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CHATraffic! Identification and Characterization of Tunneling Nanotubes for Intercellular Trafficking

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Tunneling nanotubes (TNTs) are thin membranous channels providing a direct cytoplasmic connection between remote cells. They are commonly observed in different cell cultures and increasing evidence supports their role in intercellular communication, pathogen, and amyloid proteins transfer. However, the study of TNTs presents several pitfalls (e.g., difficulty in preserving such delicate structures, possible confusion with other protrusions, structural and functional heterogeneity, etc.) and therefore requires thoroughly designed approaches. The methods described in this unit represent a guideline for the characterization of TNTs (or TNT-like structures) in cell culture. Specifically, optimized protocols to (1) identify TNTs and the cytoskeletal elements present inside them; (2) evaluate TNT frequency in cell culture; (3) unambiguously distinguish them from other cellular connections or protrusions; (4) monitor their formation in living cells; (5) characterize TNTs by a micropatterning approach; and (6) investigate TNT ultrastructure by cryo-EM are provided. Finally, this unit describes how to assess TNT-mediated cell-to-cell transfer of cellular components, which is a fundamental criterion for identifying functional TNTs.

Basic Protocol 1: (Identification of Tunneling nanotubes)

Alternate Protocol 1 : (Identifying the cytoskeletal elements present in Tunneling Nanotubes)

Alternate Protocol 2: (Distinguishing Tunneling Nanotubes from intercellular bridges formed during cell division)

Basic Protocol 2: (Deciphering Tunneling Nanotube formation and lifetime by live fluorescent microscopy)

Alternate Protocol 3: (Deciphering Tunneling Nanotube Formation Using A Live- Compatible Dye)

Basic Protocol 3: (Assessing Tunneling Nanotubes functionality in intercellular transfer)

Alternate Protocol 4 (Flow cytometry approach to quantify the rate of vesicle or mitochondria transfer)

Support Protocol 1 : (Controls to support TNT-mediated transfer)

Basic Protocol 4 (Studies of Tunneling Nanotubes by cell micropatterning)

Basic Protocol 5 (Characterization of the ultrastructure of Tunneling Nanotube by Cryo-Em)

Keywords: tunneling nanotubes (TNTs) • intercellular communication • cytoskeleton • micropatterning • correlative cryo-electron microscopy (cryo-EM)

Tunneling nanotubes (TNTs) consist of F-actin-based tubular structures that connect the cytoplasm of remote cells without touching the substratum. They represent one of the most common mechanisms of cell-to-cell communication identified in a variety of cell types, from epithelial and fibroblastic to neuronal cells. Several studies have reported evidence for their existence *in vivo* and they also appear to be implicated in essential processes such as development and immunity (Abounit and Zurzolo, 2012; Cordero Cervantes and Zurzolo, 2021; Korenkova et al., 2020). TNTs proliferate in several pathological conditions such as viral infections, neurodegenerative and cancer diseases. In cancer, functional TNTs have been shown in a variety of tumors using *in vitro* and *ex vivo* models where they could be exploited as route for the exchange of material between cancer cells or with the tumor microenvironment. As consequence of this transfer, cells can acquire new abilities as enhanced metabolic plasticity, migratory phenotype, angiogenic ability and therapy-resistance (Osswald et al., 2015; Pinto et al., 2021, 2020; Watson et al., 2023). In addition, we and others identified TNTs as effective routes for cell-to-cell spreading of protein aggregates involved in neurodegenerative diseases (NDs) (e.g. prion,

huntingtin, tau, beta-amyloid, and α -synuclein) (Abounit et al., 2016; Chastagner et al., 2020; Dilsizoglu Senol et al., 2021; Vargas et al., 2019) and in viral infection (Jansens et al., 2020; Pepe et al., 2022).

In this protocol, a well-established methodology to characterize the formation, functionality, and ultrastructure of TNTs in cellular models *in vitro* is presented. To this end, several fundamental issues have been determined: (1) identification of TNTs, (2) study of TNT formation, (3) analysis of TNT function, (4) study of the cytoskeletal composition and its role in cargo transfer, (5) differentiation of TNTs from filopodia in a controlled environment by micropatterning, and (6) study of TNT ultrastructure, for which detailed protocols are provided in order to identify specific pitfalls and suggest alternative solutions.

As extensive work has been done on TNT formation in neuronal cells, the techniques provided herein have been set up using a catecholaminergic mouse neuronal cell line (CAD cells; Qi et al., 1997), a human neuroblastoma cell line (SH-SY5Y cells; Dilsizoglu Senol et al., 2019; Pepe et al., 2023, 2022; Sartori-Rupp et al., 2019) and a human glioblastoma cell line (U-251; Pinto et al., 2021). However, the authors believe that these established protocols are useful for identifying and characterising TNT structures and functions in most other cell types.

Because TNTs are transient and fragile structures, the conditions for observation must be optimal. Therefore, all of the necessary parameters to properly observe TNTs *in vitro* are detailed in Basic Protocol 1. Alternate Protocol 1 also describes the procedures necessary to characterize the cytoskeletal content of TNTs. Finally, because cytokinetic bridges connecting two sister cells during cell division could resemble TNTs, a method has been established in Alternate Protocol 2 to discriminate TNTs from cytokinetic bridges.

To fully characterize TNTs *in vitro*, one must decipher their mechanism of formation. To date, two modes of formation have been proposed and both require cell movement and cytoskeleton remodeling. In the actin-driven mechanism, cells extend a filopodia-like protrusion that will fuse with the edge of neighboring cells resulting in TNT formation. The alternative mechanism known as cell dislodgment occurs when two cells close to each other move away in the opposite direction. During this movement, cells draw out membrane tethers, which lead to TNT formation (Fig. 1). It is not clear yet whether these two mechanisms lead to the same tubular structures and whether cells use both mechanisms to form TNTs (Ljubojevic et al., 2021; Marzo et al., 2012). Thus, Basic Protocol 2 describes a method using spinning disk confocal microscopy to monitor TNT formation and lifetime in living cells where the membrane and actin cytoskeleton were labeled. Furthermore, the use of a live-cell compatible plasma membrane dye to label TNTs is discussed in Alternate Protocol 1.

As TNTs are morphologically similar to sensory filopodia, traditional culture methods hamper the proper identification of TNTs if cells are grown in an unconstrained manner where cell-to-cell separations approach the typical length scales that most filopodia are able to achieve ($<5 \mu\text{m}$) (Ljubojevic et al., 2021; Mattila and Lappalainen, 2008; Mogilner and Rubinstein, 2005). Thus, Basic Protocol 4 describes a micropatterning strategy that could be used to investigate TNT formation, functionality, and ultrastructure.

There are two major types of TNTs depending on their cytoskeletal composition: actin-based TNTs (Type I TNTs), which are thin and have short lifetimes, typical from neuronal like cells (Sartori-Rupp et al., 2019), and microtubule-based TNTs (Type II TNTs) that are thicker and more stable. Interestingly, the latter type of TNT increases under stress conditions, such as cancer, and is involved in mitochondrial transfer as a rescue mechanism (Sáenz-de-Santa-María et al., 2017; Wang and Gerdes, 2015a). Specifically, in Alternate Protocol 1 we will describe how to investigate the cytoskeletal composition and mitochondria transfer in a glioblastoma cell line (U251) having microtubule- and actin-based TNTs.

Furthermore, once TNTs have been identified, it is important to investigate their functionality. Because several specific cargoes have been shown to transfer via TNTs (from molecules to pathogens)(Cordero Cervantes and Zurzolo, 2021), a functional assay that measures the transfer of nonspecific vesicles by fluorescence microscopy is presented in this unit. Specifically, Basic Protocol 3 details how to monitor qualitatively and quantitatively TNT-mediated vesicle transfer using a co-culture system of donor and acceptor cell populations by fluorescence microscopy. Alternate Protocol 1 details another method to quantify vesicle transfer and mitochondria by using flow cytometry (FACs). A method to rule out other cell-to-cell transfer mechanisms (secretion, endocytosis) by using a co-culture system where the two cell populations are not in physical contact (e.g., using a transwell filter) or where acceptor cells are challenged with conditioned medium of the donor cells is also provided.

The limited resolution of standard optical-based imaging approaches cannot provide definitive information about the nature and structure of TNTs. To answer fundamental questions about their structure/function and overcome fluorescence microscopy limitations, we have developed a correlative cryo-electron microscopy (cryo-EM) approach to assess TNT architecture under the closest to native conditions (Pepe et al., 2023, 2022; Sartori-Rupp et al., 2019). These techniques allowed us to assess: (1) whether cargo (e.g., organelles, amyloid proteins, viral particles) were shuttled within TNTs, or on the outside of the membrane; (2) which motor proteins are involved in the cargo transfer through TNTs; (3) discriminate their cytoskeletal composition (i.e., if they contain actin, microtubules or both); and (4) analyze the actin polarity within TNTs (Pepe et al., 2022; Sartori-Rupp et al., 2019). In Basic Protocol 6 we describe the cryo-sample preparation workflow to perform tomography on TNTs connecting neuronal cells.

BASIC PROTOCOL 1

IDENTIFICATION OF TUNNELING NANOTUBES

Two models for TNT formation exist and both require cell movement and cytoskeleton remodeling (Fig. 1). Therefore, to achieve good conditions for TNT formation, several cell culture parameters (detailed below) must be optimized.

The first key parameter that must be established is the optimal confluency of cells. Cells should be close enough (sub-confluent) to promote TNT formation. A high or low confluence of cells could impair TNT formation.

In addition to the confluence conditions, the cell growth support should also allow optimal cell motility. Cells must be adherent but motile enough to be capable of undergoing one or both mechanisms of formation. Therefore, different kinds of supports (e.g., polylysine, fibronectin, or ibidi-treated support) must be tested to meet adherence and motility requirements for TNT formation in the specific cell type.

Finally, one important aspect that explains why TNTs are difficult to observe is their highly dynamic structure. In addition, they are sensitive to light exposure, shearing force, and chemical fixation (Cordero Cervantes and Zurzolo, 2021; Rustom et al., 2004). Therefore, a special effort must be made in the fixation procedure to avoid TNT alteration or loss. Once all criteria are fulfilled, TNTs are stained and properly imaged to quantify TNT number, length, and diameter.

Since no specific TNT markers have been discovered, a lectin-binding protein is used to stain and delineate the plasma membrane (Fig. 2; Fig. 3A). Once imaged, it is important to discriminate TNTs from any cytoplasmic projections (e.g., filopodia). To do so, only projections connecting remote cells and present in middle and/or upper stacks of the picture in Z dimension are considered as TNTs. The major distinguishing feature of TNTs that connect distant cells in vitro, is the fact that they do not touch the substrate.

Materials

CAD cells (Sigma, cat. no. 08100805)
SH-SY5Y cells (ATCC CRL-2266)
CAD cell growth medium (see recipe in Reagents and Solutions)
SH-SY5Y cell growth medium (Euroclone, cat. no. ECB2000L)
Fixative 1 (see recipe in Reagents and Solutions)
Fixative 2 (see recipe in Reagents and Solutions)
1× PBS
Alexa Fluor conjugate of wheat germ agglutinin (Molecular Probes)
HCS CellMask Blue stain (Molecular Probes)
Aqua-Poly/Mount (Polysciences)
25 cm² culture flasks
37°C incubator
Hemocytometer or automated cell counter
35 mm μ-dishes, high, tissue-culture-treated (ibidi)
24 mm round coverslips
Inverted confocal fluorescence microscope
Imaging software

Protocol steps with *step annotations*

Culture and fix CAD cells for TNT observation

1. Culture CAD cells in a 25 cm² culture flask with CAD cell growth medium in a 37°C incubator until they reach 80% confluency.
Plate 500,000 to 1,000,000 cells in 7 ml culture medium to obtain 80% confluency in 72 h. However, this number depends on the cell cycle of the cell line.
2. Remove growth medium by aspiration and briefly rinse cells with 3 mL fresh growth medium.
3. Add enough growth medium to completely cover cells (~2 mL) and mechanically detach them from the plastic dish.
For other cell types, trypsin-EDTA can be used to detach cells; in which case, rinse cells with PBS before trypsinization.
4. Add 6 mL of cell growth medium and resuspend cells by pipetting up and down

several times.

The tip of the pipet should be in close contact with the bottom of the flask to separate the cells and disrupt cell clumps. Pipette up and down slowly to avoid producing bubbles.

5. Use a hemacytometer or an automated cell counter to count cells.
6. Plate different amounts of cells in 35 mm μ -dishes in a final volume of 2 mL. Incubate cells 1 to 24 h in a 37°C incubator.

