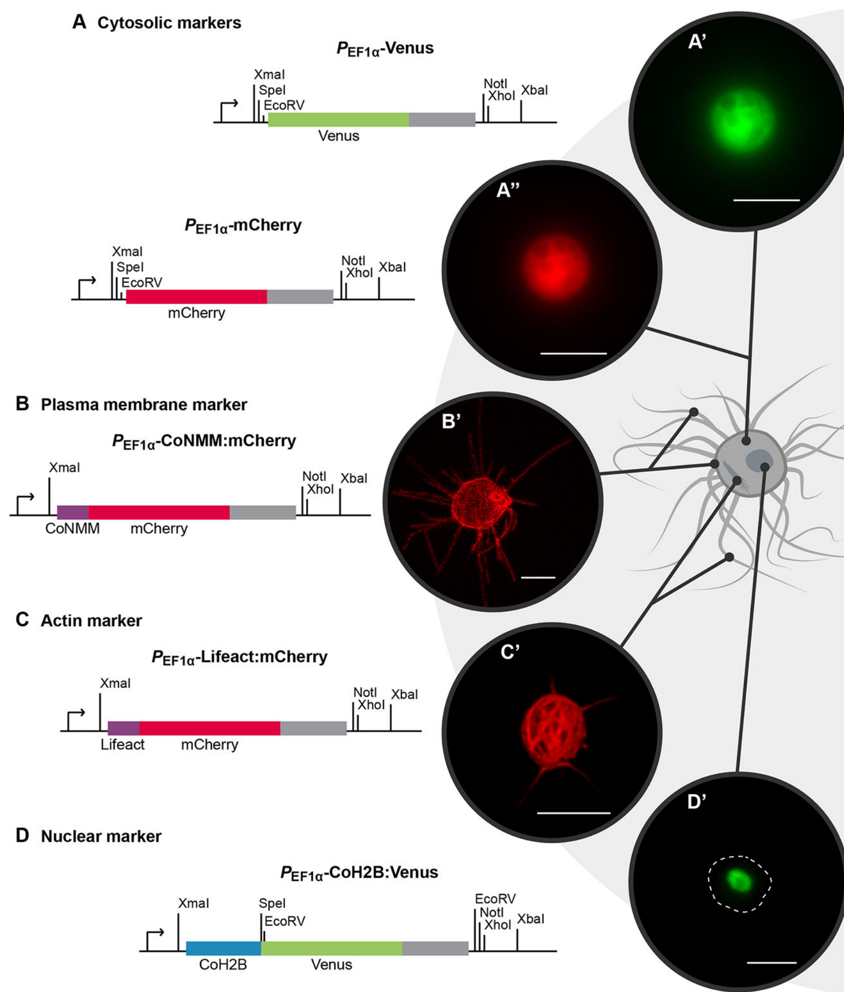

Fig. 2. *Capsaspora* expression cassettes and live imaging of transfected cells. (A) Cytosolic marker cassettes expressing mCherry (A′) or Venus (A″) fluorescent proteins. (B) Plasma membrane marker cassette containing the *Capsaspora Src2* NMM fused to *mCherry* (B′). (C) Actin marker cassette containing *Lifeact* fused to *mCherry* (C′). (D) Nuclear marker cassette containing *Capsaspora* histone *H2B* (*CoH2B*) fused to *Venus* (D′). *EF-1α* promoter (arrows) and terminator (grey boxes) and single-cut restriction enzymes are shown. Cells in (A) and (D) were imaged using wide-field fluorescence microscopy. The cell in B was imaged using a Spinning Disk confocal microscope and the cell in C was imaged using a confocal laser scanning microscope. Dashed line indicates the cell body. Scale bars: 5 µm.

Given that transfection is transient, it is of interest to know how long the expression of the reporter gene persists for. Thus, we performed three independent experiments transfecting pONSY-Venus. We analysed the percentage of positive cells by flow cytometry every 24 h for 10 days (Fig. S4 and Table S2). We observed an exponential decrease in the number of positive cells. Although there was a significant reduction in the number of positive cells after 48 h (~39% of the total of positive cells), positive cells (~3%) could still be detected by Day 10. At this point, most of the cells are expected to be in the cystic stage (Sebé-Pedrós et al., 2013b), indicating that transient expression of a gene of interest can be analysed during each of the life stages of *Capsaspora*.

We also tested whether the two different constructs could express a protein simultaneously by co-transfecting both pONSY-mCherry and pONSY-Venus at equimolar concentrations in seven independent experiments (Fig. 3E). The red (Q2) and green (Q4) positive populations were defined using their corresponding negative controls (Fig. S5). The mean relative percentage of cells showing red and green fluorescence simultaneously (Q3 in Fig. 3F) from the total number of positive cells (sum of Q2, Q3 and Q4 in Fig. 3F) was 72.909%±5.468, ranging from ~65% to ~83% (Fig. 3G, Table S3). Thus, it is possible to co-transfect two different vectors with a high probability of incorporating both of them in the same cell. This result is similar to those observed in other unicellular eukaryotes [40-80% in *Volvox carteri* (Schiedlmeier et al., 1994), 84% in *Pandorina morum* (Lerche and Hallmann, 2014) and 50-100% in *Eudorina elegans* (Lerche and Hallmann, 2013)]. Co-transfection is a useful strategy when more than one cassette is needed, such as when labelling two different cellular structures simultaneously, delivering resistance cassettes against an antibiotic with a reporter gene, or delivering different elements required for CRISPR/Cas9 assays.

Live imaging of *Capsaspora* by labelling endogenous proteins

To understand the biological role of certain genes in *Capsaspora*, it is important to subcellularly localise the protein of interest in the cell. Thus, as a means of labelling the cellular structures in *Capsaspora*, we designed three additional vectors that allowed live imaging of the plasma membrane, the actin cytoskeleton and the nucleus.

To label the plasma membrane, we built a construct expressing an endogenous membrane-binding motif fused to mCherry (pONSY-CoNMM:mCherry, Fig. 2B). We used the *N*-myristoylation motif (NMM), a well-known membrane-binding motif, present in the *Src* tyrosine kinases (Sigal et al., 1994). *Capsaspora* has two homologs of *Src*, *CoSrc1* (CAOG_02182) and *CoSrc2* (CAOG_06360), the localisation of which has been reported in filopodia (Schultheiss et al., 2012). We used *CoSrc2* NMM to create the CoNMM: mCherry fusion, which successfully localised at the plasma membrane, including filopodia (Fig. 2B′). To label the actin cytoskeleton in *Capsaspora*, we built a construct containing Lifeact fused to mCherry (pONSY-Lifeact:mCherry, Fig. 2C). Lifeact is a

