Review

THE EMBO JOURNAL

Peering into tunneling nanotubes—The path forward

Diégo Cordero Cervantes & Chiara Zurzolo*

Abstract

The identification of Tunneling Nanotubes (TNTs) and TNT-like structures signified a critical turning point in the field of cell-cell communication. With hypothesized roles in development and disease progression, TNTs' ability to transport biological cargo between distant cells has elevated these structures to a unique and privileged position among other mechanisms of intercellular communication. However, the field faces numerous challenges—some of the most pressing issues being the demonstration of TNTs *in vivo* and understanding how they form and function. Another stumbling block is represented by the vast disparity in structures classified as TNTs. In order to address this ambiguity, we propose a clear nomenclature and provide a comprehensive overview of the existing knowledge concerning TNTs. We also discuss their structure, formation-related pathways, biological function, as well as their proposed role in disease. Furthermore, we pinpoint gaps and dichotomies found across the field and highlight unexplored research avenues. Lastly, we review the methods employed to date and suggest the application of new technologies to better understand these elusive biological structures.

Keywords actin protrusions; cell communication; cell signaling; tunneling nanotubes

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Development; Signal Transduction

DOI 10.15252/embj.2020105789 | Received 1 June 2020 | Revised 21 October 2020 | Accepted 15 January 2021 | Published online 1 March 2021

The EMBO Journal (2021) 40: e105789

An overview of tunneling nanotubes

Cell-to-cell communication occurs through a variety of mechanisms which enable a diverse collection of biological messages to be shared between cells. These mechanisms include gap junctions (GJs), immunological and neural synapses, soluble factors, and extracellular vesicles. Stepping into the spotlight in 2004, thin, tubular membranous conduits rich in actin termed Tunneling nanotubes (TNTs) were first described and progressively gained recognition for allowing direct transport of cytoplasmic molecules, proteins, organelles, and even pathogens between distant cells. Although similar cellular structures called *plasmodesmata* had been described in plants (Sager & Lee, 2018), the identification of TNTs in the cells of animals was revolutionary and has incited great debate among the scientific community (Baker, 2017).

Since TNTs were first observed in cell culture (Rustom *et al*, 2004), the field of TNT-mediated intercellular communication has expanded rapidly to reveal their existence in a wide variety of cell types, primarily *in vitro*. In two-dimensional (2D) cell culture, TNTs are defined as: (I) thin (20–700 nm) and straight membranous protrusions hovering over the substrate and directly connecting two (or more) cells of both homotypic (same) and heterotypic (different) kind. (II) Internally, the presence of F-actin cytoskeletal filaments enables (III) intercellular transfer of cargo. The last and most distinctive property of *bona fide* TNTs are (IV) open-ended cell-cell contact sites at each end, allowing cytoplasmic continuity (Fig 1); however, few studies have successfully demonstrated this feature. Interestingly, a subset of TNTs described as "close-ended" protrusions facilitate electrical coupling between cells through GJs at the tip of the protrusion and recipient cell (Wang *et al*, 2010; Wang *et al*, 2012; Lock *et al*, 2016; Okafo *et al*, 2017). Notably, the functional ability to transport cargo is what makes other TNTs unique and distinct from this class of close-ended protrusion that, given the size and selective permeability of GJs, does not allow intercellular transfer of large cargoes (e.g., mitochondria). Considering that cellular processes, including TNTs, are dynamic protrusions, it is conceivable that close-ended TNTs eventually result in open-ended TNTs, indicating that at some point in their lifespan, these structures will enable large intercellular cargo transport through open ends. However, whether close-ended TNTs are different structures—and not a "time point" in their lifetime—has not yet been investigated.

One distinguishing characteristic of TNTs appears to be that they contain only actin. This fact was demonstrated through two key findings: (I) microtubule-disrupting and microtubule-stabilizing agents (nocodazole and paclitaxel, respectively) did not alter induction of TNTs (Wang *et al*, 2011), and (II) F-actin depolymerizing toxins, (*latrunculin*, *cytochalasin B*, and *cytochalasin D)* blocked TNT formation (Bukoreshtliev *et al*, 2009; Schiller *et al*, 2013; Takahashi *et al*, 2013; Dupont *et al*, 2018). In addition to thin "canonical" TNTs fulfilling the characteristics outlined above, "thick" (> 0.7 μm in diameter), microtubule-bearing TNTs have been found in macrophages (Onfelt *et al*, 2006; Souriant *et al*, 2019; Dupont

Institut Pasteur, Membrane Traffic and Pathogenesis, Paris, France
*Corresponding author. Tel: +33 1 4568-8277; E-mail: chiara.zurzolo@pasteur.fr

© 2021 The Authors

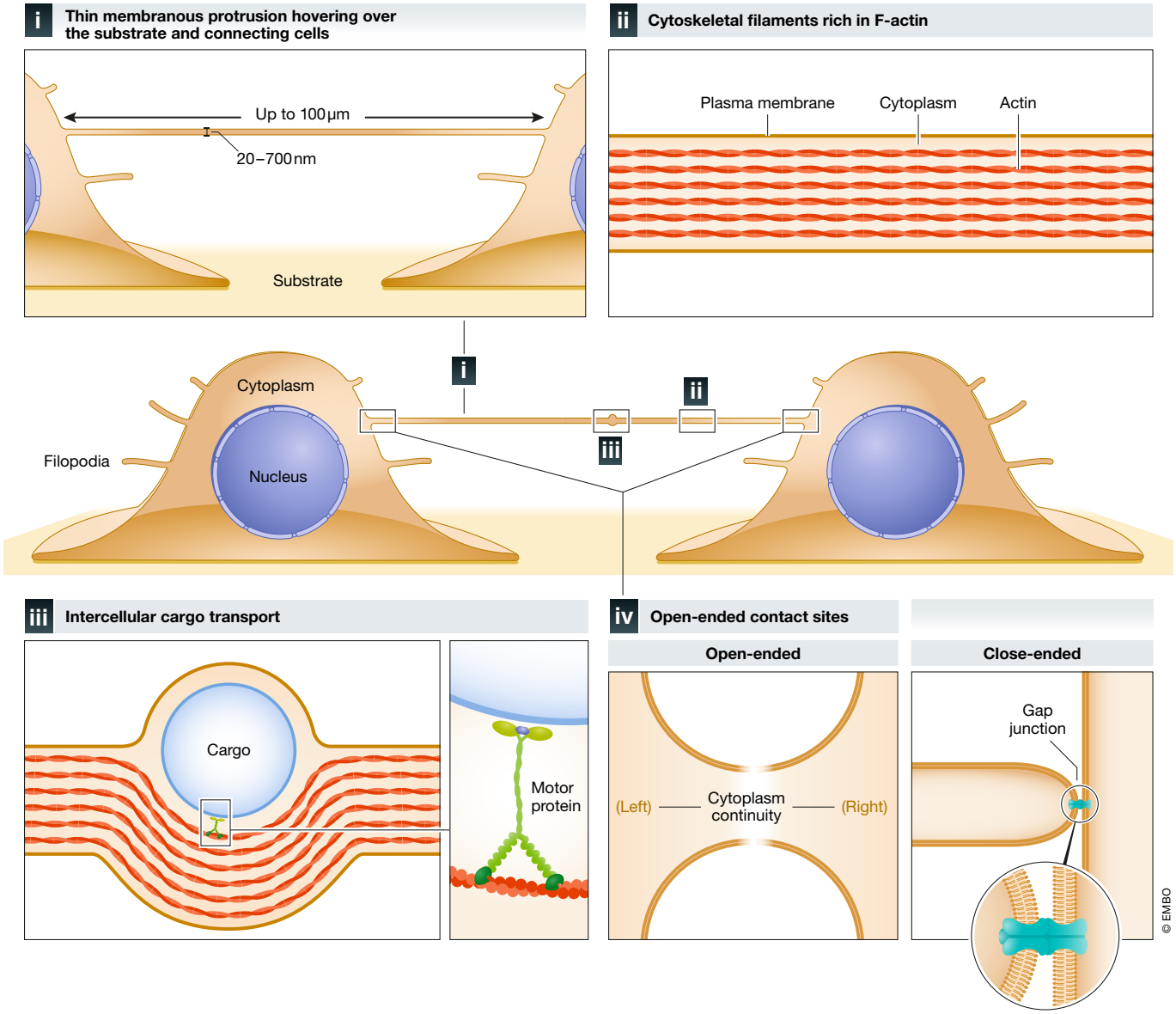


Figure 1. Distinctive properties of TNTs.

TNTs are (I) thin (20–700 nm) and long reaching (up to 100 µm in thin TNTs; < 700 nm in diameter) membranous protrusions that hover over the substrate and connect distant cells. Thick TNTs (diameter of > 700 nm) (not shown) can extend up to 250 µm in length. TNTs are (II) rich in F-actin cytoskeletal filaments, which enable (III) internal intercellular cargo transport using motor proteins. *Bona fide* TNTs feature (IV) open-ended contact sites at each end allowing for cytoplasmic continuity. A subset of TNTs contains one close-ended contact site that facilitates electrical coupling between cells through gap junctions (GJ) at the tip of the protrusion and recipient cell.

et al, 2020), urothelial cells (Onfelt *et al*, 2006; Veranic *et al*, 2008; Resnik *et al*, 2018), B cells (Osteikoetxea-Molnár *et al*, 2016); PC12 cells (Wang & Gerdes, 2015); astrocytes and cardiac myoblasts (Austefjord *et al*, 2014). These structures appear to be more resistant compared to thin TNTs, presumably from the rigidity provided by their thick architecture and microtubule cytoskeletal backbone and can reach up to 250 µm as described recently in macrophages (Dupont *et al*, 2020). They have been involved in the transfer of organelles (Eugenin *et al*, 2009; Hashimoto *et al*, 2016; Dupont *et al*, 2018) and HIV (Dupont *et al*, 2020); however, their specific structure and arrangement of their internal cytoskeleton, and whether they are open-ended has not been assessed yet.

While identifying straight membranous protrusions with no substratum contact is achievable in a sparse, 2D cell culture landscape, identifying TNTs in 3D models such as organoids (Zhang *et al*, 2018; Pulze *et al*, 2020; Whitehead *et al*, 2020), *ex vivo* explants (Chinnery *et al*, 2008), and tissues (Lou *et al*, 2012), is considerably more challenging. In these and/or other ecosystems in

 © 2021 The Authors

which cells are embedded in a natural or artificial matrix, stromal elements, such as neighboring cells, can both overshadow TNTs and force them to adopt convoluted shapes compared to those seen *in vitro* (Korenkova *et al*, 2020). In these complex circumstances, TNT detection relies solely on evaluation of intercellular cargo or signal transfer, a feat of extreme technical difficulty.

In certain cases in which structures meet only some of the criterion detailed above, we suggest authors employ the term *TNT-like structure* rather than TNT in order to establish a common language-base in the field and to avoid the use of different terms for similar TNT-like structures identified in different cell types, such as: *T-cell nanotubes* in Jurkat T cells (Sowinski *et al*, 2008) and *epithelial bridges* in epithelial cells (Zani *et al*, 2010). In other cases, TNT synonyms, such as: *bridging conduits* in macrophages (Kadiu & Gendelman, 2011), *nanoscale bridges* in endothelial cells (Connor *et al*, 2015), and *intercellular membranous nanotubes* in urothelial cells (Lokar *et al*, 2012) have also been utilized. This heterogeneity in nomenclature has reportedly "created confusion in terminology" (Ariazi *et al*, 2017; Baker, 2017; Yamashita *et al*, 2018; Korenkova *et al*, 2020) and ultimately resulted in widespread contention over differing language ascribed to what may or may not be the same biological structure.

In spite of this dissidence, TNTs and TNT-like structures still represent unique biological phenomena thought to play a role in a wide-range of physiological processes and pathological conditions. These respective roles have been carefully examined in specialized reviews spanning various TNT research domains (Table 1). However, despite remarkable advances in TNT biology over the last decade, formidable challenges in this discipline remain. The absence of a TNT-specific marker and technological limitations in the process of structural characterization have hampered our ability to (I) catalog TNTs unambiguously and determine potential existence of subclassifications, and (II) verify their existence *in vivo* and ascertain if they have the same functions as observed *in vitro*.

Here, we review methodologies previously and currently employed to study TNTs and suggest the future implementation of cutting-edge technologies to enhance and further the field. We also summarize, discuss, and critically appraise existing research to outline and define the principles on which the next generation of TNT research should be based both *in vitro* and *in vivo*.

Table 1. Classification of specialized reviews spanning various tunneling nanotube research domains.

Topic	References
Molecular machineries involved TNT formation, regulation, and architecture	Abounit and Zurzolo (2012)
	Gerdes *et al* (2007)
	Gurke *et al* (2008)
Stability and elasticity	Drab *et al* (2019)
Morphological diversity	Austefjord *et al* (2014)
	Gerdes and Carvalho (2008)
Disease and therapeutic applications	Mittal *et al* (2019)
Challenges of studying them in development, health, and disease conditions	Ariazi *et al* (2017)
	Jash *et al* (2018)
Distinctions with cytonemes, and TNT-like membranous protrusions	Korenkova *et al* (2020)
	Sherer *et al* (2007)
	Yamashita *et al* (2018)
Role in organelle transfer	Kiriyama and Nochi (2017)
	Murray and Krasnodembskaya (2019)
	Vignais *et al* (2017)
Role in neural processes and/or neurodegenerative diseases	Marzo *et al* (2012)
	Victoria and Zurzolo (2017)
	Vargas *et al* (2019)
	Nussenzveig (2019)
Role in cancer	Hekmatshoar *et al* (2018)
	Matejka and Reindl (2019)
	Roehlecke and Schmidt (2020)
	Asencio-Barría *et al* (2019)
	Pinto *et al* (2020)
	Jash *et al* (2018)
Role in immunology	Dupont *et al* (2018)
	Lin *et al* (2019)
	Onfelt *et al* (2005)
Role in Virology	Jansens *et al* (2020)

Methodology for the study of TNTs

Labeling

Given the current scarcity of a TNT-specific biomarker, the prevailing method in TNT labeling is the staining of the entire plasma membrane with fluorescently conjugated proteins which bind glycolipids and glycoproteins, such as WGA (Abounit *et al*, 2015), *FM1-43* (McCoy-Simandle *et al*, 2016), and *MemBright* (Collot *et al*, 2019). Alternatively, overexpressing proteins associated with the plasma membrane, such as H-Ras, CAAX, and Lyn also serve a similar purpose (Caneparo *et al*, 2011; Rainy *et al*, 2013; Hanna *et al*, 2019).

Although the plasma membrane composition of TNTs remains largely unexplored (Delage & Zurzolo, 2013), previous studies identified an enrichment of specific lipids, notably cholesterol and sphingolipids, in the membrane of TNTs connecting urothelial cells (Lokar *et al*, 2012), and, through indirect evidence, a role of the phosphatidylinositol triphosphate (PIP_3) in TNT formation in astrocytes (Wang *et al*, 2011) and neuronal CAD cells (Gousset *et al*, 2013) (see (Delage & Zurzolo, 2013) for review). In HeLa cells, PIP_3 and phosphatidylinositol biphosphate (PIP_2) both appear to recruit M-Sec (Kimura *et al*, 2016), a protein that shares homology with Sec6, a component of the exocyst complex, that is known to be an inducer of *de novo* TNTs (Hase *et al*, 2009). Interestingly, PIP_2 was localized on M-Sec-positive TNTs, whereas PIP_3 instead accumulated in the plasma membrane ruffles at the cell periphery. Taken together, these discoveries expose a promising avenue of research which should be further explored as it may uncover important structure/function information concerning TNT membrane composition and transport role, and TNT-specific membrane labeling hits.

Another complementary TNT-labeling approach is the targeting of their actin backbone through either fluorescently

© 2021 The Authors

conjugated Phalloidin staining in fixed cells (Abounit *et al*, 2015), expressing fluorescently conjugated actin monomers (Tardivel *et al*, 2016), or using different actin dyes in living cells (*Lifeact* fused to GFP or mCherry or the *jasplakinolide-analog dye, SiR-actin*) (Sun *et al*, 2019). Additionally, a recent report has shed light on a novel approach to simultaneously label actin-, intermediate-, and microtubule-filaments of TNTs of urothelial cells (Resnik *et al*, 2019), with potential future application for co-cytoskeletal-distribution studies. The reproducibility of this approach in TNTs in other cell types has yet to be established, but it could be used to distinguish conduits containing only actin, microtubules, or both (Resnik *et al*, 2018).

Efforts made to identify TNT-specific probes include the identification of an Aptamer, *M17A2*, which effectively labels TNT-like structures in a specific breast cancer cell line (Zhang *et al*, 2016); however, its application and reproducibility in other cells has yet to be confirmed. In another study, laser capture microdissection and mass spectroscopy enabled Gousset and colleagues to isolate and compare membranous protrusions (including TNTs) formed by neuronal CAD cells (Gousset *et al*, 2019). This study revealed interesting and unexpected proteins, such as microtubule-nucleating proteins in cells with TNTs typically deficient of microtubules. Such studies have provided our field with protein candidates to be further explored which is especially important for *in vivo* studies where the demand for a marker is much higher.

Detection by microscopy

Laser scanning confocal microscopy (LSCM) has revealed that the long (up to 100 μm) and thin (20–700 nm) topological nature of TNTs make them fragile and vulnerable to shearing forces (Sartori-Rupp *et al*, 2019) (Box 1). The preservation of TNTs through classical fixation methods has been met with countless obstacles as TNTs' heightened susceptibility to low-concentrated chemical fixatives (e.g., paraformaldehyde) and long-light exposures often results in breakage or bending.