The number of cells plated ranges from 100,000 to 700,000 cells depending on the following incubation times. For CAD cells, one should plate 100,000 to 250,000 cells for longer culture durations (12 to 24 h), or 500,000 to 700,000 cells for shorter culture durations (1 to 12 h). If other cells are used, the required amounts of cells to plate may vary. Thus, the quantity of cells plated must be standardized. It is also necessary to take into account the nature of the cell type because some cells may not be healthy being cultured at subconfluent levels. Moreover, the time-course for monitoring TNTs will depend on the rapidity of cells to adhere.

7. After the desired incubation time, add a few drops of Fixative 1 into the medium of each 35-mm μ -dish and incubate 4 min at room temperature.

After the incubation time, cells must be manipulated very carefully to avoid TNT breakage. If cells are in optimal conditions for TNT formation, it is possible to visualize some cells connected by TNTs with a brightfield microscope.

8. Remove the medium containing drops of Fixative 1 and add 500 μ l of Fixative 1. Incubate 15 min at 37°C.

The amount of fixative added should be enough to cover all cells.

9. Remove Fixative 1 and add 500 μ l of Fixative 2. Incubate 15 min at 37°C.

Since glutaraldehyde (Fixative 1) can induce photoenhancement, a step of autofluorescence quenching can be added here (e.g., sodium borohydride, toluidine blue, ammonium chloride).

10. Remove Fixative 2 by aspiration and rinse three times, 30 S each time, with 1 mL of 1 \times PBS.

Culture and fix SH-SY5Y cells for TNT observation

11. Culture SH-SY5Y cells in a 25 cm² culture flask with SH-SY5Y cell growth medium in a 37°C incubator until they reach 80% confluency.

Plate 2,000,000 cells in 5 mL culture medium to obtain 80% confluency in 48h .

12. Remove the growth medium by aspiration and briefly rinse cells with 3 mL fresh growth medium.
13. Detach them with 1 mL trypsin from the 25 cm² culture flask.

SH-SY5Y cells are detached rapidly; 1 minute incubation with trypsin at 37°C is sufficient.

14. Add 5 mL of cell growth medium to collect cells following trypsin detachment and transfer them to a 15 mL tube; centrifuge at ~~800 g~~ 1000 rpm for 5 minutes.
15. Add 5 mL of cell growth medium and resuspend cells by pipetting up and down several times.

From our experience, the tip of the pipet should be in close contact with the bottom of the tube to separate the cells and disrupt cell clumps. We suggest using a 5 mL pipette to homogenize the cellular suspension.

16. Use a hemacytometer or an automated cell counter to count cells.
17. Plate the amount of cells in 35 mm μ -dishes in a final volume of 1 mL. Incubate cells for 24 h in a 37°C incubator.

Note: To count TNTs connecting SH-SY5Y cells, plate 400,000 cells in 35 mm ibidi μ -dishes for 24 h while plate 100,000 cells on 24 mm coverslips for 24 h.

18. After a 24 h incubation time, remove the growth medium and add 500 μ L Fixative 1 to each 35 mm μ -dish and incubate 15 min at 37°C; the amount of fixative added should be enough to cover all cells.

After the incubation time, cells must be manipulated very carefully to avoid TNT breakage. If cells are in optimal conditions for TNT formation, it is possible to visualize some cells connected by TNTs with a brightfield microscope.

19. Remove Fixative 1 and add 500 μ L of Fixative 2. Incubate for 15 min at 37°C.

It is important not to carry out washes with 1 \times PBS between fixative 1 and 2 to not break TNTs.

20. Remove Fixative 2 and rinse three times, 5 min each time, with 1 mL of 1 \times PBS.

Stain membrane for TNT imaging

21. Prepare a 1:300 (v/v) dilution of fluorescent wheat germ agglutinin (WGA) (1 mg/ml stock solution) in 1 \times PBS and add 500 μ L of the diluted solution to cells. Incubate 20 min at room temperature in the dark.

WGA is a lectin-binding protein that binds glycolipids and glycoproteins. When conjugated with a fluorophore, it is used to stain the plasma membrane. Different fluorophores are coupled to WGA (e.g., Alexa Fluor 350, 488, 555, 594, and 633). The best fluorophores for TNT imaging are Alexa Fluor 488 and Alexa Fluor 594. It is strongly recommended to use glutaraldehyde in the fixative solution because it prevents internalization of WGA.

22. Wash thoroughly three times, 30 S each time, with 1 mL of 1 \times PBS.
23. Prepare a 1:10,000 (v/v) dilution of HCS CellMask Blue stain (10 mg/ml stock solution) in 1 \times PBS and add 500 μ L of the diluted solution onto cells. Incubate 20 min at room temperature in the dark.

HCS CellMask Blue is taken up by cells and stains the cytosol. This allows for cell number quantification. Additionally, DAPI (nuclear marker) can be added afterwards.

24. Wash thoroughly three times, 30 S each time, with 1 mL of 1× PBS.
25. Add 10 to 12 drops of Aqua-Poly/Mount mounting medium onto cells and place a 24mm round coverslip on top of the cells. Incubate 20 min at room temperature in the dark, then store at least 20 min at 4°C in the dark before imaging.
26. Using an ~~an~~ **inverted**-confocal microscope, image cells with a 40× objective. Choose random fields of subconfluent cells. Take several Z-stacks from the bottom to the top of the cells. Acquire at least ten pictures to get 150 to 300 cells in total.
27. Count total number of cells and the cells connected by TNTs to obtain the percentage of TNT-connected cells in the population. **Use several software (e.g. ICY and /or Fiji)** to measure length and diameter of TNTs.

Use imaging software to process the pictures (e.g., <http://icy.bioimageanalysis.org/>, imagej.nih.gov/ij/). TNT identification is based on morphological features: TNTs are protrusions connecting remote cells and do not touch the substratum.

ALTERNATE PROTOCOL 1

IDENTIFYING THE CYTOSKELETAL ELEMENTS PRESENT IN TUNNELING NANOTUBES

Classical TNTs have been described as F-actin based TNTs by Rustom et al, 2004. Following this first report, numerous studies in a wide variety of cell lines provided further evidence that F-actin is a typical component of TNTs (Dupont et al., 2018; Sartori-Rupp et al., 2019). In addition, the actin cytoskeleton seems to play a crucial role in TNT formation since actin-depolymerizing drugs such as latrunculin **A** were shown to inhibit de novo establishment of these cell-to-cell connections (Bukoreshtliev et al., 2009; Schiller et al., 2013). However, in addition to containing actin, several studies since Rustom et al, 2004 have described the existence of TNTs with microtubules and/or intermediate filaments, vimentin, and cytokeratin in cells undergoing an epithelial-mesenchymal transition (Resnik et al., 2019; Sáenz-de-Santa-María et al., 2017; Veranič et al., 2008). TNTs can be classified into two major groups: (1) Type I TNTs, also known as classical TNTs, are short (100-200 nm), very thin ($\leq 0.7 \mu\text{m}$), exhibit short lifetimes (stability, ~20 min) and are made of only actin; and (2) Type II TNTs are long (up to 250 μm), thick ($\geq 0.7 \mu\text{m}$), more stable ($\geq 1-2$ h) and are composed of microtubules and/or intermediate filaments in addition to actin. Contrary to the TNTs containing only actin, Type II TNTs are characterized by the bidirectional transfer of cellular material dependent on microtubule-based molecular motor proteins (Taiarol et al., 2021). TNTs containing microtubules have also been reported in different types of cells such as microglial cells (Chakraborty et al., 2023), in cardiomyocyte/cardiofibroblast co-cultures (He et al., 2011), and between immature hippocampal neurons and astrocytes (Wang et al., 2012). In this latter case, 35% of the TNT connections were F-actin negative. Furthermore, the presence of microtubules in TNTs is a common feature in several tumor cells (Pinto et al., 2020; Saha et al., 2022) and has been associated with stress responses and the transfer of mitochondria as a mechanism of rescue (Boukelmoune et al., 2018; Wang and Gerdes, 2015a), supporting a proposed role for microtubule-positive TNTs in tumor progression (Pinto et al., 2020). On the contrary, to date, no microtubule-containing TNTs were reported

in SH-SY5Y, CAD or HEK cells (Abounit and Zurzolo, 2012; Cordero Cervantes and Zurzolo, 2021; Ljubojevic et al., 2021; Sartori-Rupp et al., 2019). Interestingly, it has been shown that different classes of TNTs exist even within a single cell type (Veranič et al., 2008). This disparity between TNTs and the association of the presence of microtubules inside the TNTs under stress situations (Wang and Gerdes, 2015b) suggests that the different compositions may reflect a functional heterogeneity.

Identifying the cytoskeletal elements present inside cell-to-cell connections thus appears to be an important step when starting to investigate TNTs. This protocol describes the sequential staining for tubulin (the structural unit of microtubules) by immunofluorescence and F-actin using fluorescent phalloidin, an F-actin-binding and stabilizing compound (Fig. 2; Fig. 3B). Microtubule detection will also help to discriminate TNTs from cytoplasmic bridges derived from cell division, which present a typical midbody showing dense microtubule bundles (see Alternate Protocol 2). It could also be appealing to look for the presence of intermediate filaments in TNTs (Resnik et al., 2019). This can be achieved by immunodetection of intermediate filament constituents, such as cytokeratin, vimentin or glial fibrillary acidic protein (GFAP), using similar protocols to the one described here for tubulin. Yet, how the cytoskeletal composition of TNTs have a role in the transfer of different cargoes has never been addressed and remains to be clarified. Live imaging of the actin and microtubule cytoskeleton is a crucial tool for this aim. In alternate protocol 1 we describe *in vitro* techniques that have been fine-tuned to monitor actin filaments and microtubules using live imaging. As a cellular model we used a malignant glioblastoma tumor-derived U251 cell line which has both types of TNTs (Fig. 4). These approaches can be extended to most other adherent cell types. If the cells growth in suspension, such as Glioblastoma stem cells, different coating surface in 2D should be perform to make adhere to the support for easy visualization of the TNTs (Add reference, Pinto G. 10.1042/BCJ20200710)

Fixed imaging approaches to identify the composition of the cytoskeletal elements present in TNTs tunneling nanotubes.

Additional Materials (also see Basic Protocol 1)

Fixative 3 (see recipe in
Reagents and
Solutions) 100 mM NH₄Cl
in PBS
1% (v/v) Triton-X 100
2% (w/v) bovine serum albumin (BSA) in PBS
Anti- α -tubulin antibody
Alexa Fluor-conjugated secondary antibody
6.6 μ M fluorescent phalloidin (Molecular Probes) in methanol

Culture and fix U251, CAD and SH-SY5Y cells for TNT observation

1. Prepare subconfluent cultures of U251, CAD and SH-SY5Y cells in 35-mm μ -dishes as described in Basic Protocol 1.
2. Remove culture medium and add 500 μ L of Fixative 3. Incubate 15 min at room temperature.

Perform immunostaining of microtubules

3. Remove fixation buffer and replace with 500 μ L of 100 mM NH₄Cl. Incubate 10 to 30 min at room temperature to quench PFA autofluorescence.

4. Thoroughly wash three times, 30 S each time, with 1 mL of 1× PBS.
5. Add 500 μL of 0.1% Triton-X 100 and incubate 3 min at room temperature to permeabilize the membrane.

Alternatively, incubate fixed cells in a permeabilization/blocking buffer containing 0.01% (w/v) saponin and 2% BSA (w/v) in PBS for 20 min. This saponin-containing buffer must also be used to dilute primary and secondary antibodies since saponin permeabilization is reversible.

6. Thoroughly wash three times, 30 S each time, with 1 mL of 1× PBS.
7. Add 500 μL of 2% BSA (w/v) and incubate 20 min at room temperature to block nonspecific binding of the antibodies.
8. Dilute primary anti- α -tubulin antibody in 2% BSA (w/v) and add 500 μL of the dilution onto cells. Incubate 1 h at room temperature.

The optimal dilution of the primary antibody depends on several parameters (batch, sample preparation, amount of the protein of interest, etc.) and must be determined experimentally.

9. Thoroughly wash three times, 30 S each time, with 1 mL of 1× PBS.
10. Dilute the fluorescent secondary antibody in 2% BSA (w/v) and add 500 μL of the dilution onto cells. Incubate 40 min at room temperature in the dark.

Antibody dilution must be optimized. A 1:500 dilution is usually a good starting point for Alexa Fluor-conjugated antibodies.

11. Thoroughly wash three times, 30 S each time, with 1 mL of 1× PBS.

Label F-actin with phalloidin

12. Prepare a 1:250 (v/v) dilution of fluorescently tagged phalloidin in PBS containing 1% BSA (w/v) to reduce background staining. Add 500 μL of the diluted solution onto cells and incubate 30 min at room temperature in the dark.
13. Thoroughly wash three times, 30 S each time, with 1 mL of 1 × PBS.
14. Add 10 to 12 drops of Aqua-Poly/Mount mounting medium onto the cells and place a 24mm round coverslip onto cells. Incubate 20 min at room temperature in the dark, then store at least 20 min at 4°C in the dark before imaging.
15. Acquire pictures with an inverted confocal fluorescence microscope (see Basic Protocol 1).