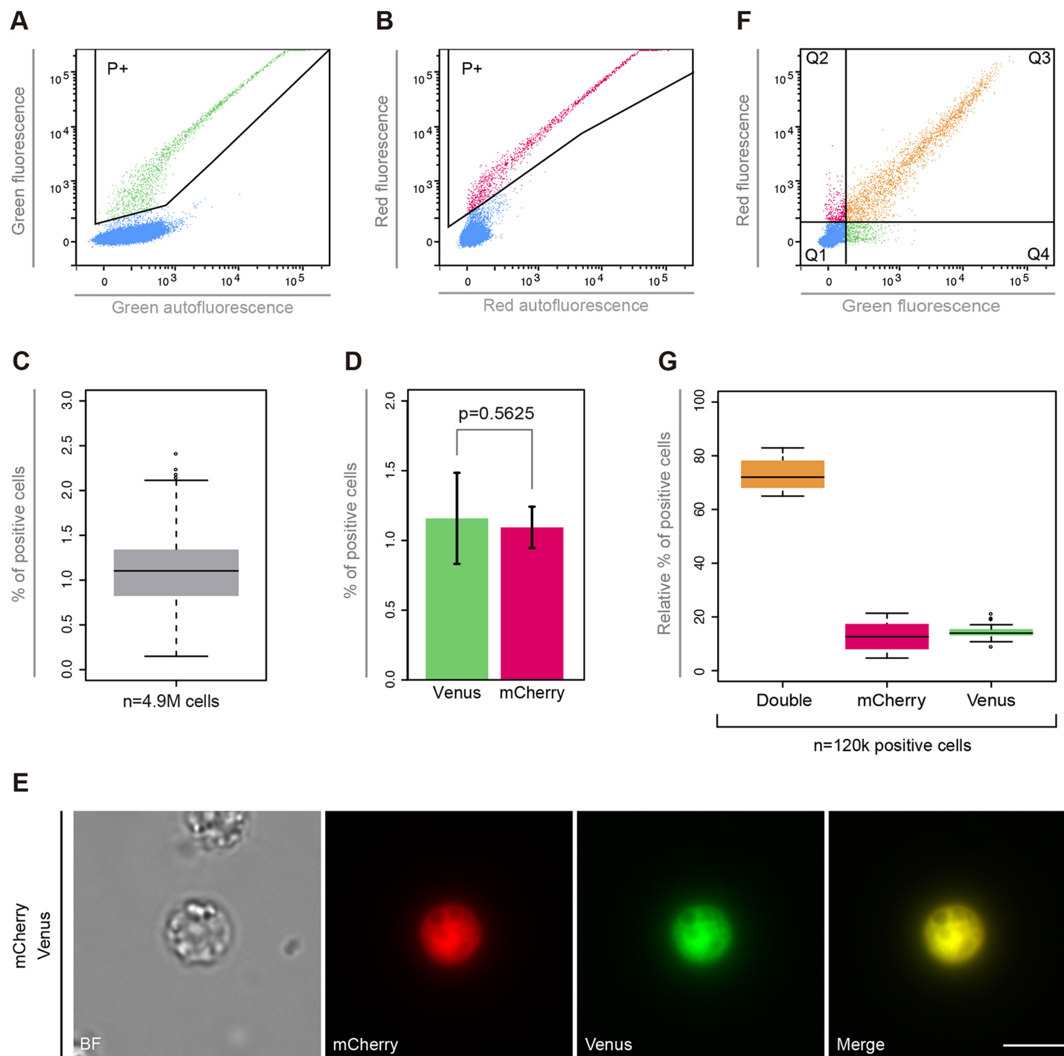

Fig. 3. Transfection efficiency analysis of *Capsaspora*. (A) Flow cytometry distribution of pONSY-Venus transfected cells. Area selected (P+) represents the Venus-positive population. (B) Flow cytometry distribution of pONSY-mCherry transfected cells. Area selected (P+) represents the mCherry-positive population. (C) Percentage of positive cells in single transfection. The box plot represents the transfection efficiency distribution over seven independent experiments with at least six technical replicates each (n=4.9 M cells). (D) Percentage of positive cells from a paired experiment with six technical replicates, transfecting either pONSY-Venus or pONSY-mCherry. Error bars represent s.d. (P=0.5625, Wilcoxon Signed Rank Test). (E) Wide-field fluorescence microscopy of a live cell co-transfected with pONSY-Venus and pONSY-mCherry. (F) Flow cytometry distribution of pONSY-Venus and pONSY-mCherry co-transfected cells. Cell population was divided into quartiles: negative cells (Q1), fluorescent cells expressing mCherry only (Q2), co-transfected cells expressing both fluorescent proteins (Q3), and fluorescent cells expressing Venus only (Q4). (G) Relative percentage of positive cells co-transfected with pONSY-Venus and pONSY-mCherry; expressing both fluorescent proteins (double), mCherry only or Venus only, calculated from the total number of positive cells in seven independent experiments with six replicates each (n=120,000 cells). Scale bar: 5 µm.

17-amino acid peptide from the N-terminal region of yeast Abp140 (Riedl et al., 2008) that works as a marker of filamentous actin. The Lifeact:mCherry fusion successfully labelled the actin cytoskeleton (Fig. 2C′). This construct also labels actin in filopodia (Fig. S7), although the signal is much weaker than that observed with the membrane marker. Finally, to label the nucleus, we built a construct containing the coding sequence of *Capsaspora* histone *H2B* (CAOG_01818) fused to Venus (pONSY-CoH2B:Venus, Fig. 2D). We confirmed nuclear localisation by staining transfected cells with DAPI (Fig. 2D′, Fig. S6).

To better understand the subcellular structures of *Capsaspora* cells, we combined the nuclear, plasma membrane and actin markers. We co-transfected pONSY-CoH2B:Venus with either pONSY-CoNMM:mCherry or pONSY-Lifeact:mCherry (Fig. 4A). Furthermore, we performed live imaging in cells transfected with either the membrane marker or the actin marker. The use of the membrane marker allowed us to observe the dynamic behaviour of filopodia on the substrate with unprecedented detail. We observed the retraction of filopodia, filopodia breakage and foci of membrane accumulation (Fig. 4B and Movie 1). In particular, we observed that filopodia are distributed around the cell body. More importantly, the projections constructed from the z-stack clearly demonstrated that the *Capsaspora* cell body is not in direct contact with the substrate, with the numerous filopodia instead holding the cell up (Fig. 4C). Moreover, we tracked a cell transfected with the actin cytoskeleton marker and observed the organisation of actin bundles around the cell body (Fig. 4D,E and Movie 2).

The accumulated knowledge on its well-annotated genome, transcriptome, proteome and phosphoproteome and histone modifications, and its key phylogenetic position as a close

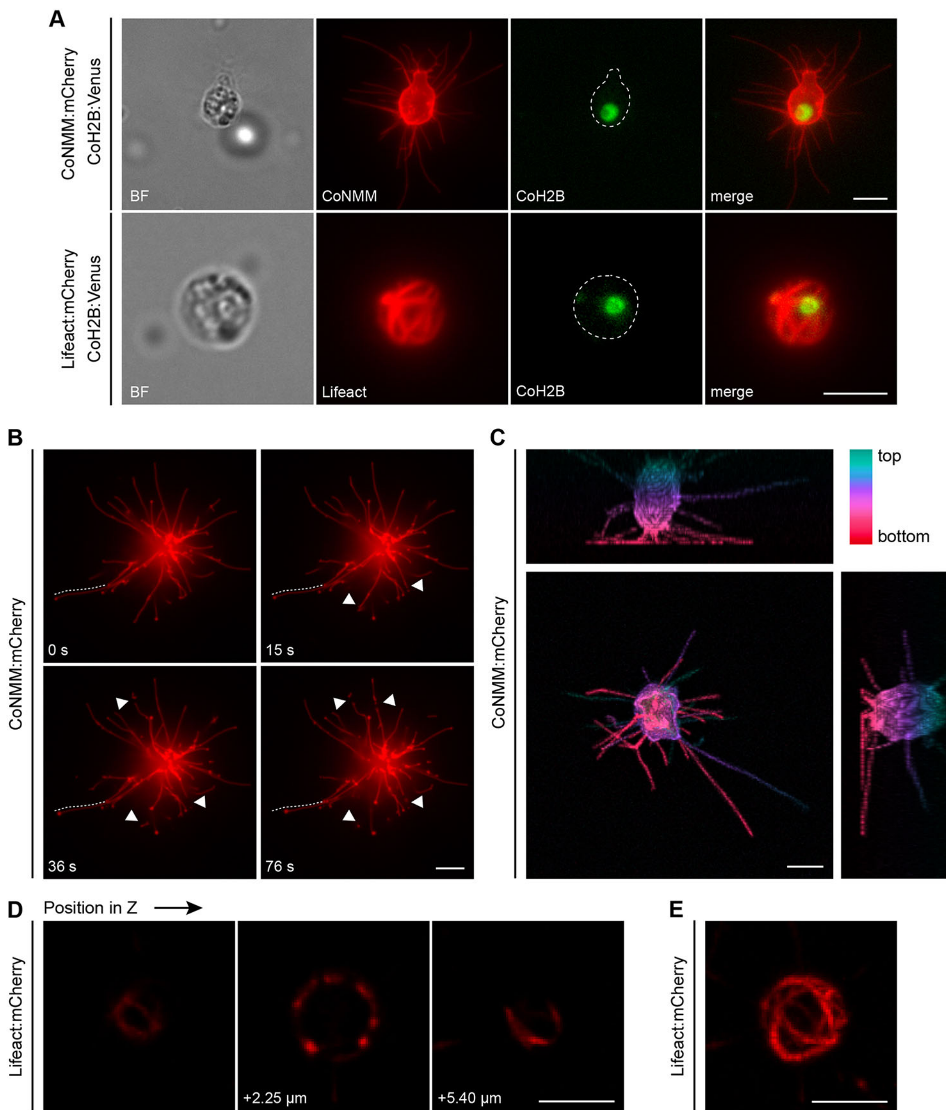

Fig. 4. Live imaging of transfected *Capsaspora* cells. (A) Wide-field fluorescence microscopy of live cells co-transfected with pONSY-CoNMM:mCherry and pONSY-CoH2B:Venus, and live cells co-transfected with pONSY-Lifeact:mCherry and pONSY-CoH2B:Venus. CoNMM:mCherry labelling is presented as a maximum projection of the cell. Dashed lines indicate cell bodies. (B) Time-points on a *Capsaspora* cell transfected with pONSY-CoNMM: mCherry imaged using wide-field fluorescence microscopy. Filopodia attached to the substrate are in focus. A retracting filopodia can be observed (dotted line), whereas four filopodia are broken (arrowheads). (C) Maximum-intensity projections in each axis of a cell transfected with pONSY-CoNMM:mCherry. Colour scale represents depth through the projection. Imaging was performed using confocal microscopy. (D) z-stack on a *Capsaspora* cell transfected with pONSY-Lifeact:mCherry imaged using confocal microscopy. Actin bundles can be observed shaping the cell in a basket-like structure that is hollow in the middle. (E) Full z-stack maximum intensity projection of cell in (D). Cells in (C-E) were imaged using a Spinning Disk confocal microscope. Scale bars: 5 µm.

unicellular relative to animals render *Capsaspora* as a powerful system to understand the emergence of multicellular animals. The reliable transfection protocol for *Capsaspora* presented here will allow us to study the function of genes that were key to the evolution of multicellularity, opening new avenues of functional research to better understand the transition to animal multicellularity.

MATERIALS AND METHODS

Cell strain and growth conditions

C. owczarzaki cell cultures (strain ATCC®30864) were grown axenically in 25 cm^2 culture flasks (Falcon® VWR, #734-0044) with 5 ml ATCC medium 1034 (modified PYNFH medium), hereafter growth medium, in a 23°C incubator (see supplementary Materials and Methods).

Construction of *Capsaspora* expression vectors

DNA from *Capsaspora* cells was extracted as in Suga et al. (2013). RNA was extracted using a Trizol reagent (Invitrogen/Thermo Fisher Scientific, #15596026). cDNA was obtained by RT-PCR using SuperScript® III Reverse Transcriptase (Invitrogen, #18080044) following the manufacturer's instructions.