To circumvent the obstacle of preserving TNTs, we and others have implemented a two-step fixation protocol leveraging extensive crosslinking via glutaraldehyde prior to paraformaldehyde fixation (Abounit *et al*, 2015; Carter *et al*, 2020). The fragility of TNTs has rendered investigations concerning membrane permeabilization experimentally cumbersome, as pores on the membrane weaken the scaffolding structure that builds TNTs. Presently, access to the lumen of TNTs is achieved through the use of milder membrane detergents (e.g., saponin) at low concentrations (Rupp *et al*, 2011; Costanzo *et al*, 2013; Zhu *et al*, 2018); however, the identification of inner TNT components with this method remains unsatisfactory due to its intrinsic inefficiency and damage it inflicts on the membrane. Expression of fluorescent proteins residing or getting transported within the lumen of TNTs has been used to image these structures using live microscopy, but this approach also generates complications due to the potential detrimental effects levied on the cell by protein overexpression. Moreover, this technique is indirect and cannot be used in all cell types given the existing variation in transfection susceptibility and ranging difficulties in protein expression.

To properly identify TNTs by microscopy, it is crucial to distinguish them from other membranous protrusions such as filopodia. Unlike peripheral filopodia (substrate-adherent filopodia), TNTs hover over the substrate and even over other cells, and unlike dorsal filopodia, TNTs must connect two or more distant cells sufficiently spaced apart in order to be visualized (Fig 1) (Abounit *et al*, 2015). This requires cells in culture to be seeded in a medium-confluent monolayer ecosystem warranting sufficient physical space between cells (Zhu *et al*, 2018). An interesting study actually found this stipulation could be overcome by cellular protease treatment (e.g., trypsin). In their study, Rustom and colleagues singularized cells by detaching them and turning them spherical, allowing for unobstructed TNT visualization (Staufer *et al*, 2018). However, it is currently unknown whether protease treatment of cells induces TNT formation via a stress response, for example (Zhang, 2011; Matejka & Reindl, 2019), and more intriguingly, if the observable connections are capable of intercellular cargo transport (Staufer *et al*, 2018). This study also highlights an intriguing conundrum in the field—the correlation between cell shape and respective capacity to form TNTs. If round cells exhibit a low membrane tension compared to a state in which cells are spreading with an increased membrane tension exerted by lamellipodial protrusions (Pontes *et al*, 2017), it is reasonable to think that round cells have a higher propensity to form TNTs. The more readily available membrane reservoir in round cells could also provide the extra membrane area necessary to give rise to new membranous protruding structures (Gauthier *et al*, 2012). However, efforts to assess the biophysical properties that mediate this relationship have not been undertaken.

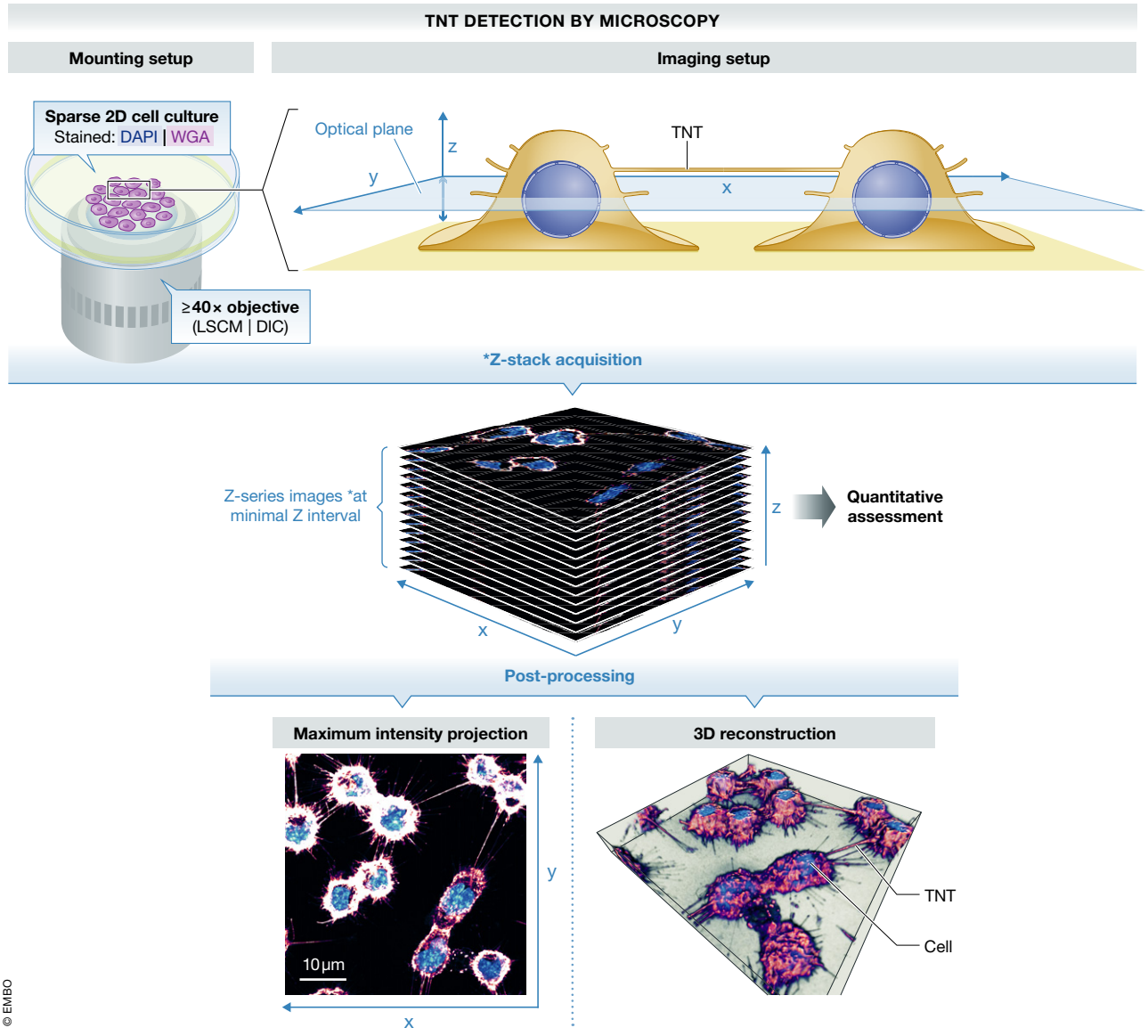
Quantitative assessment

Another aspect of TNT research not sufficiently addressed, yet vital to the reproducibility of findings, is the framework employed in quantitative analysis (see Box 2). The application of machine-learning (ML) approaches was previously proposed to identify drug targets in a single-result algorithm application using a database of inputs obtained from not only TNTs but also GJs and hemichannels (Valdebenito *et al*, 2018). Using morphological and structural features that characterize TNTs, such as: height above substrate; TNT length, diameter, and straightness; actin composition; and cargo transport (Fig 1, Box 1), we propose another ML-based approach to automate the quantification of TNTs *in vitro* by distinguishing them from other membranous protrusions (e.g., filopodia and lamellipodia) (Box 2). We withhold there is now sufficient ground truth data (LSCM micrographs) published in the field to tentatively train a neural network described above, but the achievement of this necessitates an agreement on a common TNT definition, data sharing, and the collaboration of numerous labs. International meetings held over recent years joining the academic leaders in the TNT field have shown TNT researchers are indeed ready to have these critical discussions and undertake an immense collaborative effort of this scale (Ariazi *et al*, 2017).

Quantitative analysis of TNTs could also benefit from use of controlled devices where cellular position and growth-direction of TNTs are confined to microchannels in a micro-fluidic chip (similarly to those routinely used for axonal growth studies (Neto *et al*, 2016)). This assay could in theory enhance reproducibility, throughput, and versatility of TNT studies; however, it is challenging in practice and has only been employed once thus far (Yang *et al*, 2016). As an alternative, we are establishing patterned surface arrays where cellular distance can be controlled in order to generate

 © 2021 The Authors

Box 1. Detection by microscopy



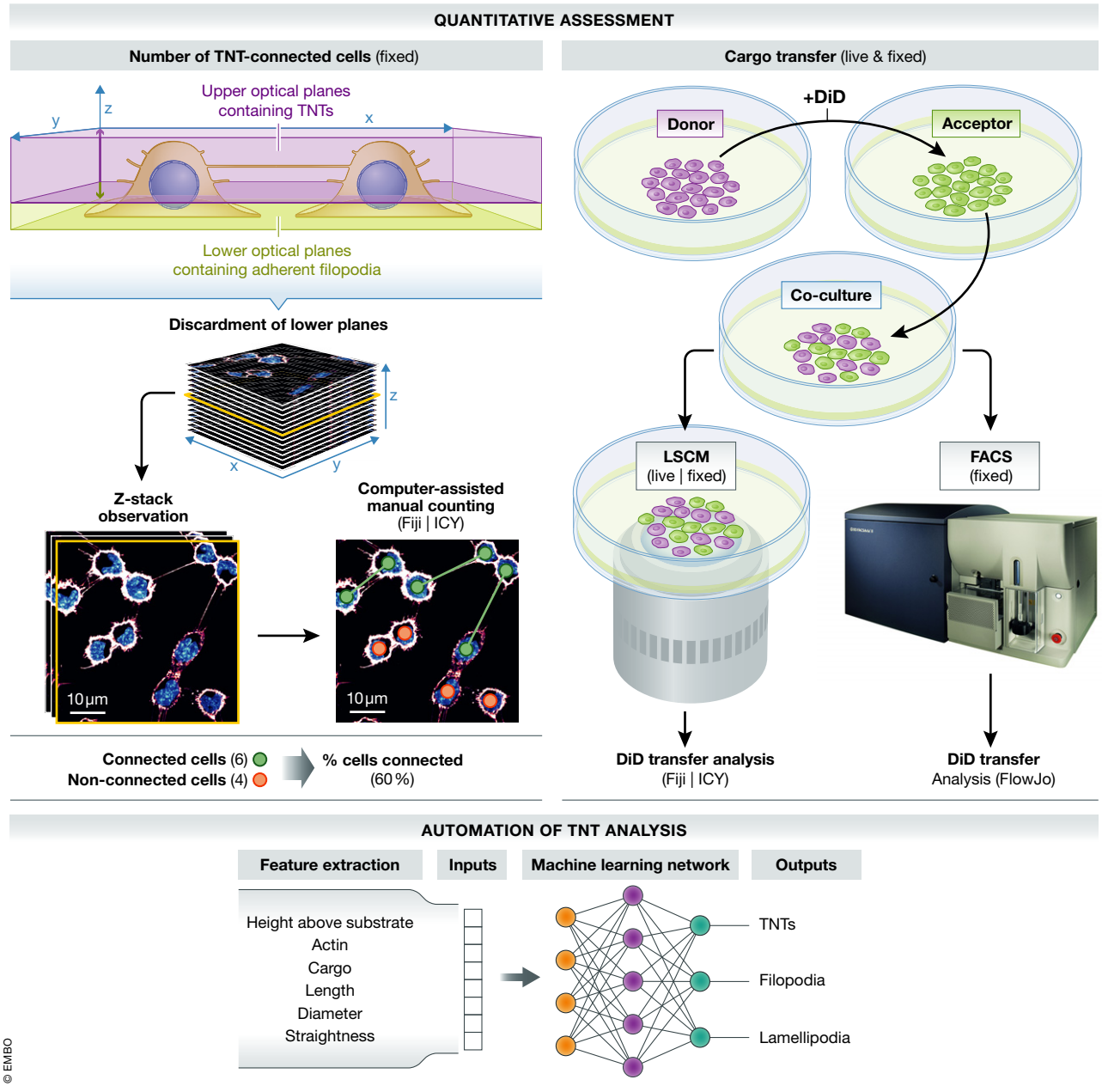
The detection of TNTs is achieved primarily through high-magnification differential interference contrast (DIC) imaging and 3D LSCM in fixed cells (Hurtig *et al*, 2010), and spinning disk microscopy in living cells. However, these methods do not grant the optical resolution necessary to image these structures.

Studies using time-gated continuous wave stimulated emission depletion (gCW-STED) (Bénard *et al*, 2015) in fixed TNTs and live-cell STED nanoscopy in living cells (Reindl *et al*, 2019), with a sub-diffraction resolution of < 100 nm, are pioneering TNT microscopy methods but have yet to reach widespread implementation in the field. Structured illumination microscopy (SIM) has also enabled us and others to observe fixed TNTs in more detail (Delage *et al*, 2016; Halász *et al*, 2018; Hanna *et al*, 2019; Vargas *et al*, 2019b). Unfortunately, without a robust membrane dye or a TNT-specific protein marker, Photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) do not offer any advantages.

The complexity of the matter only increases with the consideration of another outstanding obstacle—imaging these fragile structures in 3D cell cultures and tissues. One possible solution to this problem is to employ a lattice light sheet microscopy (LLSM) imaging system as done by Smith and colleagues in Ca^{2+}-signaling TNTs connecting human breast cancer cells (Parker *et al*, 2017). Even more recent developments in LLSM host a remarkable capacity to observe dynamic membranous protrusions in 4D with minimal bleaching and proved that even highly dynamic cellular protrusions, such as filopodia, can be observed at small time intervals without significant quenching (Chen *et al*, 2014). Implementation of tools borrowed from physics and astronomy, such as adaptive optics, may also be combined with LLSM, to allow for observation of intracellular molecules (Liu *et al*, 2018), an endeavor which would tremendously aid TNT research.

© 2021 The Authors

Box 2. Quantitative assessment



Relevant biological information from experiments is currently extracted through quantification of connected cells via TNTs using a computer-assisted manual approach in *Fiji/ImageJ* or *ICY* (via TNT-specific Annotator plugin) (Delage *et al*, 2016). However, the introduction of an effective and extensible tool to automatically and accurately count the (I) number of cells connected in a given region of interest (ROI) and (II) the number of individual tubes would improve the versatility, throughput, and reproducibility across these experiments. Recent advances in artificial intelligence (AI) and ML-based approaches have made it possible to automatically detect and quantify dendritic spines in neurons (Smirnov *et al*, 2018), but no attempts have yet been made to integrate these tools in the study of TNTs.

Accurate characterization of TNTs requires the systematic accompaniment of functional assays (i.e., transport of cargo). Cargo transfer is assessed by observing organelles (e.g., lysosomes), vesicles (or endosomes) labeled with fluorescent lipophilic cationic indocarbocyanine dyes (e.g., DiI and DiD) transfer between cells in a donor and acceptor cell co-culture system (Abounit *et al*, 2015; Sharma & Subramaniam, 2019). The amount of transfer is quantified by (I) live imaging microscopy or in fixed conditions by (II) LSCM or Fluorescence Activated Cell Sorting (FACS) (Abounit *et al*, 2015). In order to examine whether the intercellular transport of vesicles in these functional assays is TNT-dependent and not occurring via long-range EV-mediated mechanisms, these experiments must be supplemented by three controls: (I) a transwell filter that reduces (or eliminates) cell-to-cell contact; (II) assessment of supernatant transferred from donor to acceptor cells; and (III) a mixture of non-co-cultured cells to obtain a *background noise* threshold (Gousset *et al*, 2009; Abounit *et al*, 2015; Thayanithy *et al*, 2017).