Live imaging approaches to identify the composition of the cytoskeletal elements in tunneling nanotubes :

Expression of actin- and tubulin-fusion fluorescent proteins

Imaging of actin filament and microtubule dynamics has been mostly achieved by injection of fluorescently labeled actin or tubulin (technically demanding) or small amounts of fluorescently labeled phalloidin. However, the ability to express cloned proteins containing a fluorescent protein tag has become the method of choice for dynamic analysis of the cytoskeleton. Nonetheless, this approach has several limitations due to the level of expression of the actin- or tubulin-fusion protein which can compromise cytoskeletal functions (Xu and Du, 2021). To avoid artifacts produced by the overexpression of these plasmids, all experiments should be performed at low to moderate levels of actin and tubulin expression, which

have been proven to have minimal effects on actin polymerization and depolymerization, microtubule organization and do not compete with major actin-binding proteins (Riedl et al., 2008).

Materials

U-251 cell line human (Sigma, cat. No. 09063001)

Complete Cell growth medium (complete medium):

DMEM+ GlutaMAX-I (Gibco 31966-021) supplemented with 10% fetal calf serum and penicillin-streptomycin (1%).

Lentiviral particles of DNA vectors encoding chosen-fusion-fluorescent proteins

Nikon W1 Inverted Microscope Spinning Disk Confocal with Perfect Focus System with environmental control: heated stage and incubator.

Serum-free Opti-MEM (Life Technologies)

37°C humidified incubator in an atmosphere of 5% CO₂

75cm² flask

ibidi μ -Plate 24 Well Black ibiTreat (ref 82426)

Generation of stable U251 cell line expressing actin filaments and microtubules fused with fluorescent proteins.

To study the different types of TNTs formed by different components of the cytoskeleton and its role in TNT-communication, it is necessary to perform live-cell imaging in living cells. One of the main challenges is phototoxicity due to pro-longed sample illumination. Indeed, TNTs are extremely fragile and sensitive to light and can easily break under illumination, thus, it is also crucial to carefully choose the fluorescent proteins depending on the observation system and their combination according to the fluorescent emission of the fluorophore to avoid crosstalk between the different channels. The use of different fluorophores such as EGFP, mCherry, dsRED, iRFP670 or mTagBFP2 will largely depend on the filters present on the microscope.

The generation of stably transfected cell lines based on transient transfection followed by antibiotic selection has many drawbacks: it is time-consuming, transfection efficiency is limited, selection of the clonal collection is long and tedious, and consumable costs are high. However, lentiviral transduction is an easier and faster method for the delivery of transgenes to mammalian cells resulting in a more efficient and robust expression of the plasmids. Transducing the cells with fluorescent plasmids followed by sorting leads to the rapid establishment of polyclonal cell lines with selected levels of stable expression.

1. The day before infection prepare a 70% confluent U251 cell culture in 25cm² culture flask and incubate 18-20 h in a 37°C incubator.
2. Remove culture medium and replace with 5 mL of fresh culture medium.
3. Add drop-by-drop each lentiviral nanoparticle for the different constructs. The volume will depend on the concentration of the lentiviral particles. Gently swirl the plate to mix.

To increase the efficiency of transduction, use fresh lentiviral particles or perform a second infection 24 h after the first infection.

4. Incubate during 48 h in a 37°C incubator.

5. Detach and amplify the cells. In parallel seed the cells in ibidi μ -Plate 24 well to check the expression of the different plasmids by inverted spinning disk-confocal microscope. The transduction efficiency can be calculated also by FACS) *Increasing the direct-contact time between lentiviral particles and mammalian cells can improve transduction efficiency. It is necessary to establish a balance between lentiviral nanoparticles incubation time and cell viability.*

Live imaging analysis

First, we must select the optimal density that allows TNT formation but also the discrimination of filopodia-like structures. An alternative to the sparse seeding described below is seeding individual cells on micropatterns (see protocol below)

6. Seed U251 cells transduced with the actin/tubulin constructs fused with fluorescent proteins in an ibidi μ -Plate 24 well the day before the experiment to have a density of 50%.

U251 optimal confluency to study TNTs is 20,000 cells/cm²

7. Perform live imaging using a Nikon W1 Inverted Spinning Disk Confocal Microscope at 37°C and 5% CO₂. To discriminate TNTs from dorsal filopodia it is necessary to acquire movies with several Z-stacks to be sure that the TNT hovers above the substratum. The total duration of the movie and the intervals of acquisition must be set up depending on the cellular type and the compromise between the different channels, photobleaching and the number of Z-stack.

Note: To study transfer of different cargoes, labelling with different live-compatible dyes such as, DiD/DiO for vesicles, LysoTracker or mitoTracker, for lysosomes and mitochondria, respectively, can be preformed.

Live compatible dyes to stain actin filaments and microtubules.

Transfection or transduction can induce cellular stress, cell division arrest and cytotoxicity. Alternatively, expression of the transgene via plasmid or viral delivery requires significant time before enough protein is synthesized to generate a fluorescent signal suitable for live-cell imaging. As an alternative to the generation of stable cell lines expressing fluorescent protein tagged actin or tubulin, it is possible to monitor the cytoskeletal composition and the dynamics of cargo transfer using live-cell microtubule or actin fluorescent probes.

Materials

U-251 cell line human (Sigma, cat. No. 09063001)

U-251 complete cell growth medium (see recipe)

Live-staining probes to track intracellular F-actin (CellMask™ Deep Red Actin Tracking Stain, ThermoFisher A57245) and tubulin (SPY555-tubulin, Spirochrome SC203)

MitoTracker Red CMXRos (ThermoFisher, Cat. no. M7512)

LysoTracker Red (thermoFisher Cat. No. L7528)

DiO lipophilic tracer (Life Technologies)

ibidi μ -Plate 24 Well Black ibiTreat (ref 82426)

Sequential labelling of the cells with the different probes

1. The day before of the experiment prepare a 50% confluent U251 cell culture in 24 well ibidi dish and incubate 18-20 h in a 37°C incubator.
2. Remove the culture medium, add a 1x SPY555-tubulin solution (1 μ L in 1 mL complete medium for a 1000x stock solution), and incubate during 1 h in a 37°C incubator.
3. After 30 min of incubation with SPY-555 add an additional 1 μ L of CellMask Deep Red Actin (stock solution, 1000x; working solution, 1x), and the cargo dye of your choice at the recommended concentration (DiO/ LysoTracker Red/ MitoTracker Red) and incubate for 30 min more at 37°C.
4. 5 min before the end of the incubation add Hoechst 33342 to label the nuclei (stock solution, 50 mg/mL; working solution, 1 μ g/mL).
5. Wash three times with 1 \times PBS and add fresh complete medium.
6. Perform live-cell imaging using a spinning disc in a controlled atmosphere with 37°C and 5% CO₂.

ALTERNATE PROTOCOL 2

DISTINGUISHING TUNNELING NANOTUBES FROM INTERCELLULAR BRIDGES FORMED DURING CELL DIVISION

TNTs are not the only intercellular bridges observed in cell cultures. During cytokinesis, the final step of cell division, sister cells remain connected through intercellular bridges for several hours before abscission (Dambournet et al., 2011; Elia et al., 2013; Korenkova et al., 2020). The diameter of these bridges is comparable to thicker TNTs (~1 μ m) and an inexperienced observer could confuse these two structures. One major difference is the characteristic midbody present in the cytokinetic bridge connecting the sister cells (Crowell et al., 2014). The midbody is a proteinaceous structure containing tightly packed microtubules derived from the mitotic spindle along with other proteins involved in abscission. This structure is sufficiently dense and prominent to be observed by light microscopy, and tubulin immunostaining highlights typical microtubule enrichment on each side, which helps to distinguish cytokinetic bridges from TNTs (Sowinski et al., 2011). However, the most conclusive evidence to distinguish the two types of structures consists in co-culturing two cell populations labeled with distinct fluorescent markers. Connections established between cells from two different populations cannot be attributed to incomplete cytokinesis. This protocol describes how to transiently transfect cells with fluorescent fusion-proteins and establish a co-culture.

Additional Materials (also see Basic Protocol 1)

Serum-free Opti-MEM (Life Technologies)

Lipofectamine 2000 (Life Technologies)

DNA preparations of vectors encoding chosen fusion-proteins

Fluorescent protein markers to distinguish between two cell populations (see Critical Parameters)

1.5-mL microcentrifuge tubes

Transiently transfect CAD cells

1. Prepare 70% confluent CAD cell cultures in 25 cm² culture flasks.

Usually, 800,000 to 1,000,000 cells plated on the day before experiment will produce the appropriate confluency for transfection.

2. Remove culture medium and replace with 4 mL of fresh culture medium.
3. For each transfection to be performed, prepare two 1.5-mL microcentrifuge tubes with 1.5 mL serum-free Opti-MEM medium. In one tube, add DNA preparation of vector to be transfected. In the other tube, add 10 μ L Lipofectamine 2000.

The amount of DNA for optimal transfection rate and expression level must be optimized for each construct and cell type. Usually, 4 μ g is a good starting point. When performing the transfection in a smaller culture support, scale down the transfection reaction (maintain the DNA/Lipofectamine 2000 ratio).

4. Add drop-by-drop the diluted DNA to the diluted Lipofectamine 2000.
5. Incubate 5 min at room temperature.
6. Add drop-by-drop the DNA-lipid complex to cells.
7. Incubate cells 12 to 24 h in a 37°C incubator.

Prepare co-culture and acquire images.

8. Detach cells and count cells with a hemacytometer or an automated cell counter; then mix the two populations 1:1 in complete culture medium.
9. Plate cells in 35mm μ -dishes as described in Basic Protocol 1 and incubate 1 to 24 h at 37°C.
10. Fix cells as described in Basic Protocol 1.
11. (Optional) Stain membranes with WGA as described in Basic Protocol 1.
12. Add 10 to 12 drops of *Aqua-Poly/Mount mounting medium onto cells and place a 24 mm round coverslip on top of cells. Incubate 20 min at room temperature in the dark, then store at least 20 min at 4°C in the dark before imaging.*
13. Acquire pictures with an inverted confocal fluorescence microscope (see Basic Protocol 1).

BASIC PROTOCOL 2

DECIPHERING TUNNELING NANOTUBE FORMATION AND LIFETIME BY LIVE FLUORESCENT MICROSCOPY

Two main mechanisms of TNT formation have been reported to date. The first one, initially described by Rustom et al. (2004), is based on the extension of a filopodia-like protrusion toward a neighboring cell and subsequent fusion at the tip following physical contact (Abounit and Zurzolo, 2012). In the second model, membrane fusion occurs between two cells that are in close contact, and their subsequent migration in opposite directions draws out a thin thread of

membrane, leading to the formation of TNT. How (the mechanism) and at which point (when) in the formation process does cell-to-cell fusion occurs is still unclear and must be addressed. Both types of formation can be observed in the same cell line, as observed in CAD cells (Gousset et al., 2013) and SH-SY5Y (Pepe et al., 2023). However, formation of filopodia-like protrusions might be predominant in immobile cells, such as neuronal cells, compared to mobile cells such as immune cells, in which the cell-dislodgment mechanism has been described (Onfelt et al., 2006). Distinguishing the two mechanisms and deciphering TNT formation requires live cell observations of this process. This protocol describes how to monitor TNT formation in live cells expressing fluorescent protein markers. Live imaging of TNT-connected cells can also be useful to monitor the dynamic transfer of proteins or organelles through TNTs (see Basic Protocol 3), or to confirm the localization of a protein avoiding the artifacts inherent to cell fixation.

Choose an observation system and a fluorescent protein marker(s) for live imaging of TNTs.