Capsaspora expression vectors, named pONSY, bear the promoter and terminator regions from the endogenous *EF-1α* gene (CAOG_07807). To build the pONSY-Venus vector (5.849 kb), the *EF-1α* promoter (906 bp upstream from methionine) and terminator (320 bp downstream from the stop codon) were amplified from gDNA with primers 1 and 2 and primers 4 and 5, respectively (Table S4). *Venus* was amplified from a plasmid available in H.P.-A.'s lab using primers 7 and 8, which contain overlap regions with the promoter and terminator regions, respectively. The three amplicons were fused together by overlapping PCR using primers 1 and 5. The resulting $P_{\mathrm{EF1}\alpha}$-Venus-terminator cassette was digested using the *Kpn*I restriction enzyme and cloned into the pCR2.1 vector (Life Technologies, #K203001) linearised at the *Kpn*I restriction site.

To build pONSY-mCherry (5.828 kb), we followed the same strategy as described above for the promoter, and used primers 4 and 6 to amplify the terminator region. This modification was introduced to eliminate an extra *Eco*RV site that affects further cloning. *mCherry* was amplified from a plasmid available in H.P.-A.'s lab using primers 9 and 10, which contain overlap regions with both the promoter and terminator regions. The three amplicons were fused together by overlapping PCR using primers 1 and 6. The resulting $P_{\mathrm{EF1}\alpha}$-mCherry-terminator cassette was digested with *Kpn*I and *Ksp*I enzymes and cloned into the respective restriction sites of the pCR2.1 vector.

A pONSY (empty) vector (5.127 kb) was created by releasing a mCherry-terminator fragment from pONSY-mCherry using *Spe*I and *Ksp*I restriction enzymes and inserting the terminator in this backbone by Gibson Assembly® (New England Biolabs, E2611L) using primers 7 and 8.

pONSY-CoH2B:Venus (6.230 kb) was created by fusing *Capsaspora* histone *H2B* (CAOG_01818) to Venus. *CoH2B* was PCR amplified from

cDNA using primers 13 and 14 and cloned into the pONSY-Venus multicloning site using *Sma*I and *Spe*I restriction enzymes.

pONSY-CoNMM:mCherry (5.904 kb) was created by fusing an NMM to mCherry. NMM was predicted in the *Capsaspora* Src homolog CoSrc2 (CAOG_06360) using 'NMT - The MYR Predictor' online software (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm), which is based on an in-depth study of *N*-myristoyltransferase substrate proteins (Maurer-Stroh et al., 2002). The NMM predicted sequence GCSNSKPHDPSDFKVSP plus seven extra amino acids (SGVASNS) and an *mCherry* overlap region were included in primer 11. Primers 11 and 12 were used to build a CoNMM-mCherry cassette by PCR using the pONSY-mCherry vector as a template. This cassette was then cloned into pONSY (empty) using *Xma*I and *Eco*RV restriction enzymes.

pONSY-Lifeact:mCherry (5.882 kb) was created by fusing the Lifeact peptide MGVADLIKKFESISKEE(GDPP) (linker in parentheses) to mCherry using primers 15 and 16. The codons were optimised according to their usages in *C. owczarzaki* and *C. fragrantissima*. The Lifeact DNA fragment was first cloned into a pTAC-2 vector (BioDynamics Laboratory) by TA cloning, and the *Xma*I- and *Xba*I-excised fragment was cloned into the pONSY:mCherry vector.

All plasmids DNA were obtained using the plasmid GenElute™ Plasmid Midiprep Kit (Sigma, #NA0200-UKT), lyophilised and resuspended at an approximate concentration of 1 µg/µl in distilled water.

Transfection of *Capsaspora owczarzaki*

Capsaspora cells were transfected using a calcium-phosphate DNA precipitation protocol coupled with a glycerol shock. At Day 0, 2×10^6 cells were seeded in a 12-well plate (Nunc/DDBioLab, #55428) containing growth medium and grown at 23°C overnight.

At Day 1, growth medium was replaced by transfection medium (see Supplementary Materials and Methods), and incubated for 30 min at room temperature (∼18°C). During incubation, the DNA mix was prepared with 1.271 pmol of plasmid DNA for single transfection experiments or 0.636 pmol of each plasmid DNA for co-transfection experiments in 1× HBS Buffer. $CaCl_2$ was added dropwise to a final concentration of 125 mM. The DNA mix was inverted immediately twice and incubated for 10 min at 37°C. After incubation, the transfection medium was removed gradually and the DNA mix was added dropwise to the centre of each well. The cell:DNA mixes were incubated for 30 min at room temperature, after which the transfection medium was added to each well. Cells were incubated for 4 h at 23°C. After incubation, medium was removed and an osmotic shock was performed using 10% glycerol in 1× HBS buffer, for 1 min at room temperature. After the osmotic shock, the glycerol solution was replaced by growth medium and cells were incubated at 23°C overnight. Screening of positive cells was performed 18 h post transfection. More details about the transfection protocol and preparation of transfection reagents are listed in the Supplementary Materials and Methods.

Flow cytometry and FACS

Transfection efficiency was analysed 18 h post transfection. pONSY (empty) transfected cells, mock-transfected cells and nontransfected cells were used as controls for all transfection experiments to discriminate autofluorescence and to distinguish the positive population. For co-transfection experiments, pONSY-mCherry and pONSY-Venus single-transfected controls were used to correctly identify double-positive cells.

Cells were scraped and harvested by centrifugation at 1500 ×***g*** for 3 min at 18°C, washed once with 500 µl 1× PBS (Sigma, #P5368-10 PAK) and diluted to a final concentration of 1×10^6 cells ml^{-1} in a minimum volume of 300 µl 1× PBS. Samples were analysed by flow cytometry using a BD LSRFortessa analyser (Becton Dickinson).

To evaluate plasmid persistence over time, transfected cells from 12 wells per experiment were pooled to homogenise the sample, were then split again into 12 new wells and grown for 10 days. Samples were scraped and harvested by centrifugation at 1500 ×***g*** for 3 min at 18°C. Samples were fixed with 4% formaldehyde (Sigma-Aldrich, #F8775-4X25ML) in 1× PBS for 10 min at 18°C and washed once with 500 µl 1× PBS (Sigma, #P5368-10 PAK). Finally, cells were re-suspended in 400 µl 1× PBS and kept at 4°C until analysed.

SSC-A and FSC-A parameters were used to detect populations of cells (P1). Single cells were gated by FSC-H and FSC-A (P2). Around 100,000 events were recorded from P2, whenever possible. Venus-positive cells (P+ or Q1) were detected using a 488 nm laser with a 530/28 bandpass filter (green fluorescence) and differentiated from autofluorescent cells with a 670/50 bandpass filter.

mCherry-positive cells (P+ or Q4) were detected using a 561 nm laser with a 610/20 bandpass filter (red fluorescence) and differentiated from autofluorescent cells with a 780/60 bandpass filter. Around 2000 events in the population expressing both Venus and mCherry (Q2) were recorded.

For immunofluorescence validation, pONSY-Venus transfected cells were harvested as before and diluted to a concentration of 1×10^7 cells ml^{-1} in a minimum volume of 500 µl 1× PBS. Cells from nine replicates were pooled. Then, 40,000 Venus-positive cells (P+) and 1 million Venus-negative cells (P−) were sorted using a BD FACSAria II SORP flow cytometer cell sorter (Becton Dickinson) equipped with a 100 µm nozzle. The cell population (P2) was gated as before. P+ and P− were detected using a 488 nm laser with a 525/50 bandpass filter (green fluorescence) and differentiated from autofluorescent cells with a 605/40 bandpass filter. Flow cytometry data were visualised and analysed using FlowJo software (FlowJo LLC, version 9.9.3).

Immunostaining

Sorted cells were collected in 200 µl of 1× PBS and seeded in a Nunc glass-bottom dish (Thermo Fisher Scientific, #150680) previously treated with 200 µl of 20 µg ml^{-1} fibronectin (Sigma-Aldrich, #F1141-2MG) overnight at 4°C. Cells were incubated for 3 h at 23°C, then 1× PBS was substituted with 200 µl growth medium and grown overnight at 23°C.

Cells were fixed for 5 min at room temperature with 4% formaldehyde in 1× PBS and washed once with 200 µl 1× PBS. Cells were then blocked for 30 min at room temperature in blocking solution [1% bovine serum albumin (Sigma-Aldrich, #A3294-10G), 0.1% Triton-X100 (Sigma-Aldrich, X100) in 1× PBS] and incubated for 1.5 h at room temperature with 1:100 anti-green fluorescent protein (GFP) primary antibody (Abcam, ab5450, Lot GR277059-1) in blocking solution [Venus is an improved version of GFP (Nagai et al., 2002)]. Cells were washed twice for 10 min in blocking solution and incubated for 1.5 h in the dark at room temperature with 1:1000 Alexa Fluor 568 goat anti-rat IgG (Life Technologies, A11077, Lot 1512105) in the blocking solution. After three washes of 10 min in 1× PBS, the preparation was overlaid with fluorescence mounting media (DAKO/Agilent Technologies, #S3023), covered with a coverslip and sealed with nail polish.

Imaging of transfected cells

Immunostained samples were imaged using a Leica TCS SP5 II inverted confocal microscope with a 63× immersion oil objective. Acquisition settings were adjusted using Venus-positive cells without primary antibody and Venus-negative cells.

For live imaging, all samples were plated in a µ-Slide 4-well glass-bottom dish (Ibidi, #80427) and grown overnight at 23°C. In the case of pONSY-H2B:Venus transfected cell samples, plated cells were washed once with 200 µl 1× PBS, fixed for 5 min at room temperature with 4% formaldehyde in 1× PBS and washed again as before. Cells were covered using Vectashield with DAPI (Vector, #H-1200). For live imaging of the cytoskeleton, cells were plated in a µ-Slide 4-well Ph+ glass-bottom dish (Ibidi, #80447) in 800 µl of growth medium containing 0.1% low-melting agarose (Sudelab #8085). Wide-field microscopy was performed using a Zeiss Axio Observer Z.1 epifluorescence inverted microscope equipped with LED illumination and a Axiocam 503 mono camera.