© 2021 The Authors

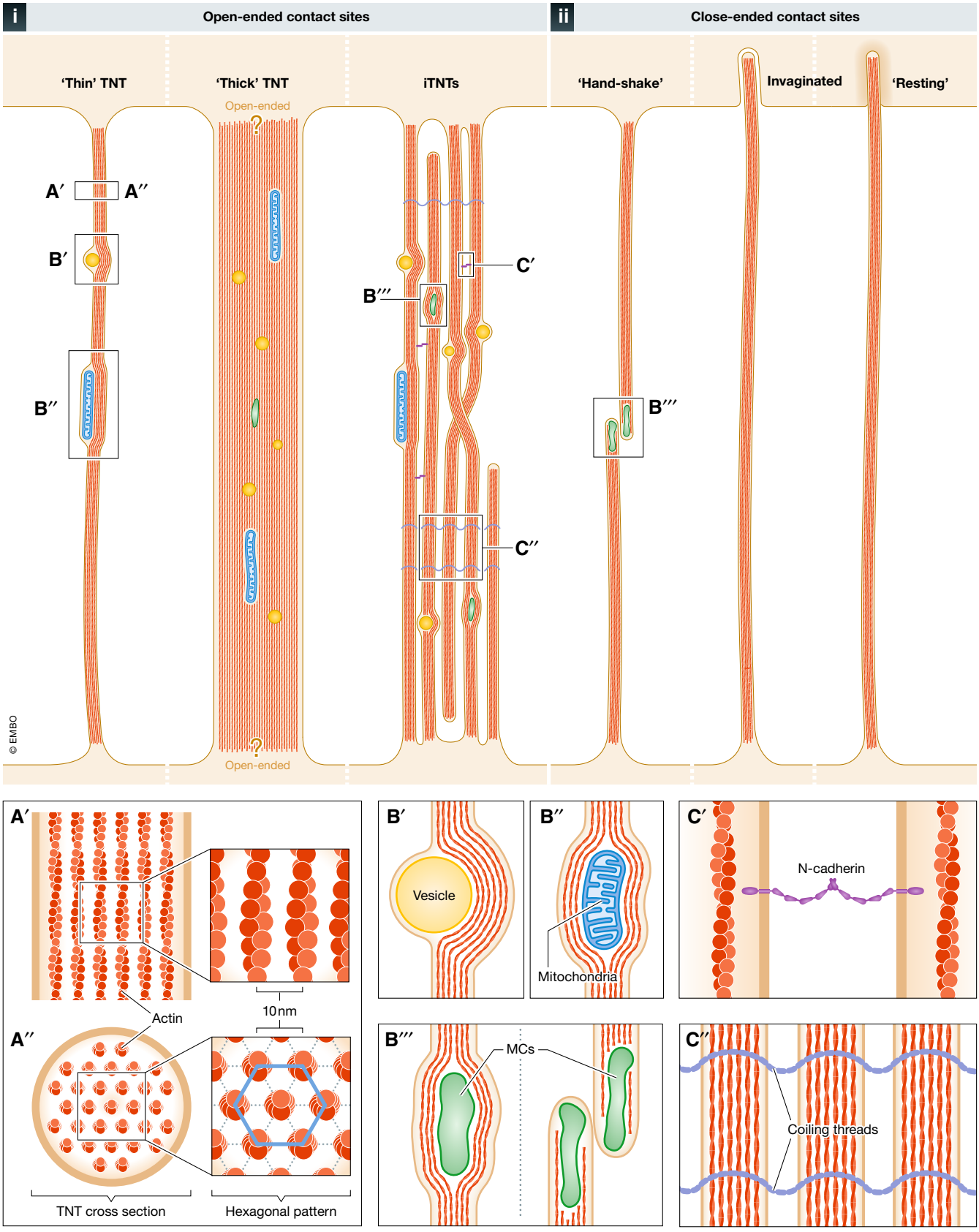

i
Open-ended contact sites
ii
Close-ended contact sites
'Thin' TNT
'Thick' TNT
iTNTs
'Hand-shake'
Invaginated
'Resting'
Open-ended
Open-ended
A′
A″
B′
B″
B‴
C′
C″
B‴
© EMBO
A′
Actin
10nm
A″
TNT cross section
Hexagonal pattern
B′
Vesicle
B″
Mitochondria
C′
N-cadherin
B‴
MCs
C″
Coiling threads

Figure 2.

Figure 2. Structural features of open- and close-ended TNTs.

Schematic representation of the 6 main types of open- and close-ended TNTs observed *in vitro* using electron microscopy (EM) approaches. i) Open-ended: "Thin" TNTs, observed in photoreceptor and stem cells via SEM and in neuronal cells via cryo-TEM/ET; "Thick" (> 700 nm) TNTs, observed in epithelial and neuronal cells with contact sites displaying questions marks to illustrate they were not demonstrated to be open; and a bundle of individual TNTs (iTNTs), a bundle of thin parallel nanotubes that get twisted together or go over and under each other, together forming one group of intertwined tubes that cannot be resolved using fluorescence microscopy; observed in neuronal and stromal cells using cryo-TEM/ET. ii) Close-ended (presumably facilitating cell–cell communication through gap junctions, which are not visible by EM approaches): "hand-shake" TNTs observed in lymphocytes via ssTEM; *invaginated* TNTs, observed in lymphocytes via ssTEM and in neuronal cells via FIB-SEM; and "resting" TNTs, observed in neuronal cells via FIB-SEM. A) Top- (A′) and cross-sectional (A′′) views of a TNT showing its internal actin arrangement: hexagonal actin arrays with a 10 nm distance between the center of adjacent actin filaments. B) Three types of cargoes observed within TNTs by EM: vesicles (B′), mitochondria (B′′), and membranous components (MCs) (B′′′), which could represent unidentified organelles. (C) Types of iTNT-iTNT interlinkage processes identified by immunogold labeling and cryo-TEM/ET: N-Cadherin adhesion molecules between iTNTs (C′) and coiling threads (C′′), whose protein and/or lipid composition are currently known.

conditions which favor TNT growth in confined spaces. This method will facilitate live imaging and quantitative analysis and could be used to characterize different transport modalities inside TNTs. A collection of TNTs from cells growing on such a functionalized pattern would allow us to characterize them with greater ease as opposed to TNTs derived from sparse culture conditions. This would improve the measurement of specific cargo transfer parameters (e.g., velocity, direction, persistence), as well as the identification of the motors involved (via overexpression and knockdown studies).

Ultra-structure of TNTs

Methods and open questions

Ultrastructural methods such as scanning and transmission electron microscopy (SEM and TEM, respectively), focused ion beam SEM (FIB-SEM), and cryo-EM, offer invaluable insights unobtainable through standard optical-based imaging approaches. Thorough structural analyses of TNTs using these imaging approaches can answer central questions concerning the morphological properties which enable their function. For example, does cargo described as "being transferred" by light microscopy get shuttled within TNTs, or on the outside of the membrane? Does the cytoskeletal backbone of TNTs contain actin, microtubules, or both? How is the cytoskeleton organized to allow cargo transfer? Are TNTs open at both ends of the structure, or are they closed, filopodia-like protrusions that mediate cargo transport via a trogocytosis-like event, (in which an entire patch of plasma membrane gets engulfed by another cell) (Dance, 2019)? These questions would be difficult to address without the implementation of EM-based methods (Figs 2 and 3).

Morphological features and cargo

Although EM has confirmed width and length measurements of TNTs obtained by light microscopy were not far off (Table 2), EM micrographs demonstrate that the diameter of TNTs varies along their length; membrane bulges, presumably created by organelles and membranous compartments (MCs) moving through TNTs can easily expand their perimeter (Reichert *et al*, 2016; Scholkmann *et al*, 2018). Serial-sectioning TEM (ssTEM) revealed MCs at the tips of close-ended TNTs in Jurkat T cells (Fig 2) (Sowinski *et al*, 2008). Given that TNTs in these cells were shown to be close-ended at the given time point in which they were fixed and observed, these observations suggest that MCs are unlikely to be transported intercellularly and may instead be used to deliver different molecules (including signaling molecules) at the end of the protrusion. By ssTEM, mitochondria in macrophages (Okafo *et al*, 2017) and ribosomes in epithelial cells (Kumar *et al*, 2017) were purportedly imaged within TNTs; however, due to the low magnification of the produced images, the veracity of this claim is not easily assessable.

Through vitrification of cells, cryo-TEM enabled us and Piwocka and colleagues to detect vesicles inside neuronal and stromal TNTs, respectively (Kolba *et al*, 2019; Sartori-Rupp *et al*, 2019). Furthermore, through cryo-correlative EM (cryo-CLEM), (identification of TNT-connected cells using fluorescence microscopy prior to freezing and EM) and cryo-electron tomography (cryo-ET) (an imaging modality of TEM that produces multi-angle, three-dimensional volume reconstructions), we found that mitochondria also transfer within the lumen of TNTs (Fig 2) (Sartori-Rupp *et al*, 2019). These tomograms demonstrated membrane bulges surrounding the cargo within, which often have a diameter equal to- or higher than the TNT containing them. These bulges not only contain vesicles and mitochondria, but also the actin cytoskeleton, which is likely responsible for cargo transportation (Fig 2).

Cytoskeletal architecture

Cross-sectional analysis proved the actin in neuronal TNTs was assembled in a similar fashion as in filopodia (Aramaki *et al*, 2016) —hexagonally arranged, with an inter-filament distance of 10 nm (Fig 2A). Unlike filopodia, where actin filaments can be branched, in TNTs, they generally appear to be straight (Sartori-Rupp *et al*, 2019). Straight actin filaments are likely accountable for TNTs' straight appearance. Given the distinct polarity of actin filaments, it is difficult to discern the underlying mechanisms of bidirectional cargo exchange unless actin filaments with opposite polarity coexist in the same actin bundle, or in the case of TNT-like structures, harbor both actin and microtubules. It is therefore essential researchers increase the resolution of cryo-TEM and -ET technologies (e.g., *TITAN Krios* cryo-TEM) in order to correctly assess the arrangement and polarity of actin filament bundles within TNTs. The application of novel computational approaches elegantly developed by Medalia and colleagues to study actin filament polarity in cryo-EM datasets will assist in this endeavor (Martins *et al*, 2021).

An intriguing discovery made by our group actually has the potential to expound upon the exact mechanisms of bidirectional TNT transport. Although historically defined as individual connections on the bases of LSCM observations, cryo-CLEM revealed that TNTs in two neuronal cell lines, (mouse CAD and human SH-SY5Y cells), generally appeared to be comprised of a bundle of several thin, individual TNTs (iTNTs) braided together (Figs 2 and 3)

 © 2021 The Authors

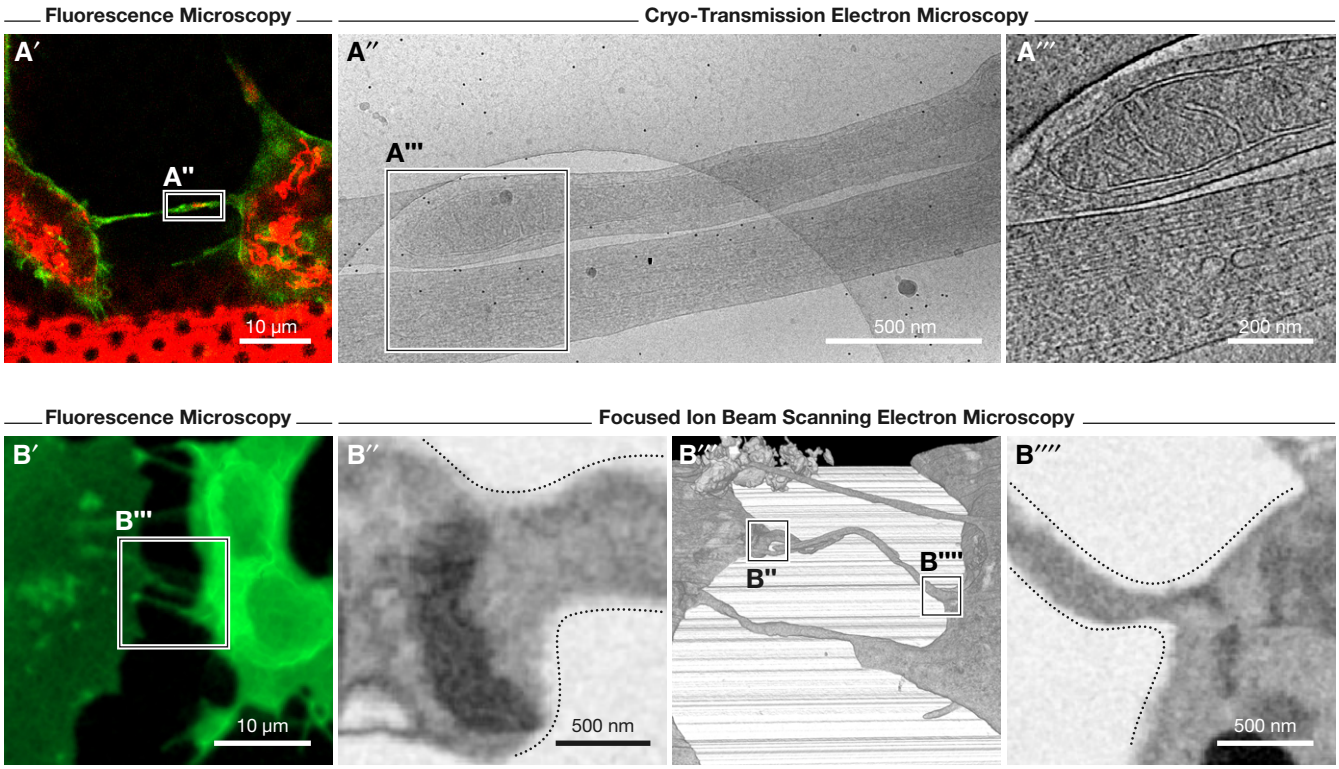


Figure 3. Observation of TNTs by various electron microscopy-based approaches.

(A) Cryo-correlative electron microscopy (cryo-CLEM) and focused ion beam scanning EM (FIB-SEM) approaches employed to study TNTs of SH-SY5Y cells; images modified from (Sartori-Rupp *et al*, 2019). (A′) Identification of TNT-connected cells is first performed through fluorescence microscopy of WGA (green)- and mitochondria (red)-labeled cells (seeded on EM finder grids). After vitrification of cells, the recorded position of TNTs acquired by fluorescence microscopy is imaged by cryo-transmission EM (cryo-TEM) and electron tomography (cryo-ET) at medium- and high-magnification (A″ and A‴, respectively). A mitochondrion can be observed within an iTNT in A‴. (B) Correlative, FIB-SEM method employed to visualize TNTs using fluorescence microscopy (cells labeled with WGA, green) and FIB-SEM to assess open-ended contact sites (B″ and B′′′′, respectively) through high-resolution 3D, volume imaging (B‴).

(Sartori-Rupp *et al*, 2019). Contrary to a single TNT, iTNTs could in reality host opposite actin polarities allowing for a bidirectional exchange of cargoes. Interestingly, iTNTs have also been observed in stromal cells, suggesting that non-neuronal TNTs could share this feature (Fig 2) (Kolba *et al*, 2019). Furthermore, even TNTs of macrophages exhibit lose-hanging, coiled, threads that could have once formed part of a bundle which partially snapped during SEM sample preparation (Hanna *et al*, 2019). Similarly, the identification of multiple processes at the edge of TNTs in another report additionally supports this notion (Okafo *et al*, 2017). SsTEM in Jurkat T cells, however, clearly depicts TNTs as singular structures (Sowinski *et al*, 2008). Intriguingly, our observations in neuronal cells have also revealed the existence of single tubes with a larger diameter (average of 500 nm). The relationship between iTNT bundles and single tubes remains unclear and will require further inquiry.

Another open question that would benefit from the use of ultrastructural methods concerns the molecular motors that drive cargo transport in TNTs, which remain largely unexplored even by light microscopy approaches. Cryo-CLEM actually enabled us to identify MyosinX (MyoX)-positive vesicles within iTNTs (Sartori-Rupp *et al*, 2019), proving correlative studies could tremendously aid in the characterization of these molecular motors.

Structural outlook

While EM has given researchers a much clearer understanding of these structures, the question of whether or not intercellular cargo transfer does indeed occur through open-ended TNTs unfortunately cannot be addressed via cryo-EM. Additionally, although SEM micrographs have previously suggested seamless surface transitions between TNTs and cells (Reichert *et al*, 2016), the images do not provide sufficient resolution to conclude whether TNTs are open- or close-ended. On the contrary, qualitative ssTEM did reveal an open TNT between PC12 cells (Rustom *et al*, 2004), but the single example shown by the authors exhibits a short and bent shape, which differs greatly from PC12 TNTs typically observed by SEM or LSCM. In immune cells, ssTEM revealed two types of close-ended TNTs: (I) a single protrusion invaginating into an opposing cell and (II) two filopodia-like protrusions meeting in the middle, making a "handshake" (Sowinski *et al*, 2008), or in a synapse-like conformation (Okafo *et al*, 2017). To test whether iTNTs in neuronal cells were in fact open-ended, we employed correlative FIB-SEM and found most iTNTs do openly connect cells, establishing cytoplasmic continuity. In addition, closed nanotubes were either observed resting on the membrane- or invaginating into other cells (Sartori-Rupp *et al*, 2019). Given that these observations were obtained using fixed

© 2021 The Authors

Table 2. Key tunneling nanotubes' structural features obtained through electron microscopy approaches.

Cell Model	Method	Diameter (nm)	Conformation	Open/ Closed	Cytoskeleton	Cargo	References
PC12 (neuronal)	SEM, ssTEM	50–200	Single	Open	N/A	N/A	Rustom *et al* (2004)
Jurkat (lymphocyte)	CL-ssTEM	180–380	Single	Closed	N/A	MCs	Sowinski *et al* (2008)
ARPE-19 (photoreceptor)	SEM	50–300	Single	Open[a]	N/A	N/A	Wittig *et al* (2012)
HSPC (stem)	SEM	< 100	Single	Open[a]	N/A	N/A	Reichert *et al* (2016)
KG1a (leukemic)	SEM	< 100	Single	N/A	N/A	N/A	Reichert *et al* (2016)
A549 (epithelial)	SEM, ssTEM	250–1,370	Single	Open	N/A	Mitochondria & ribosomes[a]	Kumar *et al* (2017)
MDCK (epithelial)	SEM, ssTEM	N/A	Single	Open	Fibers[a]	N/A	Kumar *et al* (2017)
Macrophages (phagocyte)	SEM, ssTEM	N/A	Single	Closed	Actin[a]	Mitochondria[a]	Okafo *et al* (2017)
T24 & RT4 (bladder)	SEM	100–200	Single	Open	N/A	N/A	Lu *et al* (2017)
661 W (photoreceptor)	SEM	68–110	Single	N/A	N/A	N/A	Scholkmann *et al* (2018)
CAD (neuronal)	SEM, cryo-CLEM, cryo-ET, FIB-SEM	Individual TNT Avg. 109	Single & bundle	Both	Actin, no microtubules	Vesicles, MCs	Sartori-Rupp *et al* (2019)
SH-SY5Y (neuronal)	SEM, cryo-CLEM, cryo-ET, FIB-SEM	Individual TNT Avg. 120	Single & bundle	Both	Actin, no microtubules	Vesicles, MCs, mitochondria	Sartori-Rupp *et al* (2019)
HS-5 (stromal)	SEM, cryo-CLEM, cryo-ET	~200	Single & bundle[a]	Both	Actin[a]	Vesicles	Kolba *et al* (2019)
STHdh (neuronal)	SEM, ssTEM	< 200	Single	Open	N/A	N/A	Sharma and Subramaniam (2019)
Macrophages (phagocyte)	SEM	N/A	Single	N/A	N/A	N/A	Hanna *et al* (2019)

MCs, Membranous compartments.
[a]Not assessable or confirmed due to technique and/or insufficient image resolution employed.

cells, we cannot discriminate the possibility that closed structures invaginating into apposing cells represent pre-TNT fusion events. Further studies employing high-resolution, live imaging microscopy will be required to fully investigate this point (see Box 1).