One major pitfall of live imaging is the phototoxicity due to sample illumination. In the context of TNTs, more consideration should be given to this issue, since TNTs are very sensitive to light and can break upon illumination (Rustom et al., 2004). It is therefore crucial to carefully choose both the observation system and the potential fluorescent marker(s) before starting live experiments. Differential interference contrast microscopy is minimally harmful to living cells and can be used as a first approach to monitor TNT formation. However, it does not allow one to follow specific molecules, unless it is coupled with fluorescence microscopy. Wide-field fluorescence microscopy presents the advantage of reduced phototoxicity but it might not achieve adequate resolution, depending on the question that is being addressed. A good compromise to reconcile sufficient resolution and reduced phototoxicity is the use of spinning disk confocal microscopy, which has fast image acquisition. Live imaging of TNTs can then be achieved with the further use of protein markers such as fluorescent fusion proteins targeted to the plasma membrane or the actin cytoskeleton such as actin-chromobody-GFP (Manzano et al., 2022), (see also Alternate Protocol 2). Furthermore, the entire plasma membrane of TNTs can be labelled with fluorescently conjugated proteins which bind glycolipids and glycoproteins, such as WGA (Pepe et al., 2023). However, it is important to first ensure that a satisfactory signal-to-noise ratio is obtained upon moderate sample illumination that does not alter cell physiology. Typically, a better signal-to-noise ratio will be obtained with GFP fusion proteins, compared to proteins fused with, for example, RFP, mCherry, or CFP. A first step before starting a long movie acquisition is to determine the optimal illumination parameters and the kinetics of photobleaching for a given fluorescent marker. Monitoring TNT formation requires the acquisition of at least 20min-long movies with multiple Z-stacks for each time point. Longer movies will be needed to investigate TNT stability over time or transfer of cellular material through TNTs. If the fluorescence bleaches too rapidly and it is not possible to reduce the excitation power or to increase the speed of acquisition, a solution can be to increase the time between each time point. For example, the authors usually monitor TNT formation with one timepoint taken every 20-30 S.

Materials

CAD cells (Sigma, cat. no. 08100805)

SH-SY5Y cells (ATCC CRL-2266)

SH-SY5Y cell growth medium ((see recipe in

Reagents and Solutions)

CAD cell growth medium ((see recipe in Reagents
and Solutions)

Serum-free Opti-MEM I reduced serum medium (Life Technologies)

Lipofectamine 2000 (Life Technologies)
DNA preparations of vectors encoding chosen fusion-proteins
25cm² culture flasks
37°C incubator
Hemocytometer or automated cell counter
WGA
35mm μ -dishes, high, tissue culture treated (ibidi)
Inverted spinning disk confocal fluorescence microscope equipped with live observation chamber.

Prepare culture of transiently transfected CAD cells

1. Transiently transfect CAD cells in 25cm² culture flasks with the chosen fluorescent fusion-protein as described in Alternate Protocol 2.

Although it is not essential, one may monitor TNT formation in a co-culture of two cell populations transfected with different fluorescent markers as described in Alternate Protocol 2 to avoid any confusion with cytokinetic bridges.

2. Mechanically detach cells and count them with a hemacytometer or an automated cell counter.
3. Plate between 300,000 and 500,000 cells in 35mm μ -dishes as described in Basic Protocol 1.
4. Incubate cells 15 to 30 min in a 37°C incubator.

CAD cells should be attached to the culture dish by this time and start forming TNTs.

5. During TNT formation, pre-warm the live observation chamber to 37°C and put CO₂ 5%.

It is crucial to avoid any large temperature shifts, which could negatively influence TNT formation.

Image living CAD cells

6. Place one of the 35mm μ -dishes into the live observation chamber.

Opti-MEM contains low levels of phenol red and is suitable for live imaging. If using another culture medium with higher levels of phenol red, replace it with a medium devoid of phenol red to reduce background noise and phototoxicity.

7. Select a field with a few cells that have a confluency favorable to TNT formation.
8. Start acquiring the movie.

Maximum speed for acquisition depends on the number/width of Z-stacks that are acquired. It is important to acquire most of the cell volume because TNTs can originate near the bottom or near the top of the cell.

Prepare culture of SH-SY5Y cells and perform live imaging to measure TNTs lifetime.

9. Trypsinise and count SH-SY5Y cells as described in Basic Protocol 1.

Alternatively, we can use SH-SY5Y that have been stably transfected with actin-chromobody-GFP (Manzano et al., 2022).

10. Plate 400,000 cells in 35mm μ -dishes as described in Basic Protocol 1.
11. Incubate cells O/N in a 37°C incubator.
12. After 16 h of culture, live time series images are acquired by using the inverted Eclipse Ti microscope system (Nikon Instruments, Melville, NY, USA).
13. Pre-warm the live observation chamber to 37°C and put CO₂ 5%.
14. Place the 35-mm μ -dishes into the live observation chamber.

Note, if your cells are transfected with actin-chromobody-GFP (Manzano et al., 2022), no additional fluorescent dye is required to visualize TNTs. In contrast, if the cells do not express fluorescent protein, they are labelled with WGA-Alexa Fluor (488 /546/647) in a 1:1000 dilution in 1 mL of complete cell growth medium.

15. Select the objective lens. To evaluate TNTs time life a 60 \times 1.4NA CSU oil immersion objective lens is used.

To evaluate TNTs time life a 60 \times 1.4NA CSU oil immersion objective lens is used.

16. Select the field with a few cells that have formed TNTs.
17. Select the fluorescence channel that you would visualize
18. Select the time intervals between 20 and 40 S per z-stack (with 0.35-0.5 μ m steps size).
19. Start acquiring the movie.

Note, since it is very difficult to detect newly formed TNTs, we will capture live imaging of pre-existing TNTs and calculate their lifetime using Fiji software (Pepe et al., 2023). ***Importantly***, the lifetime of TNTs may change from the use of one dye and/or fluorescent proteins to another (e.g. WGA, actin-chromobody-GFP) as shown in Manzano et al, 2022 and Pepe et al., 2023 (Manzano et al., 2022; Pepe et al., 2023).

A critical point is that if we use the WGA, we cannot perform movies longer than 40 minutes because after this time the WGA is internalized.

ALTERNATE PROTOCOL 3

DECIPHERING TUNNELING NANOTUBE FORMATION USING A LIVE-COMPATIBLE DYE

As an alternative to transient transfection with a fluorescent fusion protein, it is possible to monitor TNT formation using live-compatible fluorescent membrane dyes. This presents the main advantage of avoiding potential side effects due to the overexpression of an exogenous protein. This procedure is also significantly timesaving. Although WGA or the lipophilic tracers DiI, DiO, or DiD (dialkylcarbocyanines) have often been employed to monitor TNT formation, it is not recommended for use in this context. These dyes are quickly internalized by the cells and therefore only offer a narrow window for imaging the plasma membrane. This protocol describes how to monitor TNT formation using the CellMask plasma membrane stain developed by Molecular Probes (Invitrogen). This dye offers homogenous plasma membrane staining in CAD cells and is slowly internalized, thus being particularly suitable for 20- to 30-min long movies that are required in this context.

Additional Materials (also see Basic Protocol 2)

CellMask Orange Plasma Membrane Stain (Molecular Probes), also available in green and far red.

1. Plate between 300,000 and 500,000 cells in 35mm μ -dishes as described in Basic Protocol 1.
2. Incubate cells 15 to 30 min in a 37°C incubator.
3. Prepare a fresh 1:2000 (v/v) dilution (i.e., final concentration of 2.5 μ g/mL) of CellMask Orange Plasma Membrane Stain in warm 1 \times PBS or growth medium.

The working concentration may need to be optimized. A volume of 500 μ L of dilution is required for each 35mm ibidi μ -dish; therefore, the amount of dilution to prepare depends on the number of μ -dishes used.

4. Replace cell culture medium with staining solution from step 3 and incubate 3 min at 37°C.
5. Remove the staining solution and carefully wash the cells three times, 30 S each time, with 1 mL of warm PBS.
6. Cover cells with fresh culture medium.
7. Place one 35mm μ -dish in the pre-warmed live observation chamber.
8. Select a field with a few cells with a confluency that is favorable to TNT formation.
9. Start acquiring movie.

Do not acquire movies >90 min since significant internalization of the dye may start to occur and, as a consequence, the membrane signal is lost, making it more difficult for the recognition of TNTs.

Basic and Alternate protocol 2 can be applied for different cell types such as SH- SY5Y (Pepe *et al.*, 2023).

BASIC PROTOCOL 3

ASSESSING TUNNELING NANOTUBES FUNCTIONALITY IN INTERCELLULAR TRANSFER

Unlike other protrusions, TNTs are able to physically create continuity between the cytoplasm of relatively distant cells (Abounit and Zurzolo, 2012; Ariazi et al., 2017; Cordero Cervantes and Zurzolo, 2021). From this distinguishing feature results the major function of TNTs, which allows direct transfer of material from one cell to another. TNTs were shown to transfer several types of cargo, such as vesicles derived from various organelles (early endosome, endoplasmic reticulum, Golgi complex, and lysosome)(Kadiu and Gendelman, 2011a, 2011b; Smith et al., 2011; Wang et al., 2011), plasma membrane components (Rustom et al., 2004), cytoplasmic molecules, Ca^{2+} (Smith et al., 2011; Watkins and Salter, 2005) and mitochondria (Pasquier et al., 2013; Pinto et al., 2020; Sáenz-de-Santa-María et al., 2017; Sartori-Rupp et al., 2019; Vignais et al., 2017). In addition, TNT-like structures can transfer pathogens such as bacteria (Onfelt et al., 2006), HIV particles (Jansens et al., 2020; Sowinski et al., 2011), SARS-CoV-2 particles (Pepe et al., 2022), and proteinaceous agents such as prions (Gousset et al., 2009; Gousset and Zurzolo, 2009), huntingtin assemblies (Costanzo et al., 2013), alpha-synuclein fibrils (Abounit et al., 2016; Dilsizoglu Senol et al., 2021, 2019; Vargas et al., 2019) and tau aggregates (Chastagner et al., 2020). In addition to the high morphological heterogeneity of these structures (Cordero Cervantes and Zurzolo, 2021; Marzo et al., 2012), these studies point toward a high degree of functional diversity in TNT-like structures. To ensure that the observed structures are bona fide TNTs, it is essential to test whether they allow passage of cellular components. To do so, a co-culture system is presented, which uses a microscopy approach to show, in a qualitative and quantitative manner, TNT-mediated transfer of sub-cellular vesicles. These optimized protocols could be applied to any cell types and provide important insights into TNT functionality. Furthermore, this protocol can be used more generally to monitor fluorescent organelles and pathogen transfer.

Materials

CAD cells(Sigma, cat. no. 08100805)
CAD cell growth medium (see recipe in Reagents and Solutions)
Serum-free Opti-MEM I reduced serum medium (Life Technologies)
Lipofectamine 2000 (Life Technologies)
DNA preparation of GFP plasmid
DiD lipophilic tracer (Life Technologies)
1× PBS
Fixative 1 (see recipe in
Reagents and Solutions)
Fixative 2 (see recipe in
Reagents and Solutions)
HCS CellMask Blue stain (Molecular Probes)
Aqua-Poly/Mount (Polysciences)
37°C incubator

25 cm² flask
5 or 15 mL centrifuge tubes
1.5-mL microcentrifuge tubes
35mm μ -dishes, high, tissue culture-treated (ibidi)
24 mm round coverslips
Inverted spinning disk confocal fluorescence microscope equipped with live observation chamber
Confocal microscope

Transiently transfect acceptor CAD cells

1. Plate and transiently transfect acceptor CAD cells with GFP plasmid as described in Alternate Protocol 2.

Load donor CAD cells with a lipophilic tracer

2. The day following the transfection of acceptor cells, prepare a 1:3000 (v/v) dilution of DiD (1 mg/mL stock solution) in CAD cell growth medium

To monitor unspecific vesicular transfer, DiD, a lipophilic tracer (long chain dialkylcarbocyanine that exhibits infrared fluorescence), is routinely used. This protocol can be easily customized to follow the transfer of organelles by loading cells with organelle trackers (e.g., ER-Tracker, LysoTracker, Mito-Tracker) or fluorescent pathogens (e.g., fluorescent virus, bacteria, or proteinaceous prion-like agents).

3. From an 80% confluent 25cm² flask, remove the growth medium and add 3 mL of DiD solution. Incubate 30 min in a 37°C incubator.
4. Remove the DiD solution and add 3 mL of growth medium. Incubate 30 min in a 37°C incubator.

This step allows the internalization of the DiD-stained membrane.

5. Remove growth medium and rinse once with CAD cell growth medium or pre-warmed 1× PBS.
6. Detach, resuspend, and count cells as described in Basic Protocol 1.

Co-culture donor and acceptor CAD cells

7. At 24h post-transfection, detach, resuspend, and count GFP-vector transfected cells (as described in Basic Protocol 1).

Donor cells should be already loaded, detached, and counted.

8. Mix cells in triplicate and at a 1:1 ratio. Add the correct amount of donor and acceptor cells into each 15-mL tube and bring up to 6 mL with an appropriate volume of CAD cell growth medium.

The amount of cells to plate in the co-culture must be set up (from 100,000 to 250,000 total cell number). Transfection or other treatment could alter cell division. To be able to correlate the transfer experiment with the TNT quantification experiment (see Basic Protocol 1), the cells must be at the same confluency.

In the authors' system, the best setting for vesicular transfer is to co-culture cells at a ratio of 1:1 for 16 h. However, depending on the cell type, the ratio and the time of co-culture must be optimized.