Time-lapse videos were recorded using the same microscope. For Movie 1, acquisition was performed at 1 frame/s (fps) and video export was performed at 10 fps. For Movie 2, images were taken every 10 min and video export was performed at 2 fps. A maximum intensity projection was used of two slices from a *z*-stack.

When indicated, membrane and cytoskeleton labelling were additionally imaged using confocal microscopy with an Andor Revolution XD Spinning Disk microscope equipped with an Andor Ixon 897E Dual Mode EM-CCD camera. These images were deconvolved using The Huygens System 17.10-64 Multi-Processing edition software.

Confocal microscopy was performed with a 63× immersion oil objective using either a confocal laser scanning Leica TCS SP5 II microscope or an Andor Revolution XD Spinning Disk microscope equipped with an Andor Ixon 897E Dual Mode EM-CCD camera. These images were deconvolved using The Huygens System 17.10-64 Multi-Processing edition software.

All images were edited using Fiji Imaging Software version 2.0.0-rc-44/1.50e (Schindelin et al., 2012).

Statistical analysis

Results are shown as mean±standard deviation (s.d.) per experiment. The 95% confidence intervals were calculated using the Student's *t*-test. The significance of differences in the percentage of positive cells from single-transfection experiments were tested using the non-parametric Wilcoxon Signed Rank Test for paired samples. All statistical analyses were performed using the R Stats Package version 3.3.1 (R Core Team, 2016).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualisation: H.P.-A., N.R.-R., S.R.N., I.R.-T.; Methodology: H.P.-A., N.R.-R., A.P.-P., A.K., N.S.-P., S.R.N.; Formal analysis: H.P.-A., N.R.-R., A.P.-P., A.K., N.S.-P., S.R.N.; Resources: A.N., H.S.; Writing - original draft: H.P.-A., N.R.-R.; Writing - review & editing: H.P.-A., N.R.-R., A.P.-P., S.R.N., I.R.-T.; Supervision: I.R.-T.; Funding acquisition: I.R.-T.

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Data availability

Capsaspora expression vectors have been deposited in Addgene (www.addgene.org) under the following accession numbers: pONSY (empty) (111873), pONSY-mCherry (111874), pONSY-Venus (111875), pONSY-Lifeact: mCherry (111876), pONSY-CoH2B:Venus (111877) and pONSY-CoNMM:mCherry (111878). The Capsaspora transfection protocol has been uploaded to protocols.io under dx.doi.org/10.17504/protocols.io.p4adqse

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.162107.supplemental

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DEVELOPMENT

Supplementary Materials and Methods

Transfection of *Capsaspora* using Calcium-phosphate precipitation

Adherent stage cells at the exponential growth phase were obtained after a two-day culture passage as follows. Two days before transfection, $1x10^7$ cells were seeded in a 25 cm^2 culture flask containing 5 mL growth medium and grown overnight at 23ºC (Fig. 1A-1). Hereafter, all amounts are indicated per well.

At day 0, $2x10^6$ cells were seeded from this previous confluent culture to attain 90-95% cell confluence at the time of transfection. Cells were seeded in a 12-well plate (Nunc/DDBioLab #55428) containing 600 µL growth medium and grown overnight at 23ºC (Fig. 1A-2). Cell concentration was determined using a Neubauer Chamber Hemocytometer (DDBiolab #900505).

⚠ ***CRITICAL STEP: Adherent stage cells in confluency.*** *Cultures should be fresh to maximize transfection efficiency. Ideally, they should be maintained weekly, and used for transfection at their exponential growth phase. Do not let cultures reach higher cell densities (< $5x10^7$ cells mL^{-1}).*

At day 1, growth medium was replaced by 600 µL of transfection medium (see Reagent preparation), and incubated for 30 min at room temperature (~18ºC) (Fig. 1B-3). During incubation, 1.271 pmols of plasmid DNA for single transfection experiments or 0.636 pmols of each plasmid DNA for co-transfection experiments were diluted in sterile distilled water up to 120 µL plus an additional volume of 150 µL of 2X HBS Buffer. Next, 30 µL of 1.25 M $CaCl_2$ were added dropwise while flickering the tube carefully, reaching a final DNA mix volume of 300 µL. DNA mix was inverted immediately two times to ensure proper mixing of reagents and incubated 10 min at 37ºC (Fig. 1B-4). After incubation, transfection medium was removed and the DNA mix was added dropwise in the centre of the wells. Cells:DNA mix were incubated for 30 min at 18ºC (Fig. 1B-5).

⚠ ***CRITICAL STEP: DNA-Calcium-phosphate precipitates formation.*** *Check the cultures periodically under the microscope to check crystal size. Big cloudy precipitates may compromise transfection efficiency. Instead, verify that small grains of refractant material are spread homogeneously in the plate.*

After this period, an additional volume of 500 µL of transfection medium was added and cells were incubated for a minimum of 4 h at 23ºC (Fig. 1B-6).

⚠ ***NOTE: Transfection medium incubation.*** An incubation of less than 4 h yields lower transfection efficiency. This incubation time can be extended to 6 h.

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After incubation, the medium was removed and an osmotic shock using 110 µL 10% (v/v) glycerol in 1X HBS Buffer was performed, pouring the solution dropwise all over the well for one min at ~18ºC (Fig. 1B-7).

⚠ ***CRITICAL STEP: Glycerol shock.*** *Incubation with glycerol at this concentration should not exceed 1 min, counting from the first droplet, to avoid excessive cell death.*

After the osmotic shock, glycerol solution was removed and cells were grown at 23ºC overnight with 700 µL of growth medium (Fig. 1B-8). Screening of positive cells was performed 18 h post-transfection using fluorescence microscopy and flow cytometry analysis (Fig. 1C).

⚠ ***NOTE: Controls.*** pONSY (empty) transfected cells, mock-transfected cells and non-transfected cells were used as controls.

Transfection Reagents preparation

Growth medium (for 1 L): 10 g Peptone (BD, #211677), 10 g Yeast Extract (BD, #212750), 1 g Yeast nucleic acid (Ribonucleic Acid, Type VI from Torula Yeast) (Sigma, #R-6625), 15 mg Folic acid (Sigma, #F8758) in 880 mL distilled water. Autoclave for 15 min at 121ºC. Cool down and aseptically add 0.4 mL of Hemin stock solution* (Sigma, #H9039), 20 mL Buffer solution** and 100 mL of heat-inactivated Fetal Bovine Serum (Sigma, #F9665-100ml). Filter-sterilise through 0.22 μm and store at 4ºC.

***Hemin stock solution (for 200 mL):** 400 mg NaOH in 200 mL dH_2O. Add 500 mg of Hemin and autoclave 20 min at 121ºC. Store at 4ºC protected from the light.

****Buffer solution (for 1 L):** 18.1 g KH_2PO_4 (Sigma, #P5655), 25 g Na_2HPO_4 (Sigma, #S5136) in 1 L distilled water. Adjust final pH to 6.5 with HCl 37% and filter-sterilise through 0.22 μm. Store at 4ºC.

Transfection medium (for 1 L): 10 g Peptone, 15 mg Folic Acid in 990 mL distilled water. Autoclave for 20 min at 121ºC. Aseptically add 10 mL HEPES 1 M (Sigma, #H4034) to a final concentration of 10 mM and 2.1 g Bis-Tris methane (Sigma, #B9754) final concentration 0.21% w/w. adjust pH to 7.1 with NaOH, filter-sterilise through 0.22 μm and store at 4ºC.

2X HBS (for 250 mL): Dissolve 4 g NaCl (Sigma, #S3014), 0.18 g KCl (Sigma, #P9541), 0.05 g Na_2HPO_4 (Sigma, #S5136), 2.5 g HEPES and 0.5 g D-glucose (Sigma, #G8270) in autoclaved distilled water. Adjust pH to 7.1 with NaOH. Filter-sterilise through 0.22 μm, flash-freeze with liquid Nitrogen and store at -80ºC.

1.25M $CaCl_2$ (for 10 mL): 1.84 g $CaCl_2$ (Sigma, #C1016) in 10 mL autoclaved distilled water. Filter-sterilise through 0.22 μm, flash-freeze with liquid Nitrogen and store at -80ºC.

10% glycerol (for 4 mL): 0.8 mL of filter-sterilised 50% (v/v) glycerol (Sigma, #G7757) in 1.2 mL autoclaved distilled water and 2 mL 2X HBS. Filter-sterilise through 0.22 μm, flash-freeze with liquid Nitrogen and store at -80ºC.