Immunogold labeling of proteins on the membrane of TNTs could also pinpoint proteins involved in membrane fusion and interaction between close-ended TNTs. Using this approach, we identified N-Cadherin decorating strands between iTNTs and proposed it could play a multi-faceted role in maintaining the stability of the overall bundle through its homophilic interactions (Fig 2) (Sartori-Rupp *et al*, 2019).

The transient and fragile nature of TNTs has ultimately rendered in-depth ultrastructural analyses technically challenging, as traditional EM involves specimen-preparation steps which can seriously injure the morphology of TNTs (Sartori-Rupp *et al*, 2019). The few structural studies available (Table 2) do detail interesting considerations but overall lack sufficient sample size to draw any quantitative conclusions. This lack of statistical power impedes our judgement of whether or not the cell-specific differences in TNT size, cargo, cytoskeletal architecture, or overall topology illustrated above actually hold substantial biological meaning. Given most of our current TNT-related knowledge stems from LSCM, a *correlative* approach, such as the one employed by Davis and colleagues for the study of Jurkat T cells (Sowinski *et al*, 2008) and by us in neuronal cells (Sartori-Rupp *et al*, 2019), is recommended.

Mechanisms of TNT formation

Biogenesis

It is now well-established that TNTs emerge from one of two physicomechanical origins: the first being a *cell dislodgement mechanism*, in which a thin actin-bearing membranous tube forms after two adjacent cells fuse transiently and move apart; and the second being an *actin-driven protrusion mechanism*, by which the tip of a filopodium-like structure extrudes from one cell to fuse with another, onto either its soma or the tip of a filopodium emanating from it (Rustom *et al*, 2004; Hase *et al*, 2009; Wang *et al*, 2010).

Live imaging has revealed cell dislodgement to be the primary TNT biogenesis mechanism in a variety of cell types (Table 3). Interestingly, whole-cell patch clamping demonstrated some dislodgment-derived TNTs to be electrically coupled by Connexin 43 (Cx43) GJs (Wang *et al*, 2010). This observation is consistent with the findings of several other groups, in which immune cells known to interact by immune synapses (Finetti *et al*, 2017) form close-ended TNTs (Onfelt *et al*, 2004; Watkins & Salter, 2005; Onfelt *et al*, 2006; Davis

 © 2021 The Authors

Table 3. Tunneling nanotubes' preferred biogenesis mechanism.

Cell type	Cell dislodgement	Actin-driven	Reference
squamous cell carcinomas (SCC)	100%	–	Sáenz-de-Santa-María *et al* (2017)
neuronal crest cells (NCCs)	100%	–	Sáenz-de-Santa-María *et al* (2017)
NRK cells	100%	–	Sáenz-de-Santa-María *et al* (2017)
HEK 293	82%	18%	Wang *et al* (2010)
human primary, hematopoietic progenitors	Most*	N/A	Reichert *et al* (2016)
leukemic KG1a cells	Most*	N/A	Reichert *et al* (2016)
PC12	7%	93%	Bukoreshtliev *et al* (2009)

& Sowinski, 2008; Dustin *et al*, 2010). Whether dislodged TNTs require adhesion molecules (e.g., Cadherins) for their formation is currently unknown. The precise mechanism of actin backbone incorporation during dislodgement remains another open question. However, recent studies have demonstrated the actin-bundling protein, Fascin, found in filopodia (Vignjevic *et al*, 2006) and certain neuronal dendrites (Nagel *et al*, 2012), is absent in the dislodged TNTs of epithelial lung cells (Dubois *et al*, 2018).

In contrast to dislodgement, the actin-driven protrusion mechanism is typical of relatively immobile cells (e.g., neurons and epithelial cells) (Bukoreshtliev *et al*, 2009; Gousset *et al*, 2013). This suggests these TNTs are formed by the action of actin protein networks which promote actin filament growth, similar to other actin-based protrusions such as filopodia, or filopodia-like structures (see section below). Also in this case, TNTs do not contain fascin, where evidence in neuronal CAD cells shows that fascin overexpression has an inhibitory effect on TNT formation (Gousset *et al*, 2013). Live imaging assays additionally showed 93% of TNTs in HeLa cells originated from filopodia-like protrusion interplay (Bukoreshtliev *et al*, 2009), reinforcing this hypothesis and underlining the importance of investigating both cellular and molecular players in filopodia formation. To explore this further, here we discuss several studies focused on actin-related proteins in the context of TNT formation.

Actin cytoskeleton and actin protein regulators

Although there exists variety in both approaches and models used to study TNT formation mechanisms, there is a consensus that these mechanisms involve proteins regulating (I) actin polymerization, (II) membrane supply and recycling, and (III) stress/inflammation and survival responses. Studies also indicate different mechanisms may operate in different cellular contexts, supporting the argument that these mechanisms are cell-specific. Here, we will focus on proteins and signaling pathways linked to dynamic actin networks which function in the formation and regulation of other common membranous protrusions, such as filopodia (Ljubojevic *et al*, 2021). Molecules deployed to form TNTs via membrane recycling (e.g., different Ras-related proteins in brain (Rabs) and M-Sec, are described in detail elsewhere (Hase *et al*, 2009; Marzo *et al*, 2012; Dubois *et al*, 2018; Dupont *et al*, 2018; Zhu *et al*, 2018; Bhat *et al*, 2020)).

The Rho family of small GTPases is well-characterized in the formation and rearrangement of the actin cytoskeleton in filopodia and lamellipodia (Heasman & Ridley, 2008). CDC42 was first observed at the base of TNTs connecting immune Jurkat T cells (Lachambre *et al*, 2014) where *secramine A*, a CDC42-specific inhibitor, was shown to reduce TNT formation (Arkwright *et al*, 2010). In HeLa cells, inhibition of CDC42 by transfecting cells with a dominant negative construct of CDC42 also decreased TNT formation. Conversely, inhibition of Rac1 by overexpression of its dominant negative form had no significant effect (Hase *et al*, 2009). Treating macrophages with specific inhibitors for CDC42 and Rac1, Cox and colleagues confirmed TNTs in immune cells require both CDC42 and Rac GTPases for their formation (Hanna *et al*, 2017). Interestingly, our observations in neuronal CAD cells seem to indicate the opposite—that inhibition of CDC42 and Rac1 significantly increases the number of cells connected by TNTs (Delage *et al*, 2016). Moreover, direct inhibition of the common downstream effector of Wiskott–Aldrich syndrome protein (WASP) and WASP family verprolin-homologous 2 (WAVE2), Arp2/3, resulted in a decrease in the formation of TNTs in macrophages (Hanna *et al*, 2017) but an increase in neuronal TNTs and TNT-mediated vesicle transfer (Sartori-Rupp *et al*, 2019).

Taken together, our combined results in neuronal cells disagree with those collected in macrophages (Hanna *et al*, 2019) and suggest a cell-specific underlying pathway which is activated to generate TNTs. This was supported by other studies, most notably as seen for M-Sec, which was shown to be involved in the formation of TNTs in immune (Hanna *et al*, 2019) but not neuronal cells (Gousset *et al*, 2013).

Furthermore, recent findings indicate filopodia and TNTs use distinct mechanisms of formation. More specifically, we demonstrated that the same actin regulators have opposing effects on the two structures: (I) CDC42–VASP–IRSp53 network, which induces filopodia formation serves as a negative regulator of TNTs while the (II) epidermal growth factor receptor pathway substrate 8 (Eps8), which reduces filopodia formation, induces TNT formation and increases cargo transfer (Delage *et al*, 2016). The discovery that these same actin regulators may have antagonistic effects relative to filopodia and TNT formation could be explained by a sort of "switch" that shifts the formation of one structure over the other (Delage *et al*, 2016). However, when and how this paradigm occurs is not yet known. One possible approach to investigate this further is to perform structural studies and examine the role these actin regulators have on the cytoskeletal anatomy of TNTs and filopodia of both neuronal and immune cells. This approach could also help us understand whether the structures containing both microtubules and actin —or only actin—are similar or different in their ultrastructural architecture, open- or close-ended properties, and functional capabilities.

Wnt pathway

More recently, we have explored the *Wingless-related integration site* (Wnt) pathway due to its function in actin cytoskeleton remodeling, filopodia formation, and neuronal growth cone guidance (Onishi *et al*, 2013). Our observations show the activation of the Wnt/Ca^{2+} pathway allows Wnt ligands to regulate the formation and stability of TNTs in neuronal cells and primary neurons (Vargas

© 2021 The Authors

et al, 2019b). The Wnt/Ca^{2+} pathway achieves this through the interactional modulation of actin and the *β isoform of Ca2+/calmodulin-dependent protein kinase II* (BCaMKII). Intriguingly, activation of the Wnt pathway also influences the formation of cytonemes, a specialized type of filopodia that transport signaling proteins between distant cells in development (Kornberg & Roy, 2014; Yamashita *et al*, 2018; Korenkova *et al*, 2020); however, unlike cytonemes, TNT formation is not affected by Wnt-mediated activation of c-Jun N-terminal kinase (JNK) and B-catenin (Mattes *et al*, 2018), suggesting TNTs and cytonemes experience differential regulation (Vargas *et al*, 2019b). Future studies are necessary to accurately assess whether Wnt-induced TNTs, like cytonemes, can also transport components of the Wnt pathway (e.g., Frizzled receptor) (Stanganello & Scholpp, 2016).

MyosinX

The myosins are a superfamily of actin-based motor proteins that "walk" on actin filaments. Of all the myosins, MyoX (an unconventional myosin motor) is the most broadly distributed within cells and is a potent filopodial inducer (Sousa & Cheney, 2005). Our group additionally showed MyoX is a critical inducer of neuronal TNTs (Gousset *et al*, 2013). We demonstrated that neuronal TNT formation requires the motor and tail domains of MyoX, specifically the F2 lobe of the tail protein-ezrin-radixin-moesin (FERM) domain as essential. Given MyoX increases adherent- and dorsal filopodia formation, we postulated neuronal TNTs could arise from a specific subset of MyoX-driven dorsal filopodia, independent of integrins, (transmembrane receptors required for the formation of adherent filopodia). Consistent with our hypothesis and subsequent data (Delage *et al*, 2016), MyoX indeed acts downstream of CDC42 and induces TNT formation independent of VASP, another potent inducer of dorsal filopodia formation, which conversely gets transported to the tip of filopodia by this motor (Bohil *et al*, 2006; Kerber & Cheney, 2011). Together, these findings demonstrate the existence of different mechanisms for dorsal filopodia formation and support a distinct role of Myo10 in TNTs.

Overall, these studies indicate that differential mechanisms operate in disparate cellular contexts, reinforcing the argument for cell specificity. Additionally, the fact filopodia and TNTs do not share the same mechanisms of formation supports the notion that they are in fact distinctive cellular structures.

Transfer function of TNTs

The principal function of TNTs being intercellular cargo transport makes them unique from other membranous protrusions, such as filopodia. The transported cargo includes various organelles, proteins, but also viruses, and bacteria. Here, we focus expressly on the role of TNTs in the transfer of mitochondria, lysosomes, amyloid aggregates, and related function in disease. For reviews that describe the involvement of TNTs in bacterial and viral transmission, please refer to Table 1.

Mitochondria

Observations derived from varying cell types have demonstrated TNTs capability for intercellular mitochondrial transfer (Vignais *et al*, 2017). The biological significance of this transfer is not yet fully understood. Observations in mesenchymal stem cells of TNT transfer of healthy mitochondria to cells harboring DNA defects strongly suggest an underlying protective function of mitochondrial transfer (Lin *et al*, 2019). In addition, mitochondria transfer through TNTs has frequently been observed in the context of different cancers (Lou *et al*, 2012; Antanavičiūtė *et al*, 2014; Thayanithy *et al*, 2014; Desir *et al*, 2018), where they are proposed to sustain tumor progression and invasion (Pinto *et al*, 2020).

Mitochondria are cells' chief energy producing organelles and thus mitochondrial dysfunction can lead to an accumulation of Reactive Oxygen Species (ROS), highly charged particles long known to pose a threat to cellular integrity and viability (Dixon & Stockwell, 2014). Intriguingly, ROS have been proven to be an effective inducer of TNTs via the major form of endogenous ROS, hydrogen peroxide (H_2O_2) (Liang, 2018). In rat hippocampal astrocytes, neurons, and HEK293 cells, H_2O_2 significantly increases the number of (homotypic and heterotypic) cells connected by TNT-like structures compared to untreated cells (Wang *et al*, 2011). Notably, upon co-culturing non-stressed and stressed cells, the authors observed that H_2O_2-treated cells were the initiators of TNT formation. Subsequent studies from our group showed that H_2O_2-induced stress also increases TNT formation in neuronal cells by 70% (Gousset *et al*, 2013), indicating a ubiquitous effect on TNTs across distinct cell types.

Combined, these and other observations led Rustom to propose a mechanistic model of ROS-dependent TNT formation (Rustom, 2016). In his review, Rustom describes TNT formation directly linked to the increase of ROS level in stressed cells. The underlying reason why this occurs is not yet known; however, according to the proposed model, TNTs are formed by unstressed cells in order to establish an open communication with stressed cells as a "call for help". This event is followed by (I) the TNT-mediated transport of mitochondria to rescue the apoptotic cell or (II) removal of cells with excessive levels of ROS. This model posits TNTs as a communication tool in cellular organization and survival during stress (Rustom, 2016).

The exact mechanism underlying ROS-mediated TNT induction is not well understood; however, a study on TNTs in astrocytes indicated H_2O_2 triggers phosphorylation of the p38 mitogen-activated protein kinase (MAPK) (Zhu *et al*, 2005), a kinase responsive to stress stimuli which has previously been shown to induce actin cytoskeletal remodeling in fibroblasts (Hoffman *et al*, 2017). Other reviews have further explicated the strict relationship between physiological levels of ROS and actin-related proteins (Wilson & González-Billault, 2015) and regulated actin flow at the leading edge of cells (Taulet *et al*, 2012). These observations support the established notion that H_2O_2 affects actin cytoskeletal organization and induces the formation of actin-rich protrusions (Huot *et al*, 1998). Future studies will be required to pinpoint the effect redox imbalance can inflict on actin-associated proteins and, specifically, interrogate any correlation between ROS synthesis and actin filament oxidation. As previously reported by Wilson and González-Billault, studying this spatiotemporal relationship is a challenging quest given that both elements are short-lived and dynamic proteins (Wilson & González-Billault, 2015); however, novel genetic (Mishin *et al*, 2015) and bioluminescence-based tools (Ishimoto & Mori, 2019) might be able to help us in these endeavors.

 © 2021 The Authors

Another unexplored area of TNT-mediated mitochondrial transport is the molecular motors responsible for shuttling them. Agrawal and colleagues found that the mitochondrial Rho-GTPase, Miro1, an adaptor protein that attaches mitochondria to KIF5 motor proteins, allows for mitochondrial transport through TNTs containing microtubules (Ahmad *et al*, 2014). Our most recent correlative structural analysis identified mitochondria traveling through actin, not microtubules, suggesting that actin-driven motor proteins must also be involved (Sartori-Rupp *et al*, 2019). Furthermore, we found mitochondria travel through TNTs at an average velocity of 50 nm per second; however, their speed is not often uniform and instead appears to accelerate in straight TNT segments (Wang & Gerdes, 2015; Sartori-Rupp *et al*, 2019).

Lysosomes

Lysosomes are membrane-bound organelles whose foremost function is to digest macromolecules and break down cellular waste. Lysosomes were first observed in TNTs connecting macrophage cells, moving at a speed of 1 μm/s (Onfelt *et al*, 2006). Since then, the study of TNT-mediated intercellular transport of lysosomes has revealed they can facilitate the transport of viruses (Eugenin *et al*, 2009) and proteins linked to various neurodegenerative diseases (NDs) (Victoria & Zurzolo, 2017), which will be detailed below. Interestingly, TNTs also exhibit a protective role in allowing (I) hematopoietic stem cell (HSC)-derived macrophages to supply cystinosin-deficient cells with functional lysosomes and (II) pulling cystinosin-bearing lysosomes away from diseased cells (Naphade *et al*, 2015; Goodman *et al*, 2019). Additional studies have linked the direct transfer of lysosomes to lysosomal gradient preservation and endothelial cell viability (Yasuda *et al*, 2011). Finally, in tumor cells derived from squamous cell carcinomas, Chiara and colleagues observed that focal adhesion kinase (FAK)-deficient cells transfer lysosomes and autophagosomes to FAC-proficient cells via TNTs, supporting the relevance of a mechanism necessary to adapt to stress evoked by impaired FAK signaling (Sáenz-de-Santa-María *et al*, 2017).