9. Resuspend well the cell suspension mix of cells and place 2 mL into each 35mm μ -dish.
10. Incubate for different periods of time in a 37°C incubator.

It is necessary to perform a time-course of transfer. This must be correlated with the kinetics of TNT formation (see Basic Protocol 1). The amount of cells to plate for the time-course and the time of incubation will have to be optimized as previously described.

11. For a qualitative approach, after incubation time, image live cells with a spinning disk confocal microscope to visualize the transfer of DiD vesicles from donor to acceptor cells.

Here, differential interference contrast (DIC) microscopy (so-called Nomarski microscopy) can be used to visualize TNTs. When combined with fluorescence microscopy, one can image and record in live cells the transfer of DiD vesicles within TNTs between donor and acceptor cells.

As an alternative, acceptor cells can be transfected with a fluorescent plasma membrane or actin marker as described in Basic Protocol 2.

12. For a quantitative approach, after the incubation time, rinse with 1× PBS, then proceed to fixation, HCS CellMask Blue staining, and mounting as described in Basic Protocol 1.
13. Use a confocal microscope to image cells and acquire Z-stacks as described in Basic Protocol 1.
14. To assess the rate of transfer, software that allows automatic detection of acceptor cells containing transferred vesicles should be used.

Plugins from different software can be developed for this purpose (e.g., icy.bioimageanalysis.org/, imagej.nih.gov/ij/). As an alternative, the intensity of fluorescence can be measured in acceptor cells; however, this is less accurate because transferred vesicle number is not quantified.

This protocol can be adapted for all cell types (Dilsizoglu Senol et al., 2019; Pepe et al., 2023, 2022).

ALTERNATE PROTOCOL 4

FLOW CYTOMETRY APPROACH TO QUANTIFY THE RATE OF

VESICLE OR MITOCHONDRIA TRANSFER

Another way to investigate the intercellular transfer of vesicles or the transfer of organelles such as mitochondria in a quantitative manner is based on FACS. This method has been widely used in the literature (Bhat et al., 2020; Pinto et al., 2021; Saha et al., 2022), as it provides a more straightforward and faster quantitative analysis, although the sensitivity is lower compared to analysis by microscopy. This protocol describes how to proceed to analyze a co-culture experiment using FACS (Fig. 5). Several controls are required to confirm TNT-mediated vesicle or mitochondria transfer (see Support Protocol).

Analysis of TNT-mediated DiD-labelled vesicle transfer

Additional Materials (also see Basic Protocol 3)

- 1× PBS, ice cold
- 4% PFA (w/v), ice cold
- 37°C incubator
- 24-well plates
- 12 × 75mm tubes with cell strainer caps (Falcon)
- Flow cytometer and FACS analysis software (e.g., Kaluza, FlowJo)
- Additional reagents and equipment for transfection of acceptor CAD cells (see Alternate Protocol 2)

Transiently transfect acceptor CAD cells

1. Plate and transiently transfect acceptor CAD cells with GFP plasmid as described in Alternate Protocol 2.

Load donor CAD cells with a lipophilic tracer

2. Load the donor population of cells with DiD, as described in Basic Protocol 3, steps 2 to 6.

Co-culture donor and acceptor CAD cells

3. After 24 h transfection, detach, resuspend, and count GFP-transfected cells.
4. Prepare the mix of cells in triplicate with a ratio of 1:1. Place 150,000 donor and 150,000 acceptor cells into a 2mL microcentrifuge tube and bring up to 1.5 mL with an appropriate volume of growth medium.

The quantity of cells to plate for co-culture must be optimized.

5. Resuspend well the co-culture and place 500 μ L of the mix into each well of a 24-well plate.
6. As control 1, plate 100,000 donor cells in 6 wells of a 24-well plate.
7. As control 2, plate 100,000 acceptor cells in 6 wells of a 24-well plate.

8. Incubate all cells for different periods of time in a 37°C incubator.

As for the microscopy transfer assay, the amount of cells plated and the time of incubation must be optimized.

Fix cells for flow cytometry analysis

9. After the incubation, label twelve 12 × 75mm tubes with strainer caps: three tubes for donor cells alone; three tubes for acceptor cells alone; three tubes for the mix, and three tubes for co-culture.

For FACS analysis, single cell populations (donor and acceptor cells alone, i.e., control 1 and 2) are needed to set up acquisition parameters. In addition, a “mix” donor and acceptor cells separately cultured and instantly mixed and fixed prior to FACS is also used as a control.

10. Add 500 µL of cold 4% PFA to each tube and place the tubes on ice.
11. For donor cells alone, acceptor cells alone, and co-culture, rinse cells with cold 1× PBS.
12. Add 500 µL of cold 1× PBS and mechanically detach cells by pipetting up and down.
13. Place cell suspension into each corresponding tube by passing it through the cell strainer. Resuspend cells in PFA by pipetting up and down and store tubes at 4°C in the dark.

The final concentration of PFA is 2% (w/v). Cell detachment can be done using a solution of 1× PBS containing 1% (v/v) of serum (e.g., fetal bovine serum). By using the strainer, cells are individualized and do not form clumps. This is very important for FACS acquisition because clumped cells can clog the cytometer and create false double positive cells (doublets).

14. For the mix condition, rinse and detach each cell population following the same procedure.
15. Add donor and acceptor cells into a 12 × 75mm tube, then resuspend well and add mixed cells into each corresponding tube with strainer caps by passing the cell suspension through the cell strainer. Resuspend cells by pipetting up and down and store tubes at 4°C in the dark.

Cells are ready to be analyzed by FACS or can be stored for up to 1 week at 4°C in the dark.

For FACS acquisitions, single positive cells (donor and acceptor cells alone, i.e., control 1 and 2) will be used to set up the gate and to eliminate crosstalk between fluorophores. The “control mix” tubes will be used to monitor the transfer at time point 0 (which is considered as the nonspecific background). Then, co-culture tubes will be recorded to obtain the percentage of double- positive cells.

16. Analyze the FACS raw data with proper FACS analysis software (e.g., Kaluza, FlowJo).

It is better to use the analysis software from the same company of the FACS device used.

Analysis of TNT-mediated mitochondria transfer

Generation of stable U251 cell line expressing mitoGFP (Donor population) or cytosolic mCherry (Acceptor population) U251 cells

1. Plate and transduce U251 cells with MitoGFP (pLV-CMV-mito-GFP) and mCherry (pLV-CMV-mCherry) plasmids encoding for a fragment of the subunit VIII of human cytochrome C oxidase fused with GFP, and for cytosolic mCherry under the Cytomegalovirus (CMV) promoter, respectively, as described in alternate protocol 2.

Co-culture donor and acceptor U251 and fix cells for FACS analysis as described above.

SUPPORT PROTOCOL 1

CONTROLS TO SUPPORT TNT-MEDIATED TRANSFER

The methods described above provide insight into the intercellular transfer of vesicles and mitochondria. However, the quantitative approach is not enough to prove the involvement of TNTs in the observed transfer. This is why it is necessary to combine these experiments with robust controls that will allow for the exclusion of other cell-to-cell transfer mechanisms such as secretion, exocytosis, or endocytosis.

To achieve TNT-mediated transfer, cells must be in close contact. Thus, one way to verify that TNTs mediate vesicle/mitochondria transfer is to separate both populations (i.e., donor and acceptor cells). To do so, a protocol of co-culture impairing cellular contact between the two populations using a transwell filter is described. In addition, to investigate whether secretion could mediate the transfer, a setup where acceptor cells are only in contact with the supernatant (conditioned medium) of the donor cells has been developed. This protocol is used for monitoring vesicle/mitochondria transfer using FACS. In the case of mitochondria transfer use the same protocol described below but with stable cell populations expressing mitoGFP (Donors) and mCherry cytosolic (acceptors).

Additional Materials (also see Basic Protocol 3)

Transwell clear polyester membrane inserts for 24-well plates (6.5 mm with 0.4- μ m pores; Costar)

Transiently transfect acceptor CAD

1. Plate and transiently transfect acceptor CAD cells with GFP plasmid as described in Alternate Protocol 2.

Load donor CAD cells with a lipophilic tracer

2. Load the donor population of cells with DiD, as described in Basic Protocol 3, steps 2 to 6.

Co-culture donor and acceptor CAD cells

3. Follow the co-culture protocol described in Alternate Protocol 4, steps 1 to 5.
4. After Alternate Protocol 4, step 5, plate 50,000 acceptor cells in three wells of a 24-well plate in a final volume of 250 μ L. Allow cells to settle into the wells.

These wells are for the filter condition.

5. Plate separately 50,000 donor cells and acceptor cells in three wells of a 24-well plate in a final volume of 500 μ L.

These wells are for the supernatant condition, to be used on the next day.

6. Add a 24-mm diameter transwell filter on top of the wells containing acceptor cells (filter condition).
7. Add 50,000 donor cells into each transwell filter in a final volume of 250 μ L (filter condition).
8. Follow Alternate Protocol 4, steps 5 to 16.
9. On the next day, remove the medium of the acceptor cells from step 5 (supernatant condition).

These cells were plated for the supernatant experiment the day before.

10. Then for each well, remove the medium of donor cells and place it into each well containing the acceptor cells from step 9 (supernatant condition).

These cells were plated for the supernatant experiment, the day before.

11. On the following day, perform Alternate Protocol 4, steps 5 to 16.

BASIC PROTOCOL 4

STUDIES OF TUNNELING NANOTUBES BY CELL MICROPATTERNING

Present investigations of TNT involvement in cell-cell interactions have relied predominately on culturing cells on conventional 2D substrates. Though this method has an advantage with its ease of use, it lacks the ability to precisely control intercellular distances and cellular densities necessary to properly investigate TNT formation through actin-dependent mechanisms that drive outward protrusion formation over competing processes originating through cell dislodgment. Moreover, as TNTs are morphologically similar to sensory filopodia, traditional culture methods hamper the proper identification of TNTs if cells are grown in an unconstrained manner where cell-to-cell separations approach the typical length scales that most filopodia are able to achieve (<5 μ m)(Ljubojevic et al., 2021; Mattila and Lappalainen, 2008; Mogilner and Rubinstein, 2005). To address the challenge of TNT characterization in living cells, we present a cell patterning

protocol adapted from the earlier work of (Azioune et al., 2009) that permitted functional TNT formation (Fig. 6). As outlined below, coverslips are covalently functionalized with “cell phobic” polyethylene glycol (PEG) polymers through amino-silane chemistry, and then patterned using a chrome photomask and deep UV light irradiation before final fibronectin functionalization for cell adherence. This micropatterning approach faithfully ensures the identification of TNTs forming at distances greater than those achievable by filopodia, and that they arise through the polymerization of actin filaments and not through unwanted cell dislodgement, advantageously allowing the dissection of how TNT formation depends on cytoskeletal network organization (Henderson et al., 2022). The protocol should be easily adapted for TNT studies in other cell types.

Materials

Chrome photomask with a quartz (or fused silica) substrate (Delta Mask, Toppan Photomasks, Microtronics Photomask)

Bath sonicator

UV cleaner (e.g., from Jetlight)

Plasma cleaner (e.g., from Harrick Plasma)

25 mm diameter coverslips, #1.5 thickness

Coverslip racks

Hellmanex III cleaning concentrate (Hellma Analytics)

Ultrapure water (resistivity >18 MΩ·cm)

Ethanol

Methanol

Glacial acetic acid

(3-aminopropyl)trimethoxysilane (APTMS)

Methoxy poly(ethylene glycol) succinimidyl valerate (mPEG-SVA)

Fibronectin

Cell strainer

EGTA

1× PBS

Design and order photomask

1. Photomask designs can be generated in dedicated software such as Clewin and L-Edit, however, common vector-based drawing softwares like Adobe Illustrator, CorelDRAW, and Inkscape can be used in principle if proper dimensions are indicated for the photomask producing company. As photomasks typically are 5 x 5 inches in size, users are encouraged to: (1) initially screen differently sized patterns to optimize the adherence of their particular cell line, and (2) tune the separation distance between repeated patterns to prevent unwanted cell migration from one pattern to another yet maintains good frequency of observable cell-to-cell connections between neighbouring patterned cells. A good starting point for cell

adherence optimization is to measure the adhered area of cells plated on non-micropatterned surfaces (e.g., ibidi or MatTek dishes). In the case of CAD cells, the authors found that patterns approx. $750 \mu\text{m}^2$ and inter-pattern separation distances ranging between 15 to $30 \mu\text{m}$ worked best to robustly adhere CAD cells and to prevent their translocation between patterns while still permitting frequent TNT formation (Henderson et al, 2022).

When ordering your photomask, it is crucial that any photomask for deep UV irradiation should be transparent to wavelengths below 200 nm in order to degrade the PEG layer. Thus, be sure to indicate to the photomask company that the substrate should be composed of fused silica or synthetic quartz.