Supplementary Figures

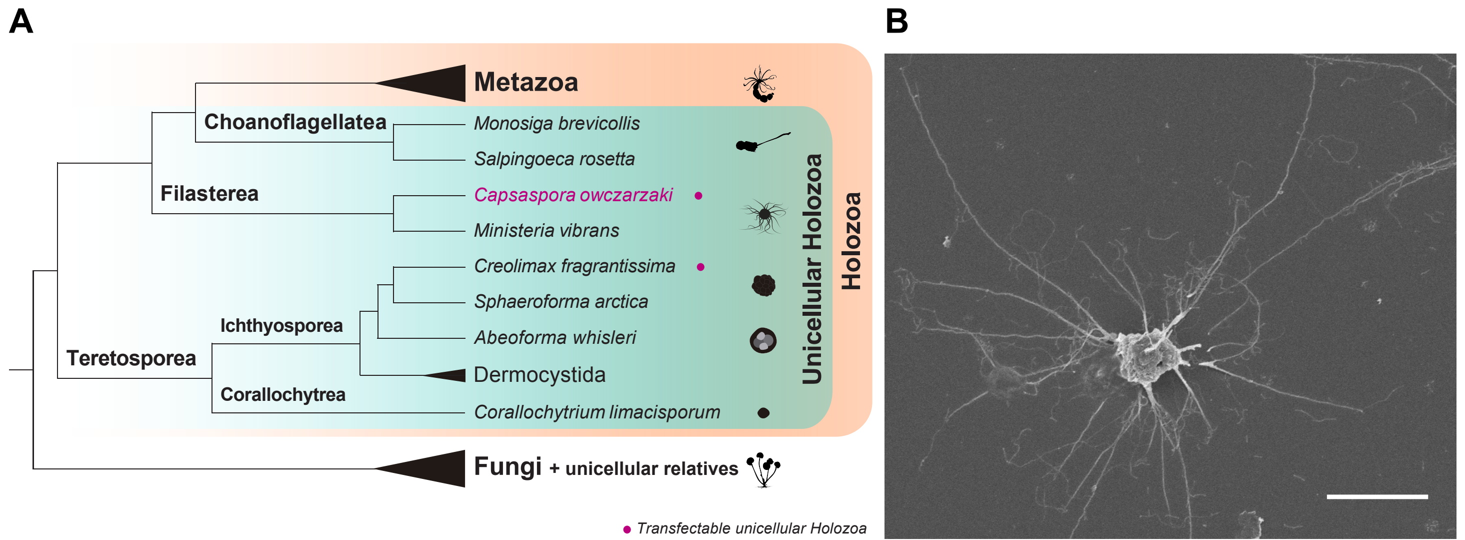


Fig. S1. Transfectable unicellular Holozoa and *Capsaspora owczarzaki*. (A) Metazoa and their unicellular relatives; Choanoflagellatea, Filasterea and Teretosporea, comprise the Holozoa clade. Transfectable unicellular Holozoa to date are *C. fragrantissima* and *C. owczarzaki.* (B) SEM image of a *Capsaspora* cell. Scale bar represents 5 µm.

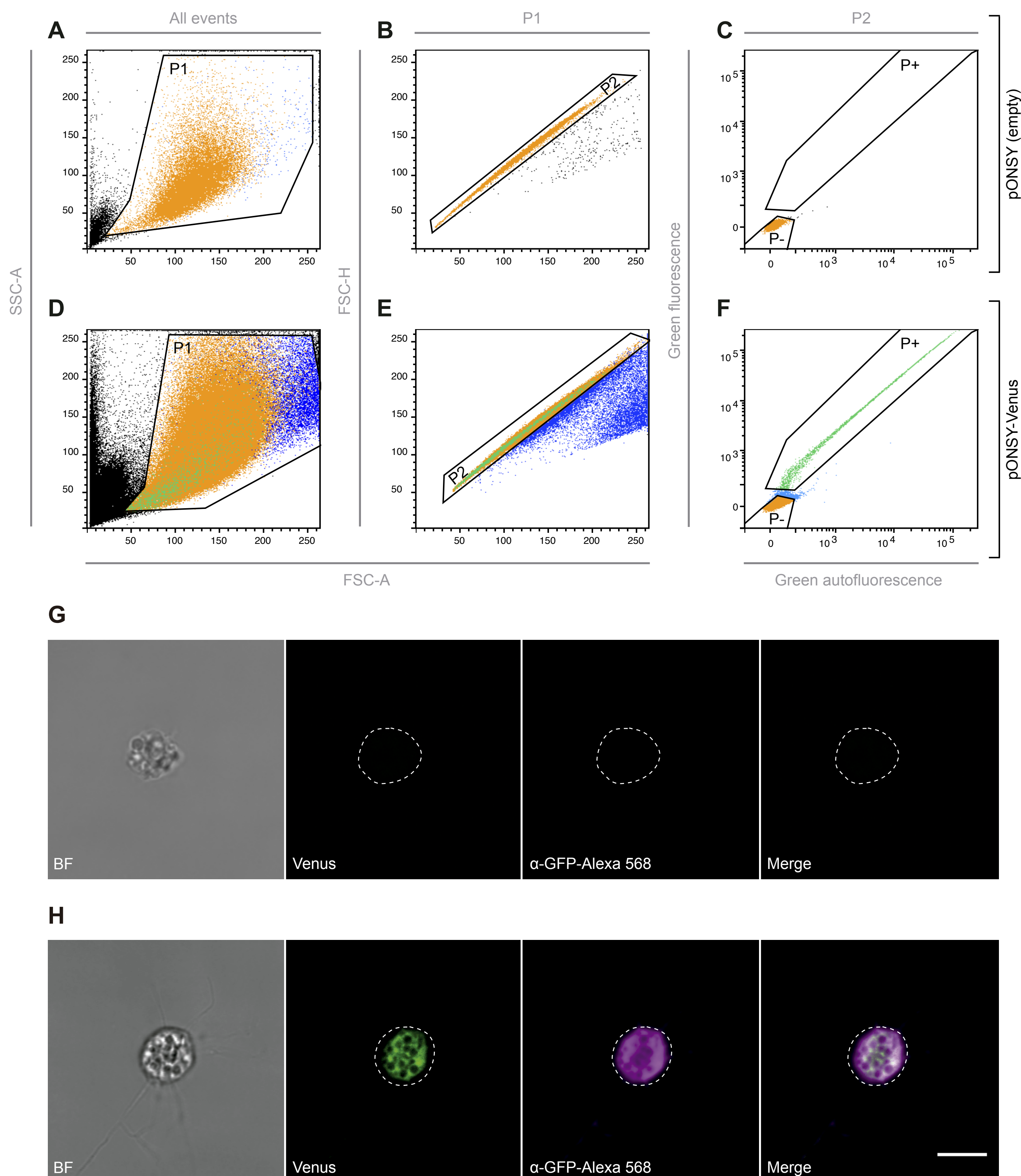


Fig. S2. FACS of *Capsaspora* transfected cells and immunofluorescence validation. (A-C) Cells transfected with pONSY (empty) as control to gate positive and negative populations. (D-F) Cells transfected with pONSY-Venus. Areas selected in (A) and (D) define total population of cells (P1). Areas selected in (B) and (E) define single cells (P2). Areas in (C) and (F) define sorted Venus positive cells (P+) and sorted Venus negative cells (P-), respectively. (G-H) Immunofluorescence validation of Venus expression of P- (G) and P+ (H) sorted populations from (F) using an anti-GFP antibody. Dashed line indicates cell body. Scale bar represents 5 µm.

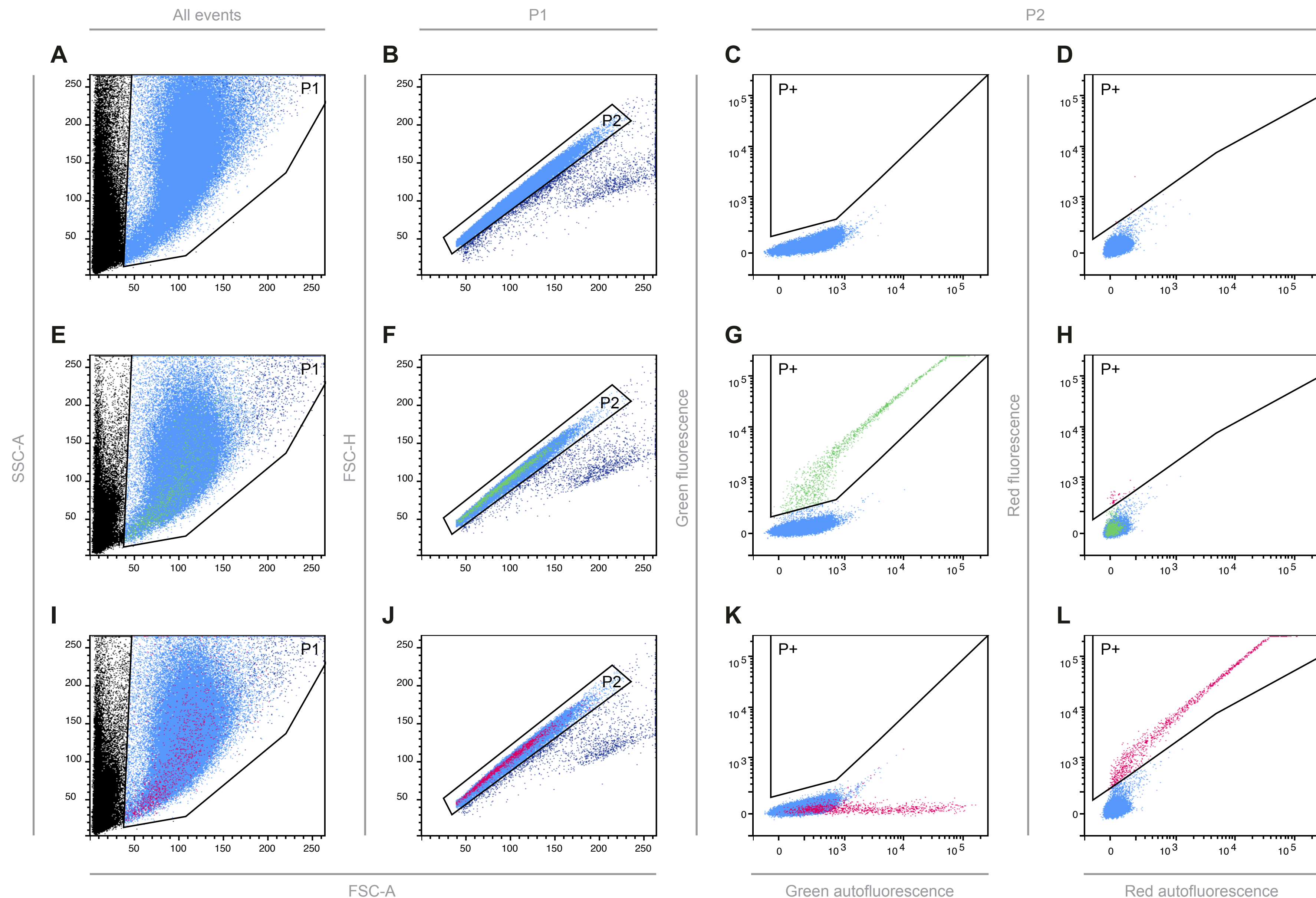


Fig. S3. Flow cytometry analysis of *Capsaspora* transfected cells. (A-D) Cells transfected with pONSY (empty) as control to gate positive and negative populations. (E-H) Cells transfected with pONSY-Venus. (I-L) Cells transfected with pONSY-mCherry. Areas selected in A, E and I define total population of cells (P1). Areas selected in B, F and J define single cells (P2). P+ in C, G and K defines positive cells in the green channel (Venus). P+ in D, H and L defines positive cells in the red channel (mCherry). Figure associated to Fig. 3A-B.