Amyloid proteins

Major NDs, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) all share the defining feature of proteinaceous aggregates which accumulate in the central nervous system (CNS) and cause neuronal cell death (Jucker & Walker, 2018). A growing body of literature supports the hypothesis linking the progression of these diseases to the spreading of these assemblies, which have the unique prion-specific ability to imprint their misfolding on soluble cellular protein (Luk *et al*, 2009; Goedert, 2015; Hasegawa *et al*, 2017; Jansen *et al*, 2017). Recent data from our laboratory and others have revealed TNT-mediated spread of amyloid protein assemblies associated with various NDs. This has led to our proposal that TNTs represent a major route for the intercellular amyloid spreading and are thus involved in the progression of these diseases (Victoria & Zurzolo, 2017; Vargas *et al*, 2019a).

In a seminal discovery, our group demonstrated that the infectious form of prion protein (PrP^{Sc}) is able to spread to other cells by hijacking TNTs (Gousset *et al*, 2009). We have also shown prions spread through TNTs within organelles of the endocytic pathway, (most likely recycling endosomes) (Zhu *et al*, 2015). TNT-mediated transport of prions occurs between neuronal cells as well as between dendritic cells and neurons, and primary neurons and astrocytes, together accounting for the progression of the prion infection from peripheral tissue to the brain, and within the brain (Gousset *et al*, 2009). Amyloid proteins' prion-like behavior of inducing misfolding of normal proteins led our group and others to investigate the role of TNTs in the spreading of amyloid proteins involved in more common diseases, namely α-synuclein with PD, huntingtin with HD, and Tau/amyloid beta (Aβ) with AD.

Alpha-synuclein

One of our studies previously documented that fibrillar α-syn could also induce cells to form TNTs and "hijack" them to efficiently spread intercellularly using lysosomal vesicles as shuttles (Abounit *et al*, 2016a). Follow-up studies from our and other groups have since shown that TNT-mediated aggregated-α-syn transfer occurs in human and mouse astrocytes (Loria *et al*, 2017; Rostami *et al*, 2017), SH-SY5Y neuronal and SW-13 epithelial cells (Dieriks *et al*, 2017; Dilsizoglu Senol *et al*, 2019), and primary brain pericytes (Dieriks *et al*, 2017). We have also recently observed α-syn-carrying TNT-like structures in primary mouse cortical neurons (Vargas *et al*, 2019b) and human neuronal precursor cells (hNPCs) derived from healthy subjects and patients carrying the early-onset PD-associated A53T mutation in the α-syn gene (Grudina *et al*, 2019).

Although our experiments in hNPCs revealing the post-internalization preferential localization of α-syn fibrils in lysosomes (Grudina *et al*, 2019), confirmed our initial demonstration in murine neuronal cells by showing that lysosomes transport α-syn aggregates through TNTs, the mechanism and pathogenetic implication of this transfer remains to be explained (Victoria & Zurzolo, 2017). The mechanism used by α-syn aggregates to recruit and induce the aggregation of soluble α-syn proteins in the cytosol of acceptor cells following TNT-mediated transfer within lysosomes also represents an exciting research avenue. Exploring this event and identifying the compartments α-syn gets directed to when they reach "acceptor" cells hold tremendous scientific and therapeutic value (Abounit *et al*, 2016a).

Dismantling TNTs to diminish or even halt the spreading of intercellular α-syn also represents a promising therapeutic avenue (Victoria & Zurzolo, 2017). Although *latrunculin B* was previously shown to abrogate TNT formation and intercellular α-syn transport (Rostami *et al*, 2017), actin depolymerizing toxins risk collapsing the entire actin scaffold of cells. In one of our recent studies, we showed that nM concentrations of the actin-targeting cyanobacterial macrolide, tolytoxin, significantly decreased the number of TNT-mediated transfers of α-syn fibrils without affecting the global actin architecture of cells (Dilsizoglu Senol *et al*, 2019).

Identifying inhibitory molecular targets for TNT-related α-syn transfer, while also avoiding effects on the actin cytoskeleton, will require further research. For example, studying the kinetics of TNT-mediated transport of α-syn through live imaging microscopy and particle tracking (Sartori-Rupp *et al*, 2019) could provide useful insights into the speed and dynamicity of α-syn spreading, including the distribution of potential molecular motors. Our study on the Wnt pathway, which demonstrates that α-syn transfer through TNTs can be modulated by the Wnt/Ca^{2+} pathway, also represents an important future area of study and is worth characterizing (Vargas *et al*, 2019b). A more in-depth look at the topological landscape governing the transfer α-syn using CLEM approaches could also

© 2021 The Authors

teach us about the interaction between α-syn, lysosomes, actin, and the TNTs containing them.

Huntingtin

In the case of HTT, several mechanisms have been proposed, including extracellular vesicles (Tang, 2018) and transneuronal (synaptic) spreading (Pecho-Vrieseling *et al*, 2014). Our early work has also shown the intercellular propagation of mHTT through TNTs (Costanzo *et al*, 2013). Primary cerebellar granule neurons could also transport mHTT in a contact-dependent manner but we did not identify any TNT-like structures due to the hectic dendritic landscape of primary neuron cell culture. It is likely that the co-labeling approach recently employed by us to identify TNT-like structures in primary cortical neurons could shed light on this intriguing possibility (Vargas *et al*, 2019b).

The precise molecular mechanism governing TNT-mediated intercellular transport of mHTT is currently unknown, and little attention has been paid to it since our initial observations. In an attempt to learn more about the molecular pathway of mHTT spreading in a brain region seriously affected by HD—the striatum (Kassubek *et al*, 2004)—Sharma and Subramaniam focused their studies on the striatum-enriched GTPase protein, *Rhes*. Using mouse normal striatal neuronal cells, the authors found Rhes induced the biogenesis of TNT-like structures and enhanced intercellular transfer of mHTT between donor and acceptor cell populations (Sharma & Subramaniam, 2019). Consistent with studies in PC12 (Bukoreshtliev *et al*, 2009) and MSCs (Zhang *et al*, 2018), TNT-like structures were abrogated with the F-actin depolymerizing toxin, cytochalasin D; however, much remains unclear about the precise molecular mechanism that underlies the TNT-mediated transport of mHTT compared to α-syn. Future studies are needed to explore the depth and specificity involved in mHTT spreading by TNTs and how this mechanism competes with others previously described (Tang, 2018). Interestingly, Rhes also mediates Tau pathology in AD mouse models, making studies with Tau mutants an interesting research avenue.

Amyloid beta and Tau

The work by Zhang and colleagues, which previously reported TNT-mediated Aβ transport in rat astrocytes and neurons, supports this notion (Wang *et al*, 2011); however, no further evidence describing the underlying mechanism or pathways involved has surfaced since then. Meanwhile, we and others have shown that extracellular monomeric and fibrillar Tau species induce the formation of TNTs, which facilitate their transfer between neuronal cells (Tardivel *et al*, 2016; Abounit *et al*, 2016b). We have also recently shown that endogenously formed Tau aggregates are found in TNTs (Chastagner *et al*, 2020). Together, these data indicate that proteins involved in Tauopathies and AD could hijack TNTs and use them as a means of spread, as previously described for Prion, α-syn, and mHTT (Victoria & Zurzolo, 2017).

Molecule and Signal transfer

Major histocompatibility complexes

Studies focused on immune cells have also demonstrated that TNTs can transport major histocompatibility complexes (MHC), molecules whose main role is to present antigens at the cell surface of certain immune cells to be recognized by T cells. Transfer or association of MHC-I class proteins with TNTs has been observed in B cells (Onfelt *et al*, 2004); HeLa (Schiller *et al*, 2013); and thymic epithelial and dendritic cells (Watkins & Salter, 2005; Millet *et al*, 2008). In NK cells, MHC class I chain-related protein A was observed to transfer to the tip of a close-ended TNT (or immune synapse) where it is postulated to interact- and activate the target cell (Chauveau *et al*, 2010). Even though it is currently unclear whether MHC molecules retain their function when transferred, or how they behave during their transfer, these studies propose an intriguing role for TNTs, including the initiation and implication of signals that can trigger an immune response. For further reading of TNT-mediated transfer of MHC complexes, we would like to point our readership in the direction of a specialized review by Bonaccorsi and colleagues (Campana *et al*, 2015).

Calcium transfer

Shortly after the discovery of TNTs, numerous studies demonstrated that Calcium (Ca^{2+}), too, could be transported via TNTs. First evidenced by observations in dendritic cells and THP-1 monocytes, and contrary to classical cellular electrical conductivity, intercellular Ca^{2+} flux propagation between cells via TNTs was not driven by an action potential (Watkins & Salter, 2005). Through GJ-specific inhibitors, the authors also showed that TNTs could transport Ca^{2+} independent of Gjs; however, the underlying mechanism of TNT-mediated Ca^{2+} transfer was only reassessed years later, when TNTs of SH-SY5Y and HEK293 cells were shown to involve IP3 receptors for the active propagation of Ca^{2+} (Smith *et al*, 2011). Since then, Ca^{2+} flux transfer through TNTs has been observed in several other cell types, including Raw cells (Hase *et al*, 2009), immature neurons (electrically coupled to distant astrocytes) (Wang *et al*, 2012), and ARPE-19 cells (Wittig *et al*, 2012). Some, but not all contained Gjs, indicating that—unless TNTs can transition between an open-ended to a GJ-mediated, close-ended state—Ca^{2+} can be transported between cells using two distinct types of TNTs. Whether and how TNTs can exist and transport Ca^{2+} signals between functional neurons connected by synapses has not yet been explored and future investigations will have to address this possibility.

Presence of TNT-like structures *in vivo*

The data outlined above demonstrate that TNTs play a role in the intercellular communication between various cells in culture (including primary cells). This has allowed researchers to speculate on their physiopathological role; however, definitive evidence stating that TNTs exist within more complex biological contexts is currently missing.

Development

Various studies have established that during gastrulation of marine animals such as Sea Urchins, actin-containing filopodial protrusions extend from ectodermal cells, and between primary and secondary mesenchyme cells (Miller *et al*, 1995). Although sightings of thin filopodia were previously made in Sea Urchins and were hypothesized to play a role in locomotion (Gustafson & Wolpert, 1963), McClay and colleagues suggest that the developmental timing, location, and rich cytoskeletal apparatus of filopodia owe their regulation to signaling organelles enabling communication of migratory cues (McClay, 1999).

During zebrafish gastrulation, long actin-rich Intercellular Bridges (IBs) are generated from dividing epiblast cells (Caneparo

 © 2021 The Authors

et al, 2011) which do not separate by abscission as daughter cells normally do during cytokinesis. The observation of fluorescent membrane-linked Dendra2 moving along IBs at a rate only possible through an active process led the authors to hypothesize that IBs could function as TNTs for intercellular signaling during embryonic patterning and development. However, recently published studies described similar protrusions as "retraction fiber projections" that host *Migrasomes* (Jiang *et al*, 2019), vesicular structures at the tips of projections that release contents into the environment through an EV-like mechanism called "migracytosis" (Ma *et al*, 2015; Huang *et al*, 2019; Tavano & Heisenberg, 2019). Although IBs and migrasome-carrying fibers resemble each other morphologically, it is currently unclear whether migrasomes are derived from IBs, or whether these two structures coexist in the complex zebrafish gastrular micro-environment as two separate processes.

The formation of stable IBs through incomplete cytokinesis was also shown in various germline and somatic tissues (Haglund *et al*, 2011). For example, actin-rich germline bridges called *Ring Canals* (RCs) are rims that connect germ cells in cysts of *Xenopus laevis* (Kloc *et al*, 2004), Drosophila (Haglund *et al*, 2010), mice (Pepling & Spradling, 1998), and several other animal models (Haglund *et al*, 2011). The function of these connections remains unknown; however, numerous reports have proposed they promote the exchange of cytoplasm and organelles, in a similar fashion to TNTs, with the important exception that TNTs do not form from cell division (Caneparo *et al*, 2011). Whether any of the structures previously identified as ring canals or IBs (such as those identified by Caneparo *et al*, in zebrafish) are instead TNTs forming between two different cell populations *in vivo,* which enable intercellular communication, synchronization of cell division, differentiation, or migration is currently unknown (Korenkova *et al*, 2020).

During development of the imaginal disk of *Drosophila*, specialized filopodia called *Cytonemes* were identified and are sometimes classified as the first demonstration of TNT-like structures *in vivo* (Ramírez-Weber & Kornberg, 1999). However, distinct from TNTs, cytonemes are close-ended, specialized filopodia (Kornberg & Roy, 2014) that play an important role in the signaling of morphogens, (e.g., Bone morphogenetic protein, BMP; Sonic hedgehog, Shh; Wingless-related integration site, Wnt, and Fibroblast growth factor, Fgf), through ligands and receptors that are currently under investigation (Kornberg, 2017; Zhang & Scholpp, 2019). Interestingly, recent evidence from our group indicates Wnt signaling is involved in the regulation of TNT formation of primary neurons (Vargas *et al*, 2019b). However, Wnt-dependent TNT formation is mediated by activation of the downstream calcium-calmodulin signaling pathway, while the formation of cytonemes appears to be mediated by the downstream activation of beta catenin (Mattes *et al*, 2018). Thus, while activation of the Wnt receptor is involved in both cytoneme and TNT formation, their downstream pathways differ, suggesting cytonemes and TNTs are in fact different structures that might have disparate developmental functions.

Prospective role of TNTs and TNT-like structures in brain development

Over the past decade, our studies in neuronal cells revealed that TNT formation and stabilization requires a variety of specific proteins and ligands. Interestingly, many of these molecules are enriched during pre- and postnatal brain development. MyoX, for example, is regulated during early postnatal development of the CNS in mice (Sousa *et al*, 2006) and is highly expressed in a brain region of active cell proliferation and migration: the external- and internal granule cell layers of the cerebellum (Raines *et al*, 2012).

During neocortical brain development, MyoX is also known to form a complex with surface N-Cadherin adhesion molecules in order to allow neuronal radial migration (Lai *et al*, 2015). Interestingly, we previously showed that MyoX can give rise to neuronal TNTs independent of its binding to N-Cadherin (Gousset *et al*, 2013) and hypothesized that N-Cadherin played a role in the formation and stability of TNTs through its' homophilic interactions with N-Cadherin molecules sitting on adjacent TNTs, "iTNTs" (Sartori-Rupp *et al*, 2019). Whether N-Cadherin, whose primary functions were classically thought to govern essential cell-to-cell interactions during neuronal morphogenesis and correct synaptic pairing by bringing apposed cell membranes into contact (Takeichi, 2007), also serves as an essential player for TNT formation during cortical neuron migration and polarity establishment will require further investigation.

Our most recent discovery showing that TNT-like structures can form between primary neurons at an early stage of development, prior to the formation of synapses, supports the notion undifferentiated neurons could utilize TNTs as a mode of intercellular communication where synapses have not been established (Vargas *et al*, 2019b). Whether this is the case in the brain requires further inquiry. Interestingly, there are numerous instances when pre-synaptic TNT-mediated communication could be employed during brain development. For example, granule cells in the cerebellum are neuronal progenitors with spherical somas and a few protruding neurites that undertake two distinctive migratory events prior to synapse formation (Komuro & Kumada, 2005). The underlying pathways that govern these events have been attributed to Bergmann glia, but continue to be investigated (Galas *et al*, 2017). The prospect of TNTs/TNT-like structures playing a role in neuronal migratory processes via the propagation of depolarization signals, to synchronize actin remodeling was previously proposed but never demonstrated (Gerdes *et al*, 2013).

Wnt ligands, (Wnt5a/7a), which induce neuronal TNTs in culture (Vargas *et al*, 2019b), participate in the maturation and function of pre-synaptic terminals and dendritic maintenance (Oliva *et al*, 2013), and, in the case of Wnt7a, get secreted by cerebellar granule cells to regulate synaptic differentiation (Hall *et al*, 2000). Formation of dendritic spines is also regulated by Eps8 (Menna *et al*, 2013; Stamatakou *et al*, 2013), another neuronal TNT inducer (see above) (Delage *et al*, 2016). In the brain, Eps8 is localized post-synaptically in the dendritic articulations of cerebellar granule neurons (Offenhäuser *et al*, 2006) and in axons of cultured hippocampal neurons, where it regulates the formation of filopodia (Menna *et al*, 2009).

Taken together, these studies provide evidence that TNT/TNT-like structures involve proteins with an elevated activity in developing brain areas, rich in neuronal precursors that are non-polarized and/or are in the process of migrating. Although these proteins were classically thought to give rise to and maintain neuronal processes (e.g., dendritic spines and axonal growth cones), our *in vitro* data proposes a novel molecular function in which these molecules also

© 2021 The Authors

regulate neuronal TNTs, which are presumably engaged during neurodevelopmental processes (e.g., neuronal precursor migration). The discovery of TNT-like structures during migratory and signaling events during early developmental processes of non-mammalian, vertebrate organisms supports this notion (Miller *et al*, 1995; Caneparo *et al*, 2011), but our hypothesis on neurodevelopmental TNTs has never been tested.

Owing to the difficulties of identifying TNTs *in vivo*—given lack of a TNT-specific marker and limitations in the resolution of light microscopy—our goal moving forward should be to focus on the implementation of long-range, structural mapping technologies (e.g., FIB-SEM) to detect TNTs in the developing brain without the need for a TNT-specific marker. The technical and conceptual strategies optimized by these investigations will serve as a stepping stone that will eventually enable us to study the presence- and physiological function of neuronal TNTs in developed mouse models commonly used to study neurodegenerative disease conditions, where we hypothesize to find an exacerbation of TNTs (Victoria & Zurzolo, 2017).