Clean coverslips

2. Load chemically resistant racks with #1.5 thick coverslips and place the racks in appropriately sized beakers.

Coverslips with #1.5 thickness (approx. $170 \mu\text{m}$) are ideal for imaging as modern objectives are designed to be used with these specific cover glasses to minimize spherical aberrations. Coverslip racks should ideally be made of Teflon as it offers superior thermal and chemical resistance, but polypropylene-composed racks are a suitable alternative.

3. Submerge the coverslips in 70% ethanol and place the beakers in a bath sonicator warmed to 35°C and sonicate the coverslips for 10–15 min.
4. Discard the ethanol and rinse the coverslips three times with ultrapure water.
5. Submerge the coverslips in a 2% Hellmanex detergent solution and sonicate the coverslips for 30 min at 35°C .
6. Discard the detergent solution and rinse the coverslips three times with ultrapure water.
7. Dry the coverslips gently using a stream of filtered nitrogen gas or dry them in an oven.

Functionalize the glass surface with amino-silanes

8. Activate the glass surfaces for 3–5 min using a plasma cleaner either with ambient air or oxygen gas.

Surface activation is critical in forming a reactive, hydrophilic surface for the coupling of the amino-silanes in the subsequent steps. If the surface is properly activated, a drop of water will immediately wet the surface and have a low contact angle.

9. Prepare a 3% (vol/vol) solution of (3-aminopropyl)trimethoxysilane (APTMS) in acidified methanol (5% vol/vol glacial acetic acid). For example, this will consist of 400 mL methanol, 20 mL acetic acid, and 12 mL of APTMS.

To prevent other, general-use glassware in the lab from being silanated, always use a dedicated container to prepare the mixture. APTMS is moisture sensitive and can be easily prone to hydrolysis if not stored properly under ambient conditions (evidenced by the emergence of a yellow discoloration over time). Purge the stock bottle with an inert gas (e.g., argon) and seal the bottle tightly with parafilm before storing the bottle in a chamber containing desiccant.

10. Submerge the coverslips in the APTMS solution and leave the reaction to proceed for at least 30 min. Longer incubation times (e.g., overnight) can also be performed with no adverse effects on the resulting micropatterning fidelity.
11. Remove the APTMS solution and rinse the coverslips three times with 70% ethanol.

In our experience for large batch preparations of coverslips, we have found that the APTMS solution can be immediately reused for another 30-min round of silanization (step 10).

12. Bake the coverslips at 100–120°C for 1 h to anneal the silanes. Let the coverslips cool to room temperature before proceeding in the protocol.

React the amines with polyethylene glycol (PEG) derivatives

13. Remove the mPEG-SVA from the -20°C, and let it reach room temperature before opening the stock bottle

mPEG-SVA is moisture sensitive. Gently purge the stock bottle with an inert gas and seal the bottle tightly using parafilm before again storing at -20°C.

14. Prepare an appropriate volume of a 100 mg/mL mPEG-SVA solution using 0.1 M NaHCO₃ (pH 8.5). Quickly centrifuge the solution at a high rpm to remove any bubbles.

The total volume depends on the number of coverslips prepared and the individual drop volume used below in step 15.

15. In parafilm-lined petri dishes, position drops of the mPEG-SVA solution (approx. 50 µL) in the dish and then place the coverslips on the drops. Be sure to have the coverslips well-spaced in the dish to prevent the coverslips from sliding and sandwiching together.
16. Leave the PEGylation reaction to proceed overnight in an enclosed, humidified environment to prevent evaporation of the solution.

After PEGylation, it is crucial to remember which side of the coverslip is now functionalized with PEG.

17. Thoroughly rinse each coverslip using ultrapure water and allow the coverslips to dry with their PEGylated side facing up.

The PEGylated coverslips can be used immediately for micropatterning or stored at room temperature for micropatterning at a later time.

Micropattern the coverslips

18. Rinse the photomask with ethanol or isopropanol and dry it using a lint-free tissue. Place the photomask in the UV cleaner with its chrome side facing up (i.e., the brown/copper colored side) towards the mercury lamps. Illuminate for 5 min to clean the photomask.
19. Place a 2.5 µL drop of ultrapure water (ideal volume for a 25 mm coverslip to provide an estimated spacing of 5 µm between the photomask and the coverslip) onto the chrome side of the photomask. Put the PEGylated side of the coverslip on the drop. Using a plastic pipette tip, press the coverslip against the photomask to remove any trapped air bubbles.

Unwanted air bubbles trapped between the photomask and the coverslip will lead to distortion of the patterns during UV illumination.

20. Place the photomask in the UV cleaner with its quartz side facing up (i.e., looking through the quartz the chrome will appear silver) towards the mercury lamps. Position the photomask roughly 5 cm away from the lamps ($\sim 10 \text{ mW/cm}^2$) and illuminate for 5 min.

Deep UV light less than 200 nm is essential for burning the exposed PEG regions through the photomask to create the desired pattern. While the minimum illumination time can vary depending on the molecular weight of the PEG and the resulting grafting density, in our hands, 5 min was sufficient for successful burning when using a 5,000 MW mPEG-SVA.

21. Apply water to the surface of the photomask and incubate for a short time for the coverslips to detach from the photomask. Using a plastic pipette tip, push them to the edge of the photomask and use a tweezer to grasp them for removal from the photomask.
22. Incubate the patterned side of the coverslip for 1 h at room temperature with 35 $\mu\text{g/mL}$ of fibronectin in 0.1 M NaHCO_3 (pH 8.5). Similarly, as in step 15, use a $\sim 50 \mu\text{L}$ drop on parafilm.

The use of fluorescently labelled fibronectin (e.g., obtained from Cytoskeleton, Inc.) is highly recommended to later observe the quality of the micropatterns. In this case, choose an appropriate dye for the labelled fibronectin so as not to interfere with the fluorescence of your transfected cells or with the immunofluorescence you plan to perform after fixation. The composition of your final fibronectin solution can be: 30 $\mu\text{g/mL}$ unlabelled fibronectin + 5 $\mu\text{g/mL}$ labelled fibronectin.

23. Rinse the functionalized coverslips in ultrapure water and place them with their patterned side facing up in 6-well dishes. Submerge the coverslips in PBS containing 0.1% sodium azide to prevent microbial growth and store them at 4°C until ready for cell plating.

In our experience, we have found that the coverslips can be stored for several weeks up to months without any appreciable impact on cell plating efficiency and cell confinement to the micropatterns.

Plate cells on the micropatterns

24. Under a cell culture hood, place the micropatterned coverslip in a sterile 6-well dish. Rinse the coverslip several times with copious amounts of $1\times$ PBS to thoroughly remove the azide storage solution. Finally, leave the coverslip submerged in $1\times$ PBS and pre-warm it in an incubator prior to cell plating.

In our experience, we have had no issue with microbial contamination when using an azide-containing storage solution. However, if users wish, they can further sterilize the coverslip in the cell culture hood using UV illumination.

25. Culture CAD cells to approximately 80% confluency as previously outlined in Basic Protocol 1.
26. Aspirate away the existing cell media and briefly rinse the cells with pre-warmed $1\times$ PBS (-/-). Mechanically detach the CAD cells in 2 mL of serum-free growth media containing 0.5 mM EGTA. Then using an additional 3 mL of the EGTA media, thoroughly rinse the culture side of the flask.

The presence of EGTA at this point helps to minimize unwanted cell clumping.

27. Strain the cell suspension through a 40 μm nylon cell strainer and determine the cell concentration using a hemacytometer or an automated cell counter.
28. Based on the concentration of your cell suspension, prepare a dilution to approx. 175,000 cells/mL. For this dilution, use complete growth media containing serum.

We found that diluting CAD cells while in suspension helps to reduce cell clumping, leading to better micropatterned cells. Using serum containing growth media at this point is necessary to ensure optimal adherence of CAD cells to the fibronectin micropatterns.

29. Plate 250,000 CAD cells in the dish and ensure the cells are evenly distributed above the coverslip. Let the cells gently settle by gravity in the culture hood for 1530 min before moving the dish to the incubator

Moving the dish to early leads to liquid swirling in the well that will concentration the suspended CAD cells in the middle of the dish. This will lead to unwanted clumping and poor micropatterning of the cells on the coverslip.

30. After approx. 4 h of cell adherence, gently aspirate the culture media to remove unattached cells, and slowly add 2 mL of pre-warmed culture media. Continue culturing the cells until the sample is ready for fixation, live imaging, etc.

BASIC PROTOCOL 5

CHARACTERIZATION OF THE ULTRASTRUCTURE OF TUNNELING NANOTUBE BY CRYO-EM

Preserving the ultrastructure of TNTs is a challenge, as classical methods used to investigate the structural identity of cellular compartments, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM), result in the breakdown of most TNTs during sample preparation. To better preserve these fragile structures and study their ultrastructure in detail, we decided to employ cryo-electron microscopy (cryo-EM). Cryo-EM can provide macromolecular structures with up to atomic resolution at an unprecedented level. This technique is powerful enough to resolve 3D structures of intracellular organelles and protein complexes in their near-native state and in their cellular environment (Weissenberger et al., 2021). However, for cryo-EM, a critical aspect is sample preservation. Preserving the integrity and stability of the samples is crucial to obtain accurate and reliable structural information. A key requirement is that the sample must be thin enough so that most electrons either pass through undeflected or undergo a single interaction with the sample (Passmore and Russo, 2016; Weissenberger et al., 2021). We developed an innovative pipeline that combines TNT preservation protocols with cryo-Fluorescent Microscopy (FM) and/or room-temperature (RT) confocal microscopy with cryo-EM and tomography (Sartori-Rupp et al., 2019) (Fig. 7 A-B).

Thanks to this unique pipeline, we have reported that TNTs of neuronal cells—that appear as a single connection by confocal microscopy—at ultrastructural levels were comprised of a bundle of individual tunneling nanotubes (iTNTs) each delimited by its own plasma membrane, and filled with parallel actin bundles (Fig. 7 C-G) on which different membrane-bound compartments and mitochondria appear to transfer (Sartori-Rupp et al., 2019). The protocol could be easily adapted for TNT studies in other cell types (Pepe et al., 2022).

Reagents and materials

CAD cells (Sigma, cat. no. 08100805)
SH-SY5Y cells (ATCC CRL-2266)
CAD cell growth medium (see recipe in Reagents and Solutions)
SH-SY5Y cell growth medium ((see recipe in Reagents and Solutions))

Fixative 1 (see recipe in Reagents and Solutions)
Fixative 2 (see recipe in Reagents and Solutions)
TEM grids (Quantifoil NH2A R2/2)
35mm μ -dishes, high, tissue culture–treated (ibidi)
24 mm round coverslips
Fibronectin
Cell membrane staining
1× PBS

Glow-discharge TEM grids

One of the most important challenges of cryo-EM lies in the efficient and reproducible preparation of samples. This is due to the sensitivity of many cell samples to the different seeding and culture conditions required for EM experiments and the fragility of EM grids. The preparation of good samples is required to have high-resolution cryo-EM acquisition.

1. Load 4 carbon-coated gold TEM grids (Quantifoil NH2A R2/2) onto 35mm μ -dishes, high, tissue culture–treated (ibidi) using fine forceps, the dark side (carbon) facing up in all grids.
2. Glow-discharge the TEM grids at 2 mA and $1.5\text{--}1.8 \times 10^{-1}$ mbar for 1 min in an ELMO (Cordouan) glow discharge system.

It is important to glow-discharge the grids to ensure that cells coat the grid smoothly and evenly (Grassucci et al., 2007).

3. Sterilize grids under UV one time for 30 min at RT.

Coat TEM grids

4. Incubate the sterilized TEM grids at 37 °C in a complete culture medium.
5. Carefully wash the grids twice in PBS and then coat them with 40 $\mu\text{g/mL}$ of fibronectin for 20 min at 37 °C.

Note: for each cell line, test the specific solution to coat the TEM grids. Specifically for CAD cells (Sartori-Rupp et al., 2019), we use fibronectin, while for SH-SY5Y, we do not use any coating solution.

6. Wash three times with 1× PBS to remove non-attached fibronectin.

Plate cells on TEM grids

7. Plate cells on TEM grids contained in 35mm μ -dishes and ensure that the cells are sparsely distributed above the TEM grids, resulting in 3 to 4 cells per grid square.

We have determined that to have approximately 4 cells per grid square, we seed 250,000 CAD (Sartori-Rupp et al., 2019) and 450,000 SH-SY5Y cells on TEM grids placed in the ibidi dishes (Fig. 7 C).

8. Incubate the cells on the TEM grids O/N at 37 °C.
9. Label the cells on the TEM grids with WGA (Alexa-488/546/647) (1:300 in PBS) for 5 min at 37 °C.