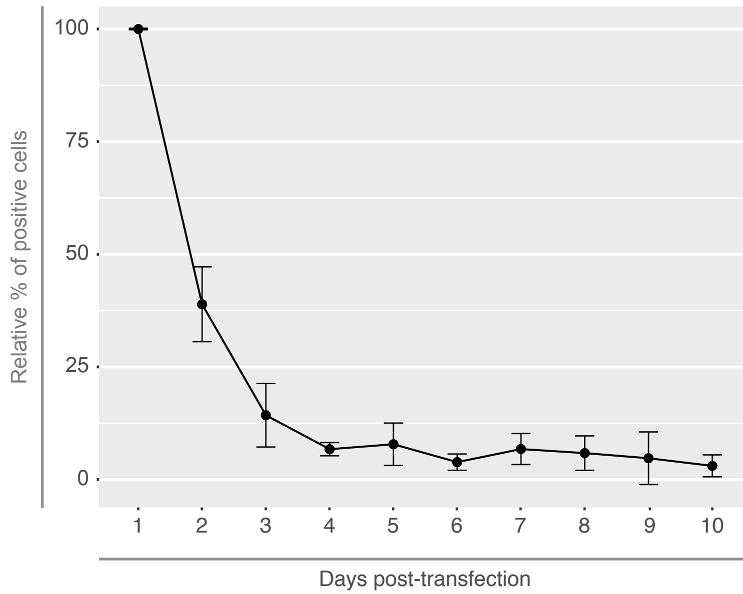


Fig. S4: Persistance of positive cells along 10 days after transfection. Percentage of positive cells transfected with pONSY-Venus, measured every 24h by flow cytometry (number of positive cells at day 1 was considered as 100%). Error bars represent s.d. Figure associated to Table S2.

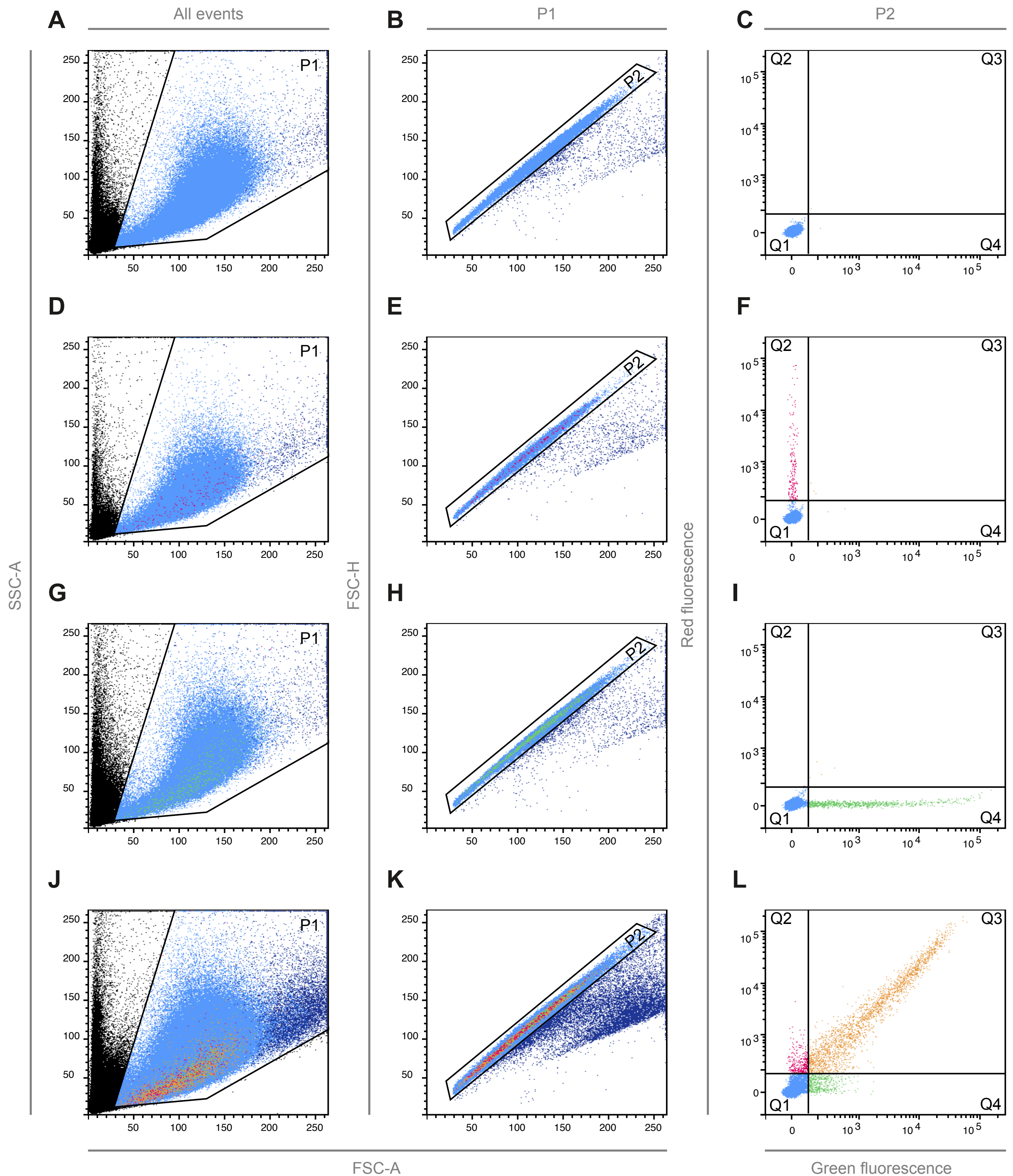


Fig. S5: *Capsaspora* co-transfected with both pONSY-mCherry and pONSY-Venus. (A-C) Cells transfected with pONSY (empty) as control. (D-F) Cells transfected with pONSY-mCherry only. (G-H) Cells transfected with pONSY-Venus only. (J-L) Cells co-transfected with both pONSY-mCherry and pONSY-Venus. Areas selected in panels A,D,G and J define total population of cells (P1). Areas selected in B, E, H, and K define single cells (P2). Quartiles define negative cells (Q1), red fluorescent cells expressing mCherry only (Q2), cells expressing both fluorescent proteins (Q3) and green fluorescent cells expressing Venus only (Q4). Figure associated to Fig. 3F.

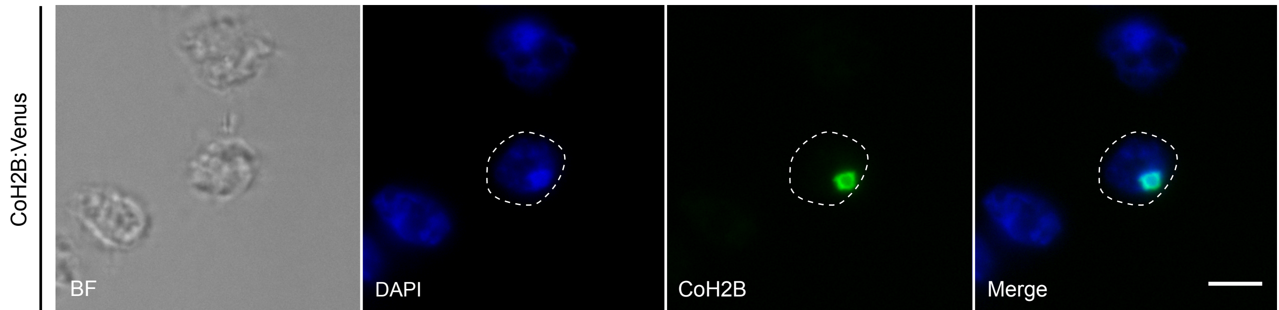


Fig. S6. Localisation of nuclear marker in *Capsaspora* transfected cells. Transfected cells with pONSY-CoH2B:Venus stained with DAPI. Dashed line indicates cell body. Scale bar represents 5 µm.