Others

In other studies, MHC class II + cells in the mouse cornea were observed to connect via TNT-like structures called membrane nanotubes (MNTs), < 800 nm in thickness and > 300 μm in length (Chinnery *et al*, 2008; Seyed-Razavi *et al*, 2013). The authors hypothesize isolated (dendritic) cells may use TNTs to communicate and form an immunological syncytium; however, this hypothesis has yet to be explored.

Intervascular bridges (IVBs) are actin-bearing fibrous strands joining neighboring capillaries in the retina of humans and mice (Mendes-Jorge *et al*, 2012). The expression of common pericyte markers (NG2 and PDGFR-b) led the authors to hypothesize IVBs are of pericyte nature. Interestingly, these bridges express Cx43, suggesting these connections allow intercellular communication. However, the precise signals transported between cells via these bridges has not yet been explored. In another more recent study, Di Polo and colleagues identified TNT-like processes (termed interpericyte TNTs or IP-TNTs) that connect pericytes on retinal capillaries and form a functional syncytium (Alarcon-Martinez *et al*, 2020). The authors show that IP-TNTs contain mitochondria and actin, and propagate intercellular calcium waves; however, their close-ended tip characterized by the presence of GJs may limit the intercellular transport of larger cargoes (Zurzolo, 2020).

Various malignant tumors, including those of lung-, ovarian-, and laryngeal cancer, mesothelioma, and neuroblastoma, were shown to form TNT-like connections (Antanavičiūtė *et al*, 2014, 2015; Ady *et al*, 2016; Desir *et al*, 2016). Mesothelioma cells forming TNTs of > 70 μm in length contained microvesicles labeled with MitoTracker (Lou *et al*, 2012). Human glioblastoma tumors implanted into a mouse brain and labeled with an astrocytoma cell GFP reporter form long (> 500 μm) and thick (> 1 μm) connections called Tumor Microtubes (TMs) between glioblastoma cells (Osswald *et al*, 2015). However, it is not clear whether TMs allow cargo exchange between cells similarly to TNTs (Pinto *et al*, 2020, 2021).

Finally, a report by Cherqui and colleagues identified TNT-like extensions connecting HSC-derived macrophages with kidney proximal tubular cells, crossing the dense basement membranes of the kidney to deliver cystinosin-containing vesicles into diseased tubular cells (Naphade *et al*, 2015; Cherqui & Courtoy, 2017).

Collectively, these findings show that TNT-like structures exist *in vivo* under certain physiological and/or pathological conditions. Future studies will need to demonstrate whether these different structures feature the TNT criteria described above (Fig 1).

Discussion and future perspectives

The growing number of TNT and TNT-like structure discoveries *in vitro* and *in vivo* usher in an exciting time for the field of TNT-mediated cell-to-cell communication research. We envision a new era in TNT biology in which the successful deployment of sophisticated labeling, imaging, and analytical technologies for the comprehensive characterization of TNTs will serve as an important benchmark for testing our *in vitro* findings *in vivo*, and most importantly, as a conduit between the bench and the promising therapeutic value that TNTs possess.

One of the primary objectives moving forward should be further refinement of the up- and downstream pathways regulating the formation of TNTs, (i.e., proteins modulating actin polymerization, membrane supply and recycling, and stress response). The outcome of these studies not only has the potential to yield a TNT-specific marker, which the field still severely lacks, but also heralds a promising therapeutic candidate that could target TNTs during disease. A rigorous characterization of TNTs' molecular architecture will undoubtedly benefit from 3D, correlative structural investigations peering into the cytoskeletal and membranous anatomy of TNTs at the nanoscale resolution.

Thorough molecular and structural studies will also shed light on the diversity found among TNTs (e.g., open- versus close-ended) and address existing dichotomies between species / cellular models. Generating a comprehensive catalog of distinct types of TNTs will enable us to label and classify them accordingly. For the time being, we encourage authors to consider employing the terms "TNT" and "TNT-like" in order to foster nomenclature consistency and eschew the creation of analogous- or cell-specific terms, which have previously led to discrepancy and confusion (Ariazi *et al*, 2017; Yamashita *et al*, 2018). Other essential aspects which remain to be uncovered regarding the operation of these complex biological machines include the kinetics and underlying biophysical mechanisms that lead to TNT fusion during formation (Abounit & Zurzolo, 2012; Dupont *et al*, 2018; Drab *et al*, 2019).

The opportunities facing us are tremendous, but to thrive, the field will have to ascertain the presence of TNTs *in vivo* using animal models, verify the functional features they exhibit in culture translate to complex biological organisms, and determine their functional role during an organism's proper or improper development. Visually accessible models like the developing zebrafish have proven to be effective systems to interrogate the presence of TNT-like structures *in vivo,* during embryogenesis. However, further studies will be required to test whether these structures also exist in a mammalian model and in the development of specific biological systems such as the CNS, which is the focus of our group.

We currently face a number of limitations, but the future prospects of TNT-mediated cell-to-cell communication during development and in disease are nonetheless very exciting.

 © 2021 The Authors

Acknowledgements

We thank Harshavardhan Khare for his helpful insights on our machine-learning strategy. We are grateful to Rio McLellan, Harshavardhan Khare, Stephen O'Shea, Olga Korenkova, and Anna Pepe for helpful comments on the manuscript. TNT research in the Zurzolo laboraotory is supported by Neurotunn (ANR-16-CE160019), Cancéropôle Ile-de-France (2018-1-PL-03-IP-1), INCEPTION (ANR-16-CONV-0005) and Institut de Convergence Q-Life awards (ANR-17-CONV-0005) grants to CZ. D.C.C. is supported by INCEPTION (ANR-16-CONV-0005) and an Institut de Convergence Q-Life award (ANR-17-CONV-0005).

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abounit S, Bousset L, Loria F, Zhu S, de Chaumont F, Pieri L, Olivo-Marin J-C, Melki R, Zurzolo C (2016a) Tunneling nanotubes spread fibrillar α-synuclein by intercellular trafficking of lysosomes. *EMBO J* 35: 2120 – 2138

Abounit S, Delage E, Zurzolo C (2015) Identification and characterization of tunneling nanotubes for intercellular trafficking. *Curr Protoc Cell Biol* 67: 12.10.1-21

Abounit S, Wu JW, Duff K, Victoria GS, Zurzolo C (2016b) Tunneling nanotubes: a possible highway in the spreading of tau and other prion-like proteins in neurodegenerative diseases. *Prion* 10: 344 – 351

Abounit S, Zurzolo C (2012) Wiring through tunneling nanotubes–from electrical signals to organelle transfer. *J Cell Sci* 125: 1089 – 1098

Ady J, Thayanithy V, Mojica K, Wong P, Carson J, Rao P, Fong Y, Lou E (2016) Tunneling nanotubes: an alternate route for propagation of the bystander effect following oncolytic viral infection. *Mol Ther Oncolytics* 3: 16029

Ahmad T, Mukherjee S, Pattnaik B, Kumar M, Singh S, Kumar M, Rehman R, Tiwari BK, Jha KA, Barhanpurkar AP *et al* (2014) Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. *EMBO J* 33: 994 – 1010

Alarcon-Martinez L, Villafranca-Baughman D, Quintero H, Kacerovsky JB, Dotigny F, Murai KK, Prat A, Drapeau P, Di Polo A (2020) Interpericyte tunnelling nanotubes regulate neurovascular coupling. *Nature* 585: 91 – 95

Antanavičiūtė I, Ereminienė E, Vysockas V, Račkauskas M, Skipskis V, Rysevaitė K, Treinys R, Benetis R, Jurevičius J, Skeberdis VA (2015) Exogenous connexin43-expressing autologous skeletal myoblasts ameliorate mechanical function and electrical activity of the rabbit heart after experimental infarction. *Int J Exp Pathol* 96: 42 – 53

Antanavičiūtė I, Rysevaitė K, Liutkevičius V, Marandykina A, Rimkutė L, Sveikatienė R, Uloza V, Skeberdis VA (2014) Long-distance communication between laryngeal carcinoma cells. *PLoS One* 9: e99196

Aramaki S, Mayanagi K, Jin M, Aoyama K, Yasunaga T (2016) Filopodia formation by crosslinking of F-actin with fascin in two different binding manners. *Cytoskelet Hoboken NJ* 73: 365 – 374

Ariazi J, Benowitz A, De Biasi V, Den Boer ML, Cherqui S, Cui H, Douillet N, Eugenin EA, Favre D, Goodman S *et al* (2017) Tunneling nanotubes and gap junctions-their role in long-range intercellular communication during development, health, and disease conditions. *Front Mol Neurosci* 10: 333

Arkwright PD, Luchetti F, Tour J, Roberts C, Ayub R, Morales AP, Rodríguez JJ, Gilmore A, Canonico B, Papa S *et al* (2010) Fas stimulation of T lymphocytes promotes rapid intercellular exchange of death signals via membrane nanotubes. *Cell Res* 20: 72 – 88

Asencio-Barría C, Defamie N, Sáez JC, Mesnil M, Godoy AS (2019) Direct intercellular communications and cancer: a snapshot of the biological roles of connexins in prostate cancer. *Cancers* 11: 1370

Austefjord MW, Gerdes H-H, Wang X (2014) Tunneling nanotubes: diversity in morphology and structure. *Commun Integr Biol* 7: e27934

Baker M (2017) How the Internet of cells has biologists buzzing. *Nature* 549: 322 – 324

Bénard M, Schapman D, Lebon A, Monterroso B, Bellenger M, Le Foll F, Pasquier J, Vaudry H, Vaudry D, Galas L (2015) Structural and functional analysis of tunneling nanotubes (TnTs) using gCW STED and gconfocal approaches. *Biol Cell* 107: 419 – 425

Bhat S, Ljubojevic N, Zhu S, Fukuda M, Echard A, Zurzolo C (2020) Rab35 and its effectors promote formation of tunneling nanotubes in neuronal cells. *Sci Rep* 10: 16803

Bohil AB, Robertson BW, Cheney RE (2006) Myosin-X is a molecular motor that functions in filopodia formation. *Proc Natl Acad Sci USA* 103: 12411 – 12416

Bukoreshtliev NV, Wang X, Hodneland E, Gurke S, Barroso JFV, Gerdes H-H (2009) Selective block of tunneling nanotube (TNT) formation inhibits intercellular organelle transfer between PC12 cells. *FEBS Lett* 583: 1481 – 1488

Campana S, De Pasquale C, Carrega P, Ferlazzo G, Bonaccorsi I (2015) Cross-dressing: an alternative mechanism for antigen presentation. *Immunol Lett* 168: 349 – 354

Caneparo L, Pantazis P, Dempsey W, Fraser SE (2011) Intercellular bridges in vertebrate gastrulation. *PLoS One* 6: e20230

Carter KP, Segall JE, Cox D (2020) Microscopic methods for analysis of macrophage-induced tunneling nanotubes. *Methods Mol Biol Clifton NJ* 2108: 273 – 279

Chastagner P, Loria F, Vargas JY, Tois J, Diamond IM, Okafo G, Brou C, Zurzolo C(2020) Fate and propagation of endogenously formed Tau aggregates in neuronal cells. *EMBO Mol Med* 12: e12025

Chauveau A, Aucher A, Eissmann P, Vivier E, Davis DM (2010) Membrane nanotubes facilitate long-distance interactions between natural killer cells and target cells. *Proc Natl Acad Sci USA* 107: 5545 – 5550

Chen B-C, Legant WR, Wang K, Shao L, Milkie DE, Davidson MW, Janetopoulos C, Wu XS, Hammer JA, Liu Z *et al* (2014) Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. *Science* 346: 1257998

Cherqui S, Courtoy PJ (2017) The renal Fanconi syndrome in cystinosis: pathogenic insights and therapeutic perspectives. *Nat Rev Nephrol* 13: 115 – 131

Chinnery HR, Pearlman E, McMenamin PG (2008) Cutting edge: membrane nanotubes in vivo: a feature of MHC class II+ cells in the mouse cornea. *J Immunol* 180: 5779 – 5783

Collot M, Ashokkumar P, Anton H, Boutant E, Faklaris O, Galli T, Mély Y, Danglot L, Klymchenko AS (2019) MemBright: a family of fluorescent membrane probes for advanced cellular imaging and neuroscience. *Cell Chem Biol* 26: 600 – 614.e7

Connor Y, Tekleab S, Nandakumar S, Walls C, Tekleab Y, Husain A, Gadish O, Sabbisetti V, Kaushik S, Sehrawat S *et al* (2015) Physical nanoscale conduit-mediated communication between tumour cells and the endothelium modulates endothelial phenotype. *Nat Commun* 6: 8671

Costanzo M, Abounit S, Marzo L, Danckaert A, Chamoun Z, Roux P, Zurzolo C (2013) Transfer of polyglutamine aggregates in neuronal cells occurs in tunneling nanotubes. *J Cell Sci* 126: 3678 – 3685

Dance A (2019) Core Concept: Cells nibble one another via the under-appreciated process of trogocytosis. *Proc Natl Acad Sci USA* 116: 17608 – 17610

© 2021 The Authors

Davis DM, Sowinski S (2008) Membrane nanotubes: dynamic long-distance connections between animal cells. *Nat Rev Mol Cell Biol* 9: 431 – 436

Delage E, Cervantes DC, Pénard E, Schmitt C, Syan S, Disanza A, Scita G, Zurzolo C (2016) Differential identity of Filopodia and Tunneling Nanotubes revealed by the opposite functions of actin regulatory complexes. *Sci Rep* 6: 39632

Delage E, Zurzolo C (2013) Exploring the role of lipids in intercellular conduits: breakthroughs in the pipeline. *Front Plant Sci* 4: 504

Desir S, Dickson EL, Vogel RI, Thayanithy V, Wong P, Teoh D, Geller MA, Steer CJ, Subramanian S, Lou E (2016) Tunneling nanotube formation is stimulated by hypoxia in ovarian cancer cells. *Oncotarget* 7: 43150 – 43161

Desir S, O'Hare P, Vogel RI, Sperduto W, Sarkari A, Dickson EL, Wong P, Nelson AC, Fong Y, Steer CJ *et al* (2018) Chemotherapy-induced tunneling nanotubes mediate intercellular drug efflux in pancreatic cancer. *Sci Rep* 8: 9484

Dieriks BV, Park TI-H, Fourie C, Faull RLM, Dragunow M, Curtis MA (2017) α-synuclein transfer through tunneling nanotubes occurs in SH-SY5Y cells and primary brain pericytes from Parkinson's disease patients. *Sci Rep* 7: 42984

Dilsizoglu Senol A, Pepe A, Grudina C, Sassoon N, Reiko U, Bousset L, Melki R, Piel J, Gugger M, Zurzolo C (2019) Effect of tolytoxin on tunneling nanotube formation and function. *Sci Rep* 9: 5741

Dixon SJ, Stockwell BR (2014) The role of iron and reactive oxygen species in cell death. *Nat Chem Biol* 10: 9 – 17

Drab M, Stopar D, Kralj-Iglič V, Iglič A (2019) Inception mechanisms of tunneling nanotubes. *Cells* 8: 626

Dubois F, Jean-Jacques B, Roberge H, Bénard M, Galas L, Schapman D, Elie N, Goux D, Keller M, Maille E *et al* (2018) A role for RASSF1A in tunneling nanotube formation between cells through GEFH1/Rab11 pathway control. *Cell Commun Signal CCS* 16: 66

Dupont M, Souriant S, Lugo-Villarino G, Maridonneau-Parini I, Vérollet C (2018) Tunneling nanotubes: intimate communication between myeloid cells. *Front Immunol* 9: 43

Dupont M, Souriant S, Balboa L, Vu Manh T-P, Pingris K, Rousset S, Cougoule C, Rombouts Y, Poincloux R, Ben Neji M *et al* (2020) Tuberculosis-associated IFN-I induces Siglec-1 on tunneling nanotubes and favors HIV-1 spread in macrophages. *Elife* 9: e52535

Dustin ML, Chakraborty AK, Shaw AS (2010) Understanding the structure and function of the immunological synapse. *Cold Spring Harb Perspect Biol* 2: a002311

Eugenin EA, Gaskill PJ, Berman JW (2009) Tunneling nanotubes (TNT) are induced by HIV-infection of macrophages: a potential mechanism for intercellular HIV trafficking. *Cell Immunol* 254: 142 – 148

Finetti F, Cassioli C, Baldari CT (2017) Transcellular communication at the immunological synapse: a vesicular traffic-mediated mutual exchange. *F1000Res* 6: 1880

Galas L, Bénard M, Lebon A, Komuro Y, Schapman D, Vaudry H, Vaudry D, Komuro H (2017) Postnatal migration of cerebellar interneurons. *Brain Sci* 7: 62

Gauthier NC, Masters TA, Sheetz MP (2012) Mechanical feedback between membrane tension and dynamics. *Trends Cell Biol* 22: 527 – 535

Gerdes HH, Bukoreshtliev NV, Barroso JFV (2007) Tunneling nanotubes: a new route for the exchange of components between animal cells. *FEBS Lett* 581: 2194 – 2201

Gerdes HH, Carvalho RN (2008) Intercellular transfer mediated by tunneling nanotubes. *Curr Opin Cell Biol* 20: 470 – 475

Gerdes H-H, Rustom A, Wang X (2013) Tunneling nanotubes, an emerging intercellular communication route in development. *Mech Dev* 130: 381 – 387

Goedert M (2015) Neurodegeneration. Alzheimer's and Parkinson's diseases: the prion concept in relation to assembled Aβ, tau, and α-synuclein. *Science* 349: 1255555