Cryo-correlative light and electron microscopy (i-CLEM) (Fig. 7-A)

Cryo-EM techniques require that the specimen is embedded in a thin layer of amorphous, non-crystalline, glassy ice (Hampton et al., 2017). To better preserve our samples, we used plunge freezing, cryo-fixation techniques where the cells seeded on the EM grid are blotted and rapidly plunged into liquid ethane. This technique is sufficient for the vitrification of samples between <100 nm and ~10 μ m thick, such as cultured cells (Dobro et al., 2010).

10. Vitrify the TEM grids containing fluorescently labeled cells (CAD and SH-SY5Y cells) using a Leica EMGP system. Cells are blotted from the back side of the grid for 8–10 S and rapidly frozen in liquid ethane (Pepe et al., 2023, 2022; Sartori-Rupp et al., 2019).

The blotting time can be varied depending on the cell confluence, the type of TEM grids, and the type of cells. Larger cells and higher cell confluence reduce blotting efficiency. From our experience, 10 S is a good time for CAD and SH-SY5Y cells.

11. Image cells are on an epifluorescent Axiovert 200 M inverted microscope (Zeiss) equipped with a cryo-correlative stage (Cryostage2, FEI), 10 \times (NA 0.3), 40 \times (NA 0.6), a 63 \times (NA 0.75, working distance 1.7 mm) long working distance air objective and with a 482/18 (Excitation), 520/28 (Emission, green) filter cube.
12. Following cryo-fluorescence imaging, acquire by cryo-TEM the vitrified samples on the TEM grids (Sartori-Rupp et al., 2019).

Correlative light and cryo-electron microscopy (ii-CLEM) (Fig. 7-B)

The main limitation of the i-CLEM pipeline is that the number of TNTs imaged by cryo-CLEM is very low. Indeed, since we are plunge freezing the cells in live conditions most of the TNTs break during the process. Therefore, we have established an alternative workflow by chemically fixing CAD and SH-SY5Y cells and imaging them by FM at room temperature (RT) prior to rapid freezing and cryo-TEM (ii-CLEM) (Fig. 7B) (Sartori-Rupp et al., 2019). In addition, by acquiring confocal images of TEM grids before vitrification, it is possible to obtain much more accurate information on the fluorescently labelled structures and their spatial organization than using the cryo-FL microscope.

13. Chemically fix CAD and SH-SY5Y cells seeded on TEM finder grids in Fixative 1 solution for 15 min at 37°C followed by Fixative 2 solution for 15 min at 37°C.
14. Wash the cells three times in PBS buffer and label them with WGA (Alexa-488/546/647) (1:300 in PBS) for 5 min at RT.

15. Identify cells of interest on the TEM grids and image the whole cellular volume by acquiring 0.5–1 μm Z-stacks with an inverted confocal microscope (Zeiss LSM 700 and/or 900) using ZEN software.
16. Following fluorescence imaging, vitrify the cells on grids using a Leica EMGP system. Cells are blotted from the back side of the grid for 8–10 S and rapidly frozen in liquid ethane as described above.
17. Acquire the regions of interest on the TEM grids (step 15) by using a cryo-EM (e.g., Titan Krios microscope) (Fig. 7 C-G).

In cryo- and chemically-fixed samples (i.e., i-CLEM vs. ii-CLEM) structural details (e.g. actin, plasma membrane) of the TNTs look indistinguishable (Sartori- Rupp et al., 2019).

REAGENTS AND SOLUTIONS.

Use deionized, distilled water in all recipes and protocol steps.

CAD cell growth medium

Opti-MEM I reduced serum medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% of penicillin-streptomycin. Store up to 1 month at 4°C.

SH-SY5Y cell growth medium

RPMI 1640 (Euroclone) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% of penicillin-streptomycin. Store up to 1 month at 4°C.

U251 cell growth medium

DMEM+ GlutaMAX-I (Gibco 31966-021) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin-streptomycin (1%). Store up to 1 month at 4°C.

Fixative 1 solution

2% (w/v) PFA
0.05% (w/v) glutaraldehyde
0.2 M HEPES
Quantity sufficient 1× PBS
Prepare fresh

Fixative 2 solution

4% (w/v) PFA
0.2 M HEPES
Quantity sufficient 1× PBS

Prepare fresh

Fixative 3 solution

4% (w/v) PFA

0.05% (w/v) glutaraldehyde

Quantity sufficient 1× PBS

Prepare fresh

Background Information

TNTs are transient membranous channels that represent a new mechanism for intercellular communication between remote cells, by establishing a direct cytoplasmic connection. They have very small diameters (20 to 500 nm) and, strikingly, can extend up to 100 μm (Abounit and Zurzolo, 2012; Cordero Cervantes and Zurzolo, 2021). Unlike other cellular protrusions, such as lamellipodia or filopodia, TNTs are not attached to the substrate in 2-D cell cultures. TNTs are typically based on F-actin cytoskeleton (Ljubojevic et al., 2021), although microtubule-containing TNTs have been described in several cell types (Onfelt et al., 2006) and in TNTs formed between tumor cells which are more stable and associated with bi-directional transfer (Kretschmer et al., 2019; Wang and Gerdes, 2015a). Indeed, the presence of microtubules in the TNTs described in several derived-tumor cells could be a mechanism to increase the lifetime of these structures and its ability to transfer different cargoes for longer periods of time under stress situation caused by chemo- or radio- therapy.

Recently, by using correlative FIB-SEM (Focused Ion Beam Scanning Electron Microscopes), light- and cryo-electron microscopy approaches, we have elucidated the structural organization of TNTs formed between neuronal cells. They are composed of a bundle of open-ended individual tunneling nanotubes (iTNTs) that are held together by threads positive for N-Cadherin proteins. iTNTs are filled with parallel actin bundles on which different membrane-bound compartments and mitochondria appear to transfer (Sartori-Rupp et al., 2019). Importantly, one of the most distinctive properties of TNTs is the open-ended cell-cell contact sites at each end, allowing cytoplasmic continuity (Sartori-Rupp et al., 2019). To explore the contact site between the cell body and the tip of the nanotube is challenging in cryo-EM as the contact site at the cell body is too thick; we have overcome this problem by using another EM approach, FIB-SEM (Sartori-Rupp et al., 2019). This open-endedness makes TNTs unique and distinguishes them from other membranous protrusions, such as filopodia (Sartori-Rupp et al., 2019) allowing the selective transfer of various cellular materials, such as: cytoplasmic molecules, plasma membrane components, vesicles derived from different organelles, whole organelles (e.g., mitochondria, lysosomes), and also viruses, bacteria and amyloid aggregates (Zurzolo, 2021).

While TNTs have been observed in a wide variety of cell types, such as neurons, astrocytes, fibroblasts, immune cells, and epithelial cells, their observation *in vivo* remains challenging as there is no specific TNT marker identified to date. However, recent studies have provided evidence for the presence of TNT-like structures in several *in vivo* and *ex vivo* models (Alarcon-Martinez et al., 2022; Chinnery et al., 2008; Lou et al., 2012b; Pasquier et al., 2013; Pinto et al., 2021). Intriguingly, by using a serial-sectioning scanning electron microscopy (ssSEM)-based connectomic approach, we have revealed open-ended connections between developing granule precursors neurons in the mouse cerebellum (Cordero Cervantes et al., 2023). This suggested that neurons can communicate directly before their synaptic connections are established (Zurzolo, 2021).

According to the literature, intercellular communication through TNTs may participate in many essential processes, such as development, immunity, tissue regeneration, and transmission of electrical signals (Korenkova et al., 2020). Furthermore, they have been implicated in the progression of several disease (Ariazi et al., 2017). TNTs can be hijacked by various pathogens, such as prion and prion-like proteins, bacteria and viruses such as SARS-CoV-2 (Cordero Cervantes and Zurzolo, 2021; Pepe et al., 2022). Increasing evidence suggests their role in cancer as well (Lou et al., 2012a; Pinto et al., 2021). Intercellular communication via TNTs between

tumor cells and other tumor cells or cells of the tumor microenvironment has been correlated with progression, evasion of apoptosis, metabolic reprogramming, and radio- and chemo-resistance (Dong et al., 2023; Nakhle et al., 2023; Osswald et al., 2015; Watson et al., 2023). We recently showed using patient-derived glioblastoma stem cells (GSLCs) exhibiting different metabolic phenotypes (isolated from the infiltrative zone of the tumor, a zone that has been proposed to be responsible for relapse), and a complex 3D-tumor organoid model, the formation of an intercellular network constituted by TNTs that is able to transfer mitochondria. In addition, the two GSLCs exhibited different responses to irradiation in terms of TNT induction and mitochondria transfer, although the correlation with the disease progression and therapy-resistance needs to be further addressed (Pinto et al., 2021).

Therefore, understanding TNT formation and subsequent transfer is an important area in cell biology, with TNTs emerging as promising therapeutical targets.

Although some molecular factors involved in TNT formation have been described in the literature, the mechanism underlying this process is far from being deciphered. Furthermore, the heterogeneity of TNT structures in different cell lines suggests that they may form through distinct mechanisms (Delage et al., 2016; Henderson et al., 2022; Pepe et al., 2023). In accordance, proteins that have been shown to be involved in this context in one cell line appear to be dispensable in others, for example, as is the case for p53 or M-Sec (Andresen et al., 2013; Gousset et al., 2013).

Most of the studies about the composition of the TNTs have been performed in fixed samples. One possible explanation is the challenge of studying actin and microtubule dynamics by live imaging. A widely used technique is the expression of actin- or tubulin fusion fluorescent proteins such as the use of “lifeAct” or “F-tractin” as actin markers or tubulin tagged with C-terminal GFP into mammalian cell lines. However, this approach has several limitations due to the level of expression of the actin-fused protein can compromise cytoskeletal functions. For instance LifeAct-GFP overexpression has been described as detrimental to actin filament organization in cardiomyocytes, leading to pericardial edema and early embryonic lethality of zebrafish embryos (Xu and Du, 2021). In this regard, it is important to demonstrate that the chimeric protein retains its functional/structural characteristics. In addition, overexpression of any protein can potentially interfere with cellular functions. This latter limitation can be overcome using inducible promoters in the plasmid construct or by establishing permanent cell lines with the desired level of expression of the chimera.

Further studies of TNT formation in different biological contexts are of crucial interest for a better understanding of these fascinating communication highways. In the last years, the development of different probes allowing the live cell microtubule or actin stain with reduced background and the improvement of the different systems of acquisition (possibility to acquire different colors at the same time, an increase of the speed of acquisition, better cameras) have been crucial proportioning the necessary tools to unravel several biological questions about the cytoskeletal dynamics performing live-cell imaging with reduced phototoxicity.

It is striking that while it is now clear that TNTs can be found in a great variety of cell types and are involved in a plethora of biological processes, these structures attracted attention only recently and were described for the first time by Gerdes and coworkers in 2004 (Rustom et al., 2004). Several reasons can explain this relatively late discovery: (1) TNTs are fragile structures, sensitive to light exposure, shearing force, and chemical fixation; (2) they are mainly observable in sub-confluent cell cultures; (3) they have very small diameters and their observation in fluorescence microscopy generally requires highly contrasted pictures. In addition, TNTs are transient and very dynamic structures, and their structural (e.g., length/diameter, presence of micro-tubules, open-endedness) and functional (e.g., type of transferred cargoes/signals)

heterogeneity among cell types further make their study more complex. It is therefore crucial to thoroughly characterize any “TNT-like” structure observed in each biological system before assuming that intercellular communication occurs through TNTs.

The protocols described in this unit provide a framework easily transferable to any cell culture for this first characterization. They allow the reliable identification of TNTs through the major criteria that have been established in the literature (see Basic Protocol 1&4), the determination of the general mechanism(s) driving TNT formation (see Basic Protocol 2), the evaluation of the transfer of cellular material through TNTs (see Basic Protocol 3) and investigation of TNTs ultrastructure (see Basic Protocol 5).

The authors believe that the information gained via these approaches will constitute a solid basis for any study on TNT involvement in a wide range of physiological contexts.

Critical Parameters

In the authors’ laboratory, mouse neuronal CAD cells (Qi et al., 1997), which can undergo differentiation upon serum deprivation, are predominantly used. Upon differentiation, the cells lose the immortalizing oncogene SV40 T antigen and form neuritis and neuritis-like protrusions (Segura-Aguilar, 2011). In addition to CAD, human neuronal SH-SY5Y cells are also used in the lab. Differentiated CAD and SH-SY5Y cells are impaired in their ability to form TNTs; it is therefore crucial to keep them in an undifferentiated state. On the other hand, stress conditions such as low temperature, pH disturbance, extended time in culture, or too high passage numbers enhance TNT formation. Hence, it is necessary to use only healthy cell cultures for TNT studies and to carefully control environmental parameters. Since cold has an inducing effect on TNT formation and breakage, it is important that all reagents coming into contact with the living cells (PBS, fixatives, etc.) are pre-warmed at 37°C. However, to preserve the structure of TNTs, it is recommended to fix the cells at 37°C. Therefore, when using different cell lines, these general parameters characteristic of the specific cell type should be analyzed and optimized.