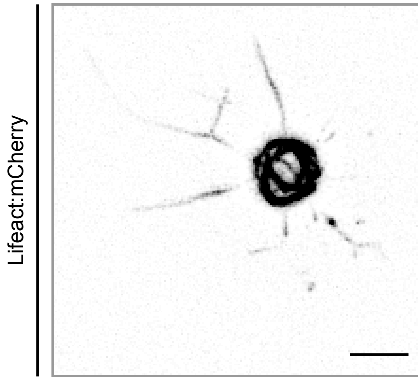
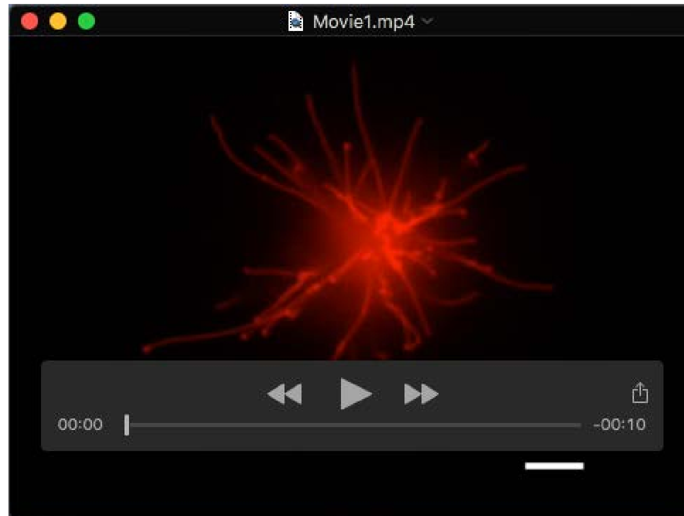
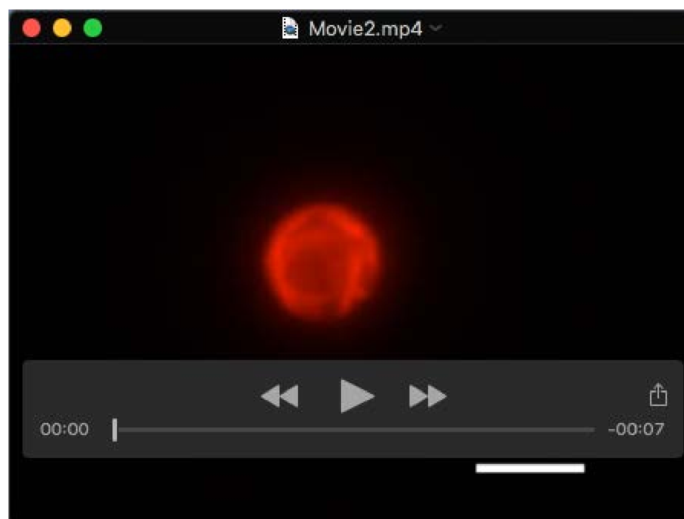


Fig. S7. Labelling the actin cytoskeleton and filopodia in *Capsaspora*. Transfected cell with pONSY-Lifeact:mCherry from Fig. 2C’. Image saturated and inverted to improve visualization of filopodia. Scale bar represents 5 µm.

Supplementary movies



Movie 1. *Capsaspora* filopodia dynamics *in vivo*. Time-lapse of a cell transfected with pONSY:CoNMM-mCherry. Images were taken every second during 100 seconds. Scale bar represents 5 μm.



Movie 2. *Capsaspora* actin cytoskeleton *in vivo*. Time-lapse of a cell transfected with pONSY:Lifeact-mCherry. Images were taken every 10 minutes during 130 minutes. Scale bar represents 5 μm.

Supplementary Tables

Tables S1-S4

Table S1. Flow Cytometry analysis of *Capsaspora* cells transfected with a single vector. Flow cytometry analysis of *Capsaspora* cells transfected with pONSY-Venus (1-7a) or pONSY-mCherry (7b) expression vectors. Results from 7 independent experiments with at least 6 replicates each (n=51) are shown. Transfection efficiency is calculated as the ratio of total number of positive cells (P+) from total number of cells (P2) and represented as mean±s.d per experiment. Table associated to Fig. 3A-D and Fig. S3.

Experiment		Number of cells		Transfection efficiency	
Number	**Sample**	**Total (P2)**	**Positive (P+)**	**(P+/P2)%**	**mean±s.d.**
1	Empty vector	100083	0	0.000	-
	Replicate 1	100152	370	0.369	0.347±0.193
	Replicate 2	100036	512	0.512	
	Replicate 3	100147	633	0.632	
	Replicate 4	100302	219	0.218	
	Replicate 5	99930	150	0.150	
	Replicate 6	100055	200	0.200	
2	Empty vector	100000	0	0.000	-
	Replicate 1	47180	1139	2.414	2.083±0.248
	Replicate 2	52604	1178	2.239	
	Replicate 3	91753	1632	1.779	
	Replicate 4	100000	2114	2.114	
	Replicate 5	100000	2146	2.146	
	Replicate 6	100000	1807	1.807	

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3	Empty vector	100000	0	0.000	-
	Replicate 1	100000	814	0.814	0.859±0.227
	Replicate 2	100000	1332	1.332	
	Replicate 3	100000	673	0.673	
	Replicate 4	100000	820	0.820	
	vReplicate 5	100000	950	0.950	
	Replicate 6	100000	827	0.827	
	Replicate 7	100000	669	0.669	
	Replicate 8	100000	1051	1.051	
	Replicate 9	100000	596	0.596	
4	Empty vector	100003	6	0.006	-
	Replicate 1	100000	1250	1.250	1.047±0.140
	Replicate 2	100000	1091	1.091	
	Replicate 3	100000	1103	1.103	
	Replicate 4	100000	1049	1.049	
	Replicate 5	100000	849	0.849	
	Replicate 6	100000	938	0.938	

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5	Empty vector	100229	9	0.009	-
	Replicate 1	100206	1048	1.046	1.204±0.128
	Replicate 2	100062	1352	1.351	
	Replicate 3	100070	1160	1.159	
	Replicate 4	100075	1368	1.367	
	Replicate 5	100067	1123	1.122	
	Replicate 6	100055	1182	1.181	
6	Empty vector	100000	0	0.000	-
	Replicate 1	100000	944	0.944	1.399±0.621
	Replicate 2	100000	472	0.472	
	Replicate 3	100000	1681	1.681	
	Replicate 4	100000	1802	1.802	
	Replicate 5	100000	2182	2.182	
	Replicate 6	100000	1315	1.315	
7a	Empty vector	12243	0	0.000	-
	Replicate 1	86084	1134	1.317	1.159±0.326
	Replicate 2	100000	559	0.559	
	Replicate 3	100000	1469	1.469	
	Replicate 4	100000	1376	1.376	
	Replicate 5	100000	1129	1.129	
	Replicate 6	100000	1101	1.101	
7b	Empty vector	12243	0	0.000	-
	Replicate 1	100000	980	0.980	1.094±0.148
	Replicate 2	100000	1151	1.151	
	Replicate 3	100000	1284	1.284	
	Replicate 4	100000	1118	1.118	
	Replicate 5	100000	1163	1.163	
	Replicate 6	100000	865	0.865	

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Table S2. Flow Cytometry analysis of *Capsaspora* transfected cells during 10 days. Cells transfected with pONSY-Venus expression vector analysed every 24 hours during 10 days after transfection. Results from 3 independent experiments are shown. Transfection efficiency was calculated as the ratio of total number of positive cells (P+) from total number of cells (P2). Ratio of positive cells was calculated as the percentage of positive cells in a particular day relative to the percentage of positive cells at day 1 and represented as mean±s.d per day. Table associated to Fig. S4.

Experiment		Number of cells		Transfection efficiency		
Days post-transfection	Sample	Total (P2)	Positive (P+)	(P+/P2)%	Ratio %	mean±s.d.
1	Empty vector	21427	0	0.000	-	-
	Replicate 1	295873	313	0.106	100.000	100.000±0.000
	Replicate 2	99871	338	0.338	100.000	
	Replicate 3	99968	79	0.079	100.000	
2	Empty vector	99944	0	0.000	-	-
	Replicate 1	99962	32	0.032	30.260	38.898±8.303
	Replicate 2	99955	134	0.134	39.612	
	Replicate 3	100000	37	0.037	46.820	
3	Empty vector	99962	1	0.001	-	-
	Replicate 1	100000	22	0.022	20.796	14.260±7.044
	Replicate 2	99953	23	0.023	6.799	
	Replicate 3	100000	12	0.012	15.185	
4	Empty vector	100000	0	0.000	-	-
	Replicate 1	100000	8	0.008	7.562	6.731±1.466
	Replicate 2	99705	17	0.017	5.038	
	Replicate 3	100000	6	0.006	7.593	
5	Empty vector	100000	0	0.000	-	-
	Replicate 1	100000	8	0.008	7.562	7.823±4.706
	Replicate 2	99942	11	0.011	3.252	
	Replicate 3	100000	10	0.010	12.654	

6	Empty vector	100000	0	0.000	-	-
	Replicate 1	100000	5	0.005	4.726	3.854±1.809
	Replicate 2	99946	6	0.006	1.744	
	Replicate 3	100000	4	0.004	5.062	
7	Empty vector	100000	0	0.000	-	-
	Replicate 1	100000	11	0.011	10.398	6.757±3.446
	Replicate 2	99973	12	0.012	3.547	
	Replicate 3	100000	5	0.005	6.327	
8	Empty vector	100000	0	0.000	-	-
	Replicate 1	100000	9	0.009	8.508	5.859±3.822
	Replicate 2	99947	5	0.005	1.478	
	Replicate 3	100000	6	0.006	7.593	
9	Empty vector	235982	0	0.000	-	-
	Replicate 1	100000	12	0.012	11.343	4.723±5.841
	Replicate 2	99983	1	0.001	0.296	
	Replicate 3	100000	2	0.002	2.531	
10	Empty vector	186504	1	0.001	-	-
	Replicate 1	100000	6	0.006	5.672	3.031±2.430
	Replicate 2	99709	3	0.003	0.889	
	Replicate 3	100000	2	0.002	2.531	

Table S3. Flow Cytometry analysis of *Capsaspora* cells co-transfected with pONSY-Venus and pONSY-mCherry. Results from 7 independent experiments with 6 replicates each (n=42) are shown. Transfection efficiency is calculated by total number of positive cells (Q2+Q3+Q4) from total number of cells (P2) and represented as mean±s.d per experiment. Relative percentages of Double, Venus and mCherry expression were calculated as number of double positive cells (Q2) or number of Venus positive cells (Q4) or number of mCherry positive cells (Q3) from total number of positive cells (Q2+Q3+Q4), respectively, and represented as mean±s.d per experiment. Table associated to Fig. 3F and G and Fig. S5.