Goodman S, Naphade S, Khan M, Sharma J, Cherqui S (2019) Macrophage polarization impacts tunneling nanotube formation and intercellular organelle trafficking. *Sci Rep* 9: 14529

Gousset K, Gordon A, Kumar Kannan S, Tovar J (2019) A novel microproteomic approach using laser capture microdissection to study cellular protrusions. *Int J Mol Sci* 20: 1172

Gousset K, Marzo L, Commere P-H, Zurzolo C (2013) Myo10 is a key regulator of TNT formation in neuronal cells. *J Cell Sci* 126: 4424 – 4435

Gousset K, Schiff E, Langevin C, Marijanovic Z, Caputo A, Browman DT, Chenouard N, de Chaumont F, Martino A, Enninga J *et al* (2009) Prions hijack tunnelling nanotubes for intercellular spread. *Nat Cell Biol* 11: 328 – 336

Grudina C, Kouroupi G, Nonaka T, Hasegawa M, Matsas R, Zurzolo C (2019) Human NPCs can degrade α-syn fibrils and transfer them preferentially in a cell contact-dependent manner possibly through TNT-like structures. *Neurobiol Dis* 132: 104609

Gurke S, Barroso JFV, Gerdes HH (2008) The art of cellular communication: tunneling nanotubes bridge the divide. *Histochem Cell Biol* 129: 539 – 550

Gustafson T, Wolpert L (1963) The cellular basis of morphogenesis and sea urchin development. *Int Rev Cytol* 15: 139 – 214

Haglund K, Nezis IP, Lemus D, Grabbe C, Wesche J, Liestøl K, Dikic I, Palmer R, Stenmark H (2010) Cindr interacts with anillin to control cytokinesis in *Drosophila melanogaster*. *Curr Biol* 20: 944 – 950

Haglund K, Nezis IP, Stenmark H (2011) Structure and functions of stable intercellular bridges formed by incomplete cytokinesis during development. *Commun Integr Biol* 4: 1 – 9

Halász H, Ghadaksaz AR, Madarász T, Huber K, Harami G, Tóth EA, Osteikoetxea-Molnár A, Kovács M, Balogi Z, Nyitrai M *et al* (2018) Live cell superresolution-structured illumination microscopy imaging analysis of the intercellular transport of microvesicles and costimulatory proteins via nanotubes between immune cells. *Methods Appl Fluoresc* 6: 45005

Hall AC, Lucas FR, Salinas PC (2000) Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell* 100: 525 – 535

Hanna SJ, McCoy-Simandle K, Miskolci V, Guo P, Cammer M, Hodgson L, Cox D (2017) The role of Rho-GTPases and actin polymerization during macrophage tunneling nanotube biogenesis. *Sci Rep* 7: 8547

Hanna SJ, McCoy-Simandle K, Leung E, Genna A, Condeelis J, Cox D (2019) Tunneling nanotubes, a novel mode of tumor cell–macrophage communication in tumor cell invasion. *J Cell Sci* 132: jcs223321

Hase K, Kimura S, Takatsu H, Ohmae M, Kawano S, Kitamura H, Ito M, Watarai H, Hazelett CC, Yeaman C *et al* (2009) M-Sec promotes membrane nanotube formation by interacting with Ral and the exocyst complex. *Nat Cell Biol* 11: 1427 – 1432

Hasegawa M, Nonaka T, Masuda-Suzukake M (2017) Prion-like mechanisms and potential therapeutic targets in neurodegenerative disorders. *Pharmacol Ther* 172: 22 – 33

Hashimoto M, Bhuyan F, Hiyoshi M, Noyori O, Nasser H, Miyazaki M, Saito T, Kondoh Y, Osada H, Kimura S *et al* (2016) Potential role of the formation of tunneling nanotubes in HIV-1 spread in macrophages. *J Immunol* 196: 1832 – 1841

Heasman SJ, Ridley AJ (2008) Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat Rev Mol Cell Biol* 9: 690 – 701

 © 2021 The Authors

Hekmatshoar Y, Nakhle J, Galloni M, Vignais M-L (2018) The role of metabolism and tunneling nanotube-mediated intercellular mitochondria exchange in cancer drug resistance. *Biochem J* 475: 2305 – 2328

Hoffman L, Jensen CC, Yoshigi M, Beckerle M (2017) Mechanical signals activate p38 MAPK pathway-dependent reinforcement of actin via mechanosensitive HspB1. *Mol Biol Cell* 28: 2661 – 2675

Huang Y, Zucker B, Zhang S, Elias S, Zhu Y, Chen H, Ding T, Li Y, Sun Y, Lou J *et al* (2019) Migrasome formation is mediated by assembly of micron-scale tetraspanin macrodomains. *Nat Cell Biol* 21: 991 – 1002

Huot J, Houle F, Rousseau S, Deschesnes RG, Shah GM, Landry J (1998) SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. *J Cell Biol* 143: 1361 – 1373

Hurtig J, Chiu DT, Onfelt B (2010) Intercellular nanotubes: insights from imaging studies and beyond. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2: 260 – 276

Ishimoto T, Mori H (2019) A new bioluminescence-based tool for modulating target proteins in live cells. *Sci Rep* 9: 18239

Jansen AHP, Batenburg KL, Pecho-Vrieseling E, Reits EA (2017) Visualization of prion-like transfer in Huntington's disease models. *Biochim Biophys Acta Mol Basis Dis* 1863: 793 – 800

Jansens RJJ, Tishchenko A, Favoreel HW (2020) Bridging the gap: virus long-distance spread via tunneling nanotubes. *J Virol* 94: e02120-19

Jash E, Prasad P, Kumar N, Sharma T, Goldman A, Sehrawat S (2018) Perspective on nanochannels as cellular mediators in different disease conditions. *Cell Commun Signal* 16: 76

Jiang D, Jiang Z, Lu D, Wang X, Liang H, Zhang J, Meng Y, Li Y, Wu D, Huang Y *et al* (2019) Migrasomes provide regional cues for organ morphogenesis during zebrafish gastrulation. *Nat Cell Biol* 21: 966 – 977

Jucker M, Walker LC (2018) Propagation and spread of pathogenic protein assemblies in neurodegenerative diseases. *Nat Neurosci* 21: 1341 – 1349

Kadiu I, Gendelman HE (2011) Macrophage bridging conduit trafficking of HIV-1 through the endoplasmic reticulum and Golgi network. *J Proteome Res* 10: 3225 – 3238

Kassubek J, Juengling FD, Kioschies T, Henkel K, Karitzky J, Kramer B, Ecker D, Andrich J, Saft C, Kraus P *et al* (2004) Topography of cerebral atrophy in early Huntington's disease: a voxel based morphometric MRI study. *J Neurol Neurosurg Psychiatry* 75: 213 – 220

Kerber ML, Cheney RE (2011) Myosin-X: a MyTH-FERM myosin at the tips of filopodia. *J Cell Sci* 124: 3733 – 3741

Kimura S, Yamashita M, Yamakami-Kimura M, Sato Y, Yamagata A, Kobashigawa Y, Inagaki F, Amada T, Hase K, Iwanaga T *et al* (2016) Distinct roles for the N- and C-terminal regions of M-Sec in plasma membrane deformation during tunneling nanotube formation. *Sci Rep* 6: 33548

Kiriyama Y, Nochi H (2017) Intra- and intercellular quality control mechanisms of mitochondria. *Cells* 7: 1

Kloc M, Bilinski S, Dougherty MT, Brey EM, Etkin LD (2004) Formation, architecture and polarity of female germline cyst in Xenopus. *Dev Biol* 266: 43 – 61

Kolba MD, Dudka W, Zaręba-Kozioł M, Kominek A, Ronchi P, Turos L, Chroscicki P, Wlodarczyk J, Schwab Y, Klejman A *et al* (2019) Tunneling nanotube-mediated intercellular vesicle and protein transfer in the stroma-provided imatinib resistance in chronic myeloid leukemia cells. *Cell Death Dis* 10: 817

Komuro H, Kumada T (2005) Ca2+ transients control CNS neuronal migration. *Cell Calcium* 37: 387 – 393

Korenkova O, Pepe A, Zurzolo C (2020) Fine intercellular connections in development: TNTs, cytonemes, or intercellular bridges? *Cell Stress* 4: 30 – 43

Kornberg TB (2017) Distributing signaling proteins in space and time: the province of cytonemes. *Curr Opin Genet Dev* 45: 22 – 27

Kornberg TB, Roy S (2014) Cytonemes as specialized signaling filopodia. *Dev Camb Engl* 141: 729 – 736

Kumar A, Kim JH, Ranjan P, Metcalfe MG, Cao W, Mishina M, Gangappa S, Guo Z, Boyden ES, Zaki S *et al* (2017) Influenza virus exploits tunneling nanotubes for cell-to-cell spread. *Sci Rep* 7: 40360

Lachambre S, Chopard C, Beaumelle B (2014) Preliminary characterisation of nanotubes connecting T-cells and their use by HIV-1. *Biol Cell* 106: 394 – 404

Lai M, Guo Y, Ma J, Yu H, Zhao D, Fan W, Ju X, Sheikh MA, Malik YS, Xiong W *et al* (2015) Myosin X regulates neuronal radial migration through interacting with N-cadherin. *Front Cell Neurosci* 9: 326

Liang D (2018) A salutary role of reactive oxygen species in intercellular tunnel-mediated communication. *Front Cell Dev Biol* 6: 2

Lin T-K, Chen S-D, Chuang Y-C, Lan M-Y, Chuang J-H, Wang P-W, Hsu T-Y, Wang F-S, Tsai M-H, Huang S-T *et al* (2019) Mitochondrial transfer of wharton's jelly mesenchymal stem cells eliminates mutation burden and rescues mitochondrial bioenergetics in rotenone-stressed MELAS fibroblasts. *Oxid Med Cell Longev* 2019: 9537504

Liu T-L, Upadhyayula S, Milkie DE, Singh V, Wang K, Swinburne IA, Mosaliganti KR, Collins ZM, Hiscock TW, Shea J *et al* (2018) Observing the cell in its native state: Imaging subcellular dynamics in multicellular organisms. *Science* 360: eaaq1392

Ljubojevic N, Henderson JM, Zurzolo C (2021) The ways of actin: why tunneling nanotubes are unique cell protrusions. *Trends Cell Biol* 31: 130 – 142

Lock JT, Parker I, Smith IF (2016) Communication of Ca2+ signals via tunneling membrane nanotubes is mediated by transmission of inositol trisphosphate through gap junctions. *Cell Calcium* 60: 266 – 272

Lokar M, Kabaso D, Resnik N, Sepčić K, Kralj-Iglič V, Veranič P, Zorec R, Iglič A (2012) The role of cholesterol-sphingomyelin membrane nanodomains in the stability of intercellular membrane nanotubes. *Int J Nanomedicine* 7: 1891 – 1902

Loria F, Vargas JY, Bousset L, Syan S, Salles A, Melki R, Zurzolo C (2017) α-Synuclein transfer between neurons and astrocytes indicates that astrocytes play a role in degradation rather than in spreading. *Acta Neuropathol* 134: 789 – 808

Lou E, Fujisawa S, Morozov A, Barlas A, Romin Y, Dogan Y, Gholami S, Moreira AL, Manova-Todorova K, Moore MAS (2012) Tunneling nanotubes provide a unique conduit for intercellular transfer of cellular contents in human malignant pleural mesothelioma. *PLoS One* 7: e33093

Lu J, Zheng X, Li F, Yu Y, Chen Z, Liu Z, Wang Z, Xu H, Yang W (2017) Tunneling nanotubes promote intercellular mitochondria transfer followed by increased invasiveness in bladder cancer cells. *Oncotarget* 8: 15539 – 15552

Luk KC, Song C, O'Brien P, Stieber A, Branch JR, Brunden KR, Trojanowski JQ, Lee VM-Y (2009) Exogenous alpha-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells. *Proc Natl Acad Sci USA* 106: 20051 – 20056

Ma L, Li Y, Peng J, Wu D, Zhao X, Cui Y, Chen L, Yan X, Du Y, Yu L (2015) Discovery of the migrasome, an organelle mediating release of cytoplasmic contents during cell migration. *Cell Res* 25: 24 – 38

Martins B, Sorrentino S, Chung W-L, Tatli M, Medalia O, Eibauer M (2021) Unveiling the polarity of actin filaments by cryo-electron tomography. *Structure* https://doi.org/10.1016/j.str.2020.12.014

Marzo L, Gousset K, Zurzolo C (2012) Multifaceted roles of tunneling nanotubes in intercellular communication. *Front Physiol* 3: 72

© 2021 The Authors

Matejka N, Reindl J (2019) Perspectives of cellular communication through tunneling nanotubes in cancer cells and the connection to radiation effects. *Radiat Oncol Lond Engl* 14: 218

Mattes B, Dang Y, Greicius G, Kaufmann LT, Prunsche B, Rosenbauer J, Stegmaier J, Mikut R, Özbek S, Nienhaus GU *et al* (2018) Wnt/PCP controls spreading of Wnt/β-catenin signals by cytonemes in vertebrates. *Elife* 7: e36953

McClay DR (1999) The role of thin filopodia in motility and morphogenesis. *Exp Cell Res* 253: 296 – 301

McCoy-Simandle K, Hanna SJ, Cox D (2016) Exosomes and nanotubes: control of immune cell communication. *Int J Biochem Cell Biol* 71: 44 – 54

Mendes-Jorge L, Llombart C, Ramos D, López-Luppo M, Valença A, Nacher V, Navarro M, Carretero A, Méndez-Ferrer S, Rodriguez-Baeza A *et al* (2012) Intercapillary bridging cells: immunocytochemical characteristics of cells that connect blood vessels in the retina. *Exp Eye Res* 98: 79 – 87

Menna E, Disanza A, Cagnoli C, Schenk U, Gelsomino G, Frittoli E, Hertzog M, Offenhauser N, Sawallisch C, Kreienkamp H-J *et al* (2009) Eps8 regulates axonal filopodia in hippocampal neurons in response to brain-derived neurotrophic factor (BDNF). *PLoS Biol* 7: e1000138

Menna E, Zambetti S, Morini R, Donzelli A, Disanza A, Calvigioni D, Braida D, Nicolini C, Orlando M, Fossati G *et al* (2013) Eps8 controls dendritic spine density and synaptic plasticity through its actin-capping activity. *EMBO J* 32: 1730 – 1744

Miller J, Fraser SE, McClay D (1995) Dynamics of thin filopodia during sea urchin gastrulation. *Development* 121: 2501 – 2511

Millet V, Naquet P, Guinamard RR (2008) Intercellular MHC transfer between thymic epithelial and dendritic cells. *Eur J Immunol* 38: 1257 – 1263

Mishin AS, Belousov VV, Solntsev KM, Lukyanov KA (2015) Novel uses of fluorescent proteins. *Curr Opin Chem Biol* 27: 1 – 9

Mittal R, Karhu E, Wang J-S, Delgado S, Zukerman R, Mittal J, Jhaveri VM (2019) Cell communication by tunneling nanotubes: implications in disease and therapeutic applications. *J Cell Physiol* 234: 1130 – 1146

Murray LMA, Krasnodembskaya AD (2019) Concise review: intercellular communication via organelle transfer in the biology and therapeutic applications of stem cells. *Stem Cells* 37: 14 – 25

Nagel J, Delandre C, Zhang Y, Förstner F, Moore AW, Tavosanis G (2012) Fascin controls neuronal class-specific dendrite arbor morphology. *Development* 139: 2999 – 3009

Naphade S, Sharma J, Gaide Chevronnay HP, Shook MA, Yeagy BA, Rocca CJ, Ur SN, Lau AJ, Courtoy PJ, Cherqui S (2015) Brief reports: lysosomal cross-correction by hematopoietic stem cell-derived macrophages via tunneling nanotubes. *Stem Cells* 33: 301 – 309

Neto E, Leitão L, Sousa DM, Alves CJ, Alencastre IS, Aguiar P, Lamghari M (2016) Compartmentalized microfluidic platforms: the unrivaled breakthrough of in vitro tools for neurobiological research. *J Neurosci Off J Soc Neurosci* 36: 11573 – 11584

Nussenzveig HM (2019) Are cell membrane nanotubes the ancestors of the nervous system? *Eur Biophys J* 48: 593 – 598

Offenhäuser N, Castelletti D, Mapelli L, Soppo BE, Regondi MC, Rossi P, D'Angelo E, Frassoni C, Amadeo A, Tocchetti A *et al* (2006) Increased ethanol resistance and consumption in Eps8 knockout mice correlates with altered actin dynamics. *Cell* 127: 213 – 226

Okafo G, Prevedel L, Eugenin E (2017) Tunneling nanotubes (TNT) mediate long-range gap junctional communication: Implications for HIV cell to cell spread. *Sci Rep* 7: 16660

Oliva CA, Vargas JY, Inestrosa NC (2013) Wnts in adult brain: from synaptic plasticity to cognitive deficiencies. *Front Cell Neurosci* 7: 224

Önfelt B, Nedvetzki S, Yanagi K, Davis DM (2004) Cutting edge: membrane nanotubes connect immune cells. *J Immunol* 173: 1511 – 1513

Önfelt B, Purbhoo MA, Nedvetzki S, Sowinski S, Davis DM (2005) Long-distance calls between cells connected by tunneling nanotubules. *Sci STKE* 2005: pe55