Furthermore, as mentioned in Basic Protocol 1, cell confluence is one of the most critical parameters when studying TNTs. Some cells, such as CADs and SH-SY5Y, tend to form clumps if they are too confluent or not properly separated following their detachment. In this case, the plating will be heterogeneous, with cells too close to each other to form TNTs, thus creating a bias in TNT number evaluation. To avoid the formation of clumps, the cell suspension can be filtered using cell strainers. However, in order to characterize TNTs and solve the problems described above (e.g., confluent cells not properly distributed), we have described a micropatterning approach in Basic Protocol 4 that will make it possible to identify TNTs from filopodia and the TNTs that arise through a filopodia like protrusion mechanisms and not cell dislodgment.

The fixative solutions described in Basic Protocol 1 contain both PFA and glutaraldehyde. The addition of glutaraldehyde is beneficial because this agent is known to have a better propensity to cross-link proteins. Although methanol is widely used as a fixative prior to immunodetection (because it preserves protein immunogenicity), it can produce high background and disrupt actin filaments and TNTs. Methanol-free formaldehyde is therefore the best fixative in this context. Permeabilization with Triton-X is well suited for cytoskeleton observation because it can partially extract free actin or tubulin monomers (often present at high level in the cytoplasm) without altering the polymeric structure, thus improving the resolution of cytoskeleton filaments. However, if detection of a soluble or membrane protein has to be performed in parallel, a milder detergent such as saponin is preferred.

Since most of the protocols described in this unit rely on optical techniques (microscopy and FACS) based on fluorescent labeling of different cell features, the choice of the fluorophore is particularly important. All examples provided in this unit have proved to offer strong and stable signals, allowing easy observation of TNTs and vesicle transfer. Other dyes or fluorescent tags can be employed if necessary, keeping in mind that when several fluorophores are used on the same sample, their excitation/emission spectra must be far enough for their signal to be easily discriminated.

Although any fluorescent protein or protein-fusion can be transiently transfected to distinguish the two cell populations (given that the fluorescent signals will be easily discriminated), it is important to choose proteins that will not significantly affect cell physiology, and especially TNT formation. This can consist of fluorescent soluble proteins, such as GFP and RFP for example, or fluorescent nuclear markers, such as H2B-GFP or mCherry fusion-proteins. In the latter case, cells must be labeled with the fluorescent WGA to detect TNTs (see Basic Protocol 1). An alternative consists of transfecting cells with GFP-actin on one hand and mCherry-actin on the other hand. This will allow for the observation of intercellular connections without additional membrane staining and might give some indications on which cell originated each TNT (by looking at the predominant fluorescence). However, the expression level of the actin- or tubulin-fused proteins should be an intermediate-low expression to avoid aberrant cytoskeletal dynamic behaviours (Riedl et al., 2008; Xu and Du, 2021). On the other hand, cells could also be transfected with plasma membrane markers, such as fluorescent proteins fused to a GPI-attachment signal (e.g., GFP-GPI) (Gousset et al., 2009; Sowinski et al., 2008) or a farnesylation signal (e.g., EGFP-f) (Rustom et al., 2004).

Importantly, one of the main issues in the field of TNTs is that most of the information on TNTs has been described using mainly low-resolution imaging methods such as fluorescence microscopy (FM). Our correlative cryo-EM pipeline allowed us to obtain the first description of the ultrastructure of TNTs connecting neuronal cells. The protocol described in Basic Protocol 5 illustrates the pipeline we have developed (Pepe et al., 2023, 2022; Sartori-Rupp et al., 2019) and provides detailed information. One of the greatest challenges in cryo-EM is the sample preparation. Firstly, the grids are fragile, and break easily. Secondly, cells such as CAD and SH-SY5Y cells tend to form clumps and consequently do not distribute well on the grids. However, to solve this problem, we could micropattern the TEM grids.

Troubleshooting

Low basal TNT number

CAD and SH-SY5Y cells cultured under normal conditions form a relatively high number of TNTs but this may not be the case for other cell lines. However, a low number of TNT-forming cells in basal conditions does not necessarily imply that the cell line is unable to form TNTs, or that TNT do not exert important functions. These structures are dynamic and can be induced by specific conditions, such as environmental stress or pathogen infection. The protocols described in this unit must be performed in conditions inducing TNT formation.

Inefficient cell transfection

For efficient cell transfection, in addition to the DNA/Lipofectamine[®] 2000 ratio, cell confluency also needs to be optimized. For CAD cells, it appears that a confluency much lower than the one recommended by the manufacturer gives better results. When performing a transient transfection,

the transfection rate and expression level vary with time following the transfection, depending on the construct and the cell type. It is recommended to start with a time-course in order to determine the optimal time post-transfection for analysis of transfected cells.

Very low rate of transfer

When the ratio of co-culture is not optimal, the rate of transfer observed can be very low or nonexistent (vesicles, organelles, or pathogens). This can happen when the loading of the donor cells is not efficient and/or when the rate of transfection of the acceptor cells is too low. To solve the loading problems of the donor cells, a time course of uptake and clearance should be done to know at which time point the cells are well loaded. This is important to set up to get the optimal time of co-culture. For the acceptor cells, a better transfection rate can be obtained by using stably transfected clones. In this case, the transfer experiment should be done without the antibiotic of selection.

Overexpression of actin fusion fluorescent proteins

When the expression of the plasmids affects the morphology and normal functionality of the cytoskeletal components it is necessary to select cells with an intermediate–low level of expression of the constructs. Sorting the cells permits enrichment of the population of cells displaying normal cytoskeletal function. As a control, the use of live-compatible dyes to stain the actin filaments and microtubules should be performed.

Low quality of cryo-EM image

In cryo-EM, good sample preparation is the key to getting the best ultrastructural information. For example, cells do not distribute well on the TEM grids, grids are damaged, vitrified cells are too thick and ice crystal contamination on top of the vitrified sample drastically compromise the high-resolution acquisition of cryo-EM images.

Since cellular samples are very fragile, it is vital to set-up the cellular parameters such as cell time replication, optimal cellular confluence on EM grids and cell ability to adhere on grids before to start ultrastructural studies. Set the vitrification parameters (e.g., blotting times) in advance, as the cells need to be frozen quickly in order for the water to transform into amorphous or vitreous ice. If water becomes crystallized ice upon freezing, fine structures are easily destroyed. Importantly, the sample must be thin and small to enable rapid freezing.

Understanding Results

In the authors' hands, the basal number of TNT forming cells observed for CAD cells ranges between 40% and 60%, and for SH-SY5Y cells between 30% and 35%. Depending on the experimental conditions, up to 40% increase or 80% decrease of TNT forming cells can be observed. The basal rate of vesicle transfer after 16 h co-culture is ~80% when evaluated by microscopy or 20% when evaluated by FACS. This difference is due to the higher sensitivity of microscopy compared to FACS to detect transferred vesicles or proteins. Thus, it is recommended, whenever possible, to use both techniques to assess TNT-mediated transfer. In SH-SY5Y cells the basal rate of vesicle transfer after 16 h co-culture is 25% when evaluated by microscopy. Results about TNT counting and DiD transfer on micropatterns are found in Henderson et al., 2022 (Henderson et al., 2022). The basal rate of transfer of

mitochondria in U251 GBM cells after 2 days of 2D co-culture is 1.25%; this percentage increases over time, for example, Glioblastoma stem cells can reach 8.37% of acceptors receiving mitochondria after 23 days of culture in 3D-tumor organoids (Pinto et al., 2021).

Time Considerations

In CAD and SH-SY5Y cells presenting an appropriate confluency, TNT formation occurs fast following plating. A significant number of TNT forming cells can be noticed after 30 min or even less. Regarding vesicle transfer, longer cultures are usually required: a 3-hr co-culture represents a minimum time to obtain significant transfer and a 16-h co-culture appears to be an optimal incubation time. Mitochondria transfer request longer time (e.g., 48h). The time window to study TNTs is thus relatively wide and the experiment planning must be thoroughly adapted to the question that is addressed.

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CONFLICT OF INTEREST STATEMENT (*required*):

The authors declare no conflict of interest.

Authors are responsible for disclosing all financial and personal relationships between themselves and others that might bias their work. Authors who work for organizations that sell or provide reagents used in the protocol should note that here.

DATA AVAILABILITY STATEMENT (*required*):

The data, tools, and material (or their source) that support the protocol are available from the corresponding author upon reasonable request.

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(old) Figure 1 Schematic representation of the two models of TNT formation. TNTs can form by filopodia-like extensions directed toward a neighboring cell (A) or when two cells previously in contact move in opposite directions, while retaining a thin thread of membrane (B).

(old) Figure 2 Membrane and actin cytoskeleton staining in CAD cells. CAD cells plated on ibidi 35-mm μ -dishes were cultured overnight and fixed as described in Basic Protocol 1. (A) Fluorescent wheat germ agglutinin staining of TNTs. Fixed cells were labeled with Alexa Fluor 488 conjugate of WGA for 20 min to dye membranes and detect TNTs. Scale bar = 10 μ m. (B) Fluorescent phalloidin staining of TNTs. Fixed cells were labeled with Alexa Fluor 546 conjugate of phalloidin for 30 min to dye the F-actin cytoskeleton. Scale bar = 10 μ m.

(New) Figure 3. Membrane and actin cytoskeleton staining in SH-SY5Y cells. (A). Bottom (left) and upper (right) sections of WGA Alexa 488 labelled SH-SY5Y cells. Yellow arrows indicate TNTs visible only in upper sections (right). Scale bar: 5 μ m. (B) Representative confocal micrograph shows actin and microtubule cytoskeleton labelling with rhodamine-phalloidin and beta-tubulin respectively and DAPI for nuclei of SH-SY5Y cells; white arrows indicate TNTs positive for rhodamine- phalloidin. Scale bar: 10 μ m.

(New) Figure 4: Live-imaging approaches to identify the cytoskeletal composition. (A) Expression actin- and tubulin fusion fluorescent proteins. U251 expressing lifeAct-670 and GFP-tubulin. (B) Live-compatibles dyes to stain actin filaments and microtubules. U251 labelled with Cell Mask Deep Red Actin Tracking stain and SPY555-tubulin. Insets (right) shows a magnified image of TNTs. White arrows indicate the presence of only actin positive TNTs. White asterisk indicates TNTs with microtubules. Scale bars 10 μ m

(New) Figure 5: Flow cytometry approach to quantify the rate of mitochondria transfer. (A) Schematic representation of the co-culture experiment. U251 expressing mitoGFP (as Donors) were co-cultured with U251 expressing mCherry cytosolic (as acceptors) in a ratio 1:1 for 2 days in 2D-culture in two different conditions: Direct contact or separated for a filter, which allows the pass of mitochondria but impairs the direct contact between donors and acceptors. (B) Representative FACSdot plots. Y and X axis indicate positive events for mitoGFP and mCherry, respectively. The gate in red indicates double- positive cells (Acceptors mCherry with mitoGFP inside). (C) Graph of the quantification of acceptor cells receiving mitochondria in U251 by FACs. A minimum of 100.000 events were analyzed.

(New) Figure 6: Surface micropatterning using deep UV light for TNT studies. (A) Scheme depicting the deep UV micropatterning approach to produce hexagonally arranged circles of fibronectin (spaced equidistantly) for cell patterning and TNT identification. (B) Reaction scheme for the functionalization of glass coverslips with high molecular weight PEG polymers. (C) Representative surface and upper stack maximum intensity projections showing substrate detached TNT connections between CAD cells adhered on neighbouring fibronectin micropatterns (30 μm spacing). Several DiD-labelled vesicles within the TNTs could be observed, confirming their functionality for cargo transfer.

(New) Figure 7. Schematic of correlative light and cryo-electron microscopy and ultrastructure of TNT connecting SH-SY5Y cells. Cells are seeded on TEM grids. After 24 h, the labelled cells (A) are cryo-fixed and acquired with a cryo-FM microscope before being observed with cryo-EM. (B) Labelled cells are chemical fixed and acquired using a confocal microscope. Then cryo-Fix and acquired using cryo-EM. (C) Confocal micrograph of SH-SY5Y cells stained with WGA (green). (D) Low and (E) intermedia magnification electron micrograph corresponding to the TNT showed in (C). (F) High-magnification micrographs corresponding to yellow square in (E). (G) Sum slices of the tomograms corresponding to yellow squares in D. Scale bars: C-E, 10 μm , F-G, 100 nm.