Experiment		**Number of cells**						**Transfection efficiency**		**Relative % over total number of positive cells**		
Number	**Sample**	**Total (P2)**	**Negative (Q1)**	**mCherry (Q2)**	**Double (Q3)**	**Venus (Q4)**	**Total positive (Q2+Q3+Q4)**	**% Total positive**	**% Double (Q3/P2)%**	**% Double**	**% Venus**	**% mCherry**
1	Empty vector	100000	99996	1	2	1	4	0.004				
	Control Venus	100000	98177	1	2	1820	1823	1.823				
	Control mCherry	100000	99689	310	1	0	311	0.311				
	Replicate 1	520488	516878	706	2346	558	3610	0.694	0.451	64.986	15.457	19.557
	Replicate 2	358753	355200	731	2354	468	3553	0.990	0.656	66.254	13.172	20.574
	Replicate 3	322368	319598	501	1818	451	2770	0.859	0.564	65.632	16.282	18.087
	Replicate 4	408411	404855	620	2339	597	3556	0.871	0.573	65.776	16.789	17.435
	Replicate 5	426129	422602	592	2357	578	3527	0.828	0.553	66.827	16.388	16.785
	Replicate 6	380788	377503	501	2305	479	3285	0.863	0.605	70.167	14.581	15.251
	Mean±s.d.							0.851±0.095	0.567±0.068	66.607±1.850	15.445±1.364	17.948±1.918
2	Empty vector	100000	99988	2	5	5	12	0.012				
	Control Venus	100000	98648	7	2	1343	1352	1.352				
	Control mCherry	100000	98879	1116	2	3	1121	1.121				
	Replicate 1	209708	206792	191	2247	478	2916	1.391	1.071	77.058	16.392	6.550
	Replicate 2	249962	247044	230	2217	471	2918	1.167	0.887	75.977	16.141	7.882
	Replicate 3	231155	228463	180	2154	358	2692	1.165	0.932	80.015	13.299	6.686
	Replicate 4	342982	340145	222	2220	395	2837	0.827	0.647	78.252	13.923	7.825
	Replicate 5	231457	228666	222	2203	366	2791	1.206	0.952	78.932	13.114	7.954
	Replicate 6	348075	345308	233	2167	367	2767	0.795	0.623	78.316	13.263	8.421
	Mean±s.d.							1.092±0.233	0.852±0.179	78.091±1.416	14.355±1.508	7.553±0.755
3	Empty vector	100677	100662	4	9	2	15	0.015				
	Control Venus	100712	99079	5	8	1620	1633	1.621				
	Control mCherry	100673	99677	990	5	1	996	0.989				
	Replicate 1	374093	371723	186	1881	303	2370	0.634	0.503	79.367	12.785	7.848
	Replicate 2	380307	377928	145	1940	294	2379	0.626	0.510	81.547	12.358	6.095
	Replicate 3	292266	289888	132	1972	274	2378	0.814	0.675	82.927	11.522	5.551
	Replicate 4	218046	215653	112	1910	371	2393	1.097	0.876	79.816	15.504	4.680
	Replicate 5	279028	276622	138	1937	331	2406	0.862	0.694	80.507	13.757	5.736
	Replicate 6	179012	176633	140	1892	347	2379	1.329	1.057	79.529	14.586	5.885
	Mean±s.d.							0.894±0.275	0.719±0.215	80.616±1.386	13.419±1.480	5.966±1.043

4	Empty vector	100000	100000	0	0	0	0	0				
	Control Venus	100000	99020	5	3	972	980	0.980				
	Control mCherry	100000	99794	205	1	0	206	0.206				
	Replicate 1	459409	456258	383	2359	409	3151	0.686	0.513	74.865	12.980	12.155
	Replicate 2	412692	409512	375	2388	417	3180	0.771	0.579	75.094	13.113	11.792
	Replicate 3	837030	833626	480	2436	488	3404	0.407	0.291	71.563	14.336	14.101
	Replicate 4	402481	399267	481	2387	346	3214	0.799	0.593	74.269	10.765	14.966
	Replicate 5	332644	329608	357	2352	327	3036	0.913	0.707	77.470	10.771	11.759
	Replicate 6	582324	579008	502	2398	416	3316	0.569	0.412	72.316	12.545	15.139
	Mean±s.d.							0.691±0.180	0.516±0.147	74.263±2.118	12.418±1.410	13.319±1.597
5	Empty vector	100000	99986	8	5	1	14	0.014				
	Control Venus	100000	99573	4	2	421	427	0.427				
	Control mCherry	100000	99983	16	1	0	17	0.017				
	Replicate 1	464014	461024	294	2300	396	2990	0.644	0.496	76.923	13.244	9.833
	Replicate 2	425876	423064	259	2300	253	2812	0.660	0.540	81.792	8.997	9.211
	Replicate 3	616927	613484	411	2300	732	3443	0.558	0.373	66.802	21.261	11.937
	Replicate 4	598473	595286	342	2300	545	3187	0.533	0.384	72.168	17.101	10.731
	Replicate 5	491626	488340	355	2300	631	3286	0.668	0.468	69.994	19.203	10.803
	Replicate 6	801753	798352	434	2300	667	3401	0.424	0.287	67.627	19.612	12.761
	Mean±s.d.							0.581±0.095	0.425±0.093	72.551±5.805	16.57±4.623	10.879±1.309
6	Empty vector	100262	100248	9	4	1	14	0.014				
	Control Venus	100194	99427	3	0	764	767	0.766				
	Control mCherry	100000	99676	324	0	0	324	0.324				
	Replicate 1	1172129	1168353	697	2517	562	3776	0.322	0.215	66.658	14.883	18.459
	Replicate 2	780326	776622	645	2520	539	3704	0.475	0.323	68.035	14.552	17.414
	Replicate 3	1036442	1032768	645	2513	516	3674	0.354	0.242	68.400	14.045	17.556
	Replicate 4	725427	721735	692	2511	489	3692	0.509	0.346	68.012	13.245	18.743
	Replicate 5	757172	753425	709	2531	507	3747	0.495	0.334	67.547	13.531	18.922
	Replicate 6	697333	693820	643	2460	410	3513	0.504	0.353	70.026	11.671	18.303
	Mean±s.d.							0.443±0.083	0.302±0.059	68.113±1.113	13.654±1.148	18.233±0.620
7	Empty vector	12243	12230	7	5	1	13	0.106				
	Control Venus	100000	98740	15	9	1236	1260	1.260				
	Control mCherry	100000	98809	1185	4	2	1191	1.191				
	Replicate 1	161940	160433	322	981	204	1507	0.931	0.606	65.096	13.537	21.367
	Replicate 2	155693	154319	172	1000	202	1374	0.883	0.642	72.780	14.702	12.518
	Replicate 3	123025	121624	186	1000	215	1401	1.139	0.813	71.378	15.346	13.276
	Replicate 4	163206	162117	174	783	132	1089	0.667	0.480	71.901	12.121	15.978
	Replicate 5	258248	256791	233	1000	224	1457	0.564	0.387	68.634	15.374	15.992
	Replicate 6	122506	121096	202	1000	208	1410	1.151	0.816	70.922	14.752	14.326
	Mean±s.d.							0.889±0.240	0.624±0.173	70.119±2.826	14.305±1.260	15.576±3.165

Table S4. List of primers used to build *Capsaspora* expression vectors with reporter genes. Restriction enzymes sites are underlined. CoNMM sequence plus 7 extra aminoacids is highlighted in red.

Region/Gene	*Capsaspora* gene ID	Primer name	Sequence 5'-3'
CoEF1α promoter	CAOG_07807	1	CTGGTACCAAATGCACAGTTAGCAACGACC
		2	GATATCACTAGTCCCGGGATCCTGTGAAGGTTGTTCTG
		3	AAATGCACAGTTAGCAACGACC
CoEF1α terminator	CAOG_07807	4	GAGCTGTACAAGTAAATTTTGTGTTTGCCAAG
		5	CATTGCTAGTGCTGTTCTCACC
		6	GACCGCGGTGAGAACAGCACTAGCAATG
		7	CCCGGGACTAGTGATATCTGAATTTTGTGTTTGCCAAGACAC
		8	CGCCAGTGTGATGGATTGAAAGCTTCCGCGGTGA
mCherry/Venus	-	9	CCCGGGACTAGTGATATCATGGTGAGCAAGGGCG
		10	CTTGGCAAACACAAAATTTACTTGTACAGCTC
CoSrc2 NMM	CAOG_06360	11	TATACCCGGG**ATGGGCTGCTCCAACTCTAAACCGCACGACCCGTCCGATTTCAAGGTTTCCCCTTCTGGCGTTGCGTCCAACAGC**ATGGTGAGCAAGGGCGAGGAG
		12	TTACTTGTACAGCTCGTCCATG
CoH2B	CAOG_01818	13	TACCCGGGATGCCGCCGAAGGTC
		14	TAACTAGTCTTGGCGCCGGAGGT
Lifeact	-	15	CCCGGGACCATGGGTGTGGCAGACCTGATTAAGAAGTTCGAGAGCATT
		16	TCTAGATGGTGGGTCACCCTCCTCCTTGCTAATGCTCTCGAACTTCTT