Önfelt B, Nedvetzki S, Benninger RKP, Purbhoo MA, Sowinski S, Hume AN, Seabra MC, Neil MAA, French PMW, Davis DM (2006) Structurally distinct membrane nanotubes between human macrophages support long-distance vesicular traffic or surfing of bacteria. *J Immunol* 177: 8476 – 8483

Önishi K, Shafer B, Lo C, Tissir F, Goffinet AM, Zou Y (2013) Antagonistic functions of Dishevelleds regulate Frizzled3 endocytosis via filopodia tips in Wnt-mediated growth cone guidance. *J Neurosci Off J Soc Neurosci* 33: 19071 – 19085

Osswald M, Jung E, Sahm F, Solecki G, Venkataramani V, Blaes J, Weil S, Horstmann H, Wiestler B, Syed M *et al* (2015) Brain tumour cells interconnect to a functional and resistant network. *Nature* 528: 93 – 98

Osteikoetxea-Molnár A, Szabó-Meleg E, Tóth EA, Oszvald Á, Izsépi E, Kremlitzka M, Biri B, Nyitray L, Bozó T, Németh P *et al* (2016) The growth determinants and transport properties of tunneling nanotube networks between B lymphocytes. *Cell Mol Life Sci* 73: 4531 – 4545

Parker I, Evans KT, Ellefsen K, Lawson DA, Smith IF (2017) Lattice light sheet imaging of membrane nanotubes between human breast cancer cells in culture and in brain metastases. *Sci Rep* 7: 11029

Pecho-Vrieseling E, Rieker C, Fuchs S, Bleckmann D, Esposito MS, Botta P, Goldstein C, Bernhard M, Galimberti I, Müller M *et al* (2014) Transneuronal propagation of mutant huntingtin contributes to non-cell autonomous pathology in neurons. *Nat Neurosci* 17: 1064 – 1072

Pepling ME, Spradling AC (1998) Female mouse germ cells form synchronously dividing cysts. *Development* 125: 3323 – 3328

Pinto G, Brou C, Zurzolo C (2020) Tunneling nanotubes: the fuel of tumor progression? *Trends Cancer* 6: 874 – 888

Pinto G, Saenz-de-Santa-Maria I, Chastagner P, Perthame E, Delmas C, Toulas C, Moyal-Jonathan-Cohen E, Brou C, Zurzolo C (2021) Patient-derived glioblastoma stem cells transfer mitochondria through tunneling nanotubes in tumor organoids. *Biochem J* 478: 21 – 39

Pontes B, Monzo P, Gole L, Le Roux A-L, Kosmalska AJ, Tam ZY, Luo W, Kan S, Viasnoff V, Roca-Cusachs P *et al* (2017) Membrane tension controls adhesion positioning at the leading edge of cells. *J Cell Biol* 216: 2959 – 2977

Pulze L, Congiu T, Brevini TAL, Grimaldi A, Tettamanti G, D'Antona P, Baranzini N, Acquati F, Ferraro F, de Eguileor M (2020) MCF7 Spheroid development: new insight about spatio/temporal arrangements of TNTs, amyloid fibrils, cell connections, and cellular bridges. *Int J Mol Sci* 21: 5400

Raines AN, Nagdas S, Kerber ML, Cheney RE (2012) Headless Myo10 is a negative regulator of full-length Myo10 and inhibits axon outgrowth in cortical neurons. *J Biol Chem* 287: 24873 – 24883

Rainy N, Chetrit D, Rouger V, Vernitsky H, Rechavi O, Marguet D, Goldstein I, Ehrlich M, Kloog Y (2013) H-Ras transfers from B to T cells via tunneling nanotubes. *Cell Death Dis* 4: e726

Ramírez-Weber FA, Kornberg TB (1999) Cytonemes: cellular processes that project to the principal signaling center in *Drosophila* imaginal discs. *Cell* 97: 599 – 607

Reichert D, Scheinpflug J, Karbanová J, Freund D, Bornhäuser M, Corbeil D (2016) Tunneling nanotubes mediate the transfer of stem cell marker CD133 between hematopoietic progenitor cells. *Exp Hematol* 44: 1092 – 1112.e2

Reindl J, Shevtsov M, Dollinger G, Stangl S, Multhoff G (2019) Membrane Hsp70-supported cell-to-cell connections via tunneling nanotubes revealed by live-cell STED nanoscopy. *Cell Stress Chaperones* 24: 213 – 221

© 2021 The Authors

Resnik N, Erman A, Veranič P, Kreft ME (2019) Triple labelling of actin filaments, intermediate filaments and microtubules for broad application in cell biology: uncovering the cytoskeletal composition in tunneling nanotubes. *Histochem Cell Biol* 152: 311 – 317

Resnik N, Prezelj T, De Luca GMR, Manders E, Polishchuk R, Veranič P, Kreft ME (2018) Helical organization of microtubules occurs in a minority of tunneling membrane nanotubes in normal and cancer urothelial cells. *Sci Rep* 8: 17133

Roehlecke C, Schmidt MHH (2020) Tunneling nanotubes and tumor microtubes in cancer. *Cancers* 12: 857

Rostami J, Holmqvist S, Lindström V, Sigvardson J, Westermark GT, Ingelsson M, Bergström J, Roybon L, Erlandsson A (2017) Human astrocytes transfer aggregated alpha-synuclein via tunneling nanotubes. *J Neurosci Off J Soc Neurosci* 37: 11835 – 11853

Rupp I, Sologub L, Williamson KC, Scheuermayer M, Reininger L, Doerig C, Eksi S, Kombila DU, Frank M, Pradel G (2011) Malaria parasites form filamentous cell-to-cell connections during reproduction in the mosquito midgut. *Cell Res* 21: 683 – 696

Rustom A (2016) The missing link: does tunnelling nanotube-based supercellularity provide a new understanding of chronic and lifestyle diseases? *Open Biol* 6: 160057

Rustom A, Saffrich R, Markovic I, Walther P, Gerdes H-H (2004) Nanotubular highways for intercellular organelle transport. *Science* 303: 1007 – 1010

Sáenz-de-Santa-María I, Bernardo-Castiñeira C, Secades P, Bernaldo-de-Quirós S, Rodrigo JP, Astudillo A, Chiara M-D (2017) Clinically relevant HIF-1α-dependent metabolic reprogramming in oropharyngeal squamous cell carcinomas includes coordinated activation of CAIX and the miR-210/ISCU signaling axis, but not MCT1 and MCT4 upregulation. *Oncotarget* 8: 13730 – 13746

Sager RE, Lee J-Y (2018) Plasmodesmata at a glance. *J Cell Sci* 131: jcs209346

Sartori-Rupp A, Cordero Cervantes D, Pepe A, Gousset K, Delage E, Corroyer-Dulmont S, Schmitt C, Krijnse-Locker J, Zurzolo C (2019) Correlative cryo-electron microscopy reveals the structure of TNTs in neuronal cells. *Nat Commun* 10: 342

Schiller C, Diakopoulos KN, Rohwedder I, Kremmer E, von Toerne C, Ueffing M, Weidle UH, Ohno H, Weiss EH (2013) LST1 promotes the assembly of a molecular machinery responsible for tunneling nanotube formation. *J Cell Sci* 126: 767 – 777

Scholkmann F, Seidel D, Funk RHW, Roehlecke C (2018) Ultrastructural features (bulges) of membrane nanotubes between cone-like photoreceptor cells: an investigation employing scanning electron microscopy. *Matters* https://doi.org/10.19185/matters.201802000011

Seyed-Razavi Y, Hickey MJ, Kuffová L, McMenamin PG, Chinnery HR (2013) Membrane nanotubes in myeloid cells in the adult mouse cornea represent a novel mode of immune cell interaction. *Immunol Cell Biol* 91: 89 – 95

Sharma M, Subramaniam S (2019) Rhes travels from cell to cell and transports Huntington disease protein via TNT-like protrusion. *J Cell Biol* 218: 1972 – 1993

Sherer NM, Lehmann MJ, Jimenez-Soto LF, Horensavitz C, Pypaert M, Mothes W (2007) Retroviruses can establish filopodial bridges for efficient cell-to-cell transmission. *Nat Cell Biol* 9: 310 – 315

Smirnov MS, Garrett TR, Yasuda R (2018) An open-source tool for analysis and automatic identification of dendritic spines using machine learning. *PLoS One* 13: e0199589

Smith IF, Shuai J, Parker I (2011) Active generation and propagation of Ca2+ signals within tunneling membrane nanotubes. *Biophys J* 100: L37 – L39

Souriant S, Balboa L, Dupont M, Pingris K, Kviatcovsky D, Cougoule C, Lastrucci C, Bah A, Gasser R, Poincloux R *et al* (2019) Tuberculosis exacerbates HIV-1 infection through IL-10/STAT3-dependent tunneling nanotube formation in macrophages. *Cell Rep* 26: 3586 – 3599.e7

Sousa AD, Berg JS, Robertson BW, Meeker RB, Cheney RE (2006) Myo10 in brain: developmental regulation, identification of a headless isoform and dynamics in neurons. *J Cell Sci* 119: 184 – 194

Sousa AD, Cheney RE (2005) Myosin-X: a molecular motor at the cell's fingertips. *Trends Cell Biol* 15: 533 – 539

Sowinski S, Jolly C, Berninghausen O, Purbhoo MA, Chauveau A, Köhler K, Oddos S, Eissmann P, Brodsky FM, Hopkins C *et al* (2008) Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. *Nat Cell Biol* 10: 211 – 219

Stamatakou E, Marzo A, Gibb A, Salinas PC (2013) Activity-dependent spine morphogenesis: a role for the actin-capping protein Eps8. *J Neurosci Off J Soc Neurosci* 33: 2661 – 2670

Stanganello E, Scholpp S (2016) Role of cytonemes in Wnt transport. *J Cell Sci* 129: 665 – 672

Staufer O, Hernandez BJE, Rustom A (2018) Protease-resistant cell meshworks: an indication of membrane nanotube-based syncytia formation. *Exp Cell Res* 372: 85 – 91

Sun YY, Bradley JM, Keller KE (2019) Phenotypic and functional alterations in tunneling nanotubes formed by glaucomatous trabecular meshwork cells. *Invest Ophthalmol Vis Sci* 60: 4583 – 4595

Takahashi A, Kukita A, Li Y, Zhang J, Nomiyama H, Yamaza T, Ayukawa Y, Koyano K, Kukita T (2013) Tunneling nanotube formation is essential for the regulation of osteoclastogenesis. *J Cell Biochem* 114: 1238 – 1247

Takeichi M (2007) The cadherin superfamily in neuronal connections and interactions. *Nat Rev Neurosci* 8: 11 – 20

Tang BL (2018) Unconventional secretion and intercellular transfer of mutant huntingtin. *Cells* 7: 59

Tardivel M, Bégard S, Bousset L, Dujardin S, Coens A, Melki R, Buée L, Colin M (2016) Tunneling nanotube (TNT)-mediated neuron-to neuron transfer of pathological Tau protein assemblies. *Acta Neuropathol Commun* 4: 117

Taulet N, Delorme-Walker VD, DerMardirossian C (2012) Reactive oxygen species regulate protrusion efficiency by controlling actin dynamics. *PLoS One* 7: e41342

Tavano S, Heisenberg C-P (2019) Migrasomes take center stage. *Nat Cell Biol* 21: 918 – 920

Thayanithy V, Dickson EL, Steer C, Subramanian S, Lou E (2014) Tumor-stromal cross talk: direct cell-to-cell transfer of oncogenic microRNAs via tunneling nanotubes. *Transl Res J Lab Clin Med* 164: 359 – 365

Thayanithy V, O'Hare P, Wong P, Zhao X, Steer CJ, Subramanian S, Lou E (2017) A transwell assay that excludes exosomes for assessment of tunneling nanotube-mediated intercellular communication. *Cell Commun Signal CCS* 15: 46

Valdebenito S, Lou E, Baldoni J, Okafo G, Eugenin E (2018) The novel roles of connexin channels and tunneling nanotubes in cancer pathogenesis. *Int J Mol Sci* 19: 1270

Vargas JY, Grudina C, Zurzolo C (2019a) The prion-like spreading of α-synuclein: From in vitro to in vivo models of Parkinson's disease. *Ageing Res Rev* 50: 89 – 101

Vargas JY, Loria F, Wu Y-J, Córdova G, Nonaka T, Bellow S, Syan S, Hasegawa M, van Woerden GM, Trollet C *et al* (2019b) The Wnt/Ca2+ pathway is involved in interneuronal communication mediated by tunneling nanotubes. *EMBO J* 38: e101230

Veranic P, Lokar M, Schütz GJ, Weghuber J, Wieser S, Hägerstrand H, Kralj-Iglic V, Iglic A (2008) Different types of cell-to-cell connections mediated by nanotubular structures. *Biophys J* 95: 4416 – 4425

© 2021 The Authors

Victoria GS, Zurzolo C (2017) The spread of prion-like proteins by lysosomes and tunneling nanotubes: Implications for neurodegenerative diseases. *J Cell Biol* 216: 2633 – 2644

Vignais M-L, Caicedo A, Brondello J-M, Jorgensen C (2017) Cell connections by tunneling nanotubes: effects of mitochondrial trafficking on target cell metabolism, homeostasis, and response to therapy. *Stem Cells Int* 2017: 6917941

Vignjevic D, Kojima S, Aratyn Y, Danciu O, Svitkina T, Borisy GG (2006) Role of fascin in filopodial protrusion. *J Cell Biol* 174: 863 – 875

Wang X, Bukoreshtliev NV, Gerdes H-H (2012) Developing neurons form transient nanotubes facilitating electrical coupling and calcium signaling with distant astrocytes. *PLoS One* 7: e47429

Wang X, Gerdes H-H (2015) Transfer of mitochondria via tunneling nanotubes rescues apoptotic PC12 cells. *Cell Death Differ* 22: 1181 – 1191

Wang X, Veruki ML, Bukoreshtliev NV, Hartveit E, Gerdes H-H (2010) Animal cells connected by nanotubes can be electrically coupled through interposed gap-junction channels. *Proc Natl Acad Sci USA* 107: 17194 – 17199

Wang Y, Cui J, Sun X, Zhang Y (2011) Tunneling-nanotube development in astrocytes depends on p53 activation. *Cell Death Differ* 18: 732 – 742

Watkins SC, Salter RD (2005) Functional connectivity between immune cells mediated by tunneling nanotubules. *Immunity* 23: 309 – 318

Whitehead J, Zhang J, Harvestine JN, Kothambawala A, Liu G, Leach JK (2020) Tunneling nanotubes mediate the expression of senescence markers in mesenchymal stem/stromal cell spheroids: TNTs mediate senescence in MSC spheroids. *Stem Cells* 38: 80 – 89

Wilson C, González-Billault C (2015) Regulation of cytoskeletal dynamics by redox signaling and oxidative stress: implications for neuronal development and trafficking. *Front Cell Neurosci* 9: 381

Wittig D, Wang X, Walter C, Gerdes H-H, Funk RHW, Roehlecke C (2012) Multi-level communication of human retinal pigment epithelial cells via tunneling nanotubes. *PLoS One* 7: e33195

Yamashita YM, Inaba M, Buszczak M (2018) Specialized intercellular communications via cytonemes and nanotubes. *Annu Rev Cell Dev Biol* 34: 59 – 84

Yang H, Borg TK, Ma Z, Xu M, Wetzel G, Saraf LV, Markwald R, Runyan RB, Gao BZ (2016) Biochip-based study of unidirectional mitochondrial transfer from stem cells to myocytes via tunneling nanotubes. *Biofabrication* 8: 15012

Yasuda K, Khandare A, Burianovskyy L, Maruyama S, Zhang F, Nasjletti A, Goligorsky MS (2011) Tunneling nanotubes mediate rescue of prematurely senescent endothelial cells by endothelial progenitors: exchange of lysosomal pool. *Aging* 3: 597 – 608

Zani BG, Indolfi L, Edelman ER (2010) Tubular bridges for bronchial epithelial cell migration and communication. *PLoS One* 5: e8930

Zhang C, Scholpp S (2019) Cytonemes in development. *Curr Opin Genet Dev* 57: 25 – 30

Zhang J, Whitehead J, Liu Y, Yang Q, Leach JK, Liu G-Y (2018) Direct observation of tunneling nanotubes within human mesenchymal stem cell spheroids. *J Phys Chem B* 122: 9920 – 9926

Zhang N, Bing T, Shen L, Song R, Wang L, Liu X, Liu M, Li J, Tan W, Shangguan D (2016) Intercellular connections related to cell-cell crosstalk specifically recognized by an aptamer. *Angew Chem Int Ed Engl* 55: 3914 – 3918

Zhang Y (2011) Tunneling-nanotube: A new way of cell-cell communication. *Commun Integr Biol* 4: 324 – 325

Zhu D, Tan KS, Zhang X, Sun AY, Sun GY, Lee JC-M (2005) Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes. *J Cell Sci* 118: 3695 – 3703

Zhu S, Bhat S, Syan S, Kuchitsu Y, Fukuda M, Zurzolo C (2018) Rab11a–Rab8a cascade regulates the formation of tunneling nanotubes through vesicle recycling. *J Cell Sci* 131: jcs215889

Zhu S, Victoria GS, Marzo L, Ghosh R, Zurzolo C (2015) Prion aggregates transfer through tunneling nanotubes in endocytic vesicles. *Prion* 9: 125 – 135

Zurzolo C (2020) Evidence that tunnelling nanotube-like structures connect cells in mice. *Nature* 585: 32 – 33

 © 2021 The Authors