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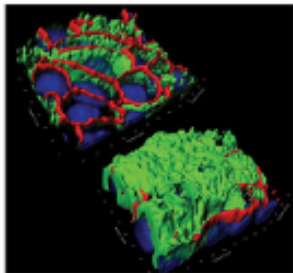
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Tunneling nanotubes

Diversity in morphology and structure

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Tunneling nanotubes (TNTs) are recently discovered thin membranous tubes that interconnect cells. During the last decade, research has shown TNTs to be diverse in morphology and composition, varying between and within cell systems. In addition, the discovery of TNT-like extracellular protrusions, as well as observations of TNTs *in vivo*, has further enriched our knowledge on the diversity of TNT-like structures. Considering the complex molecular mechanisms underlying the formation of TNTs, as well as their different functions in intercellular communication, it is important to decipher how heterogeneity of TNTs is established, and to address what roles the compositional elements have in the execution of various functions. Here, we review the current knowledge on the morphological and structural diversity of TNTs, and address the relation between the formation, the structure, and the function of TNTs.

In 2004, Rustom and colleagues reported *in vitro* findings of a thin structure connecting single cells over long distances, which facilitated the transfer of membrane vesicles.¹ This structure, coined a tunneling nanotube (TNT), was hovering above the substrate, and contained a straight, continuous actin rod enclosed in a lipid bilayer. TNTs and similar structures have since been reported in many different cell systems²⁻⁷ and have been shown to act as conduits for intercellular transfer of a range of cellular compounds^{1,8} and transmission of depolarization signals.⁹⁻¹² Furthermore, TNTs have been shown to be involved in the spread of pathogens^{3,7} and transfer of aberrant cellular proteins responsible for disease, such as prions and misfolded huntingtin.^{13,14} In addition to this wide range of functions, studies performed during the last decade have revealed a high level of heterogeneity in TNT morphology and structure, even within the same cell line.³ In this review, we summarize current knowledge on the diversity of TNTs and TNT-like structures, and address the relationship between their structure and function.

Diversity of the Morphology and Composition of TNTs

To date, no TNT-specific protein markers are known. Therefore, morphological properties remain the main criteria for TNT identification. The property that most clearly separates TNTs from other cellular protrusions *in vitro* is their straight, bridge-like structure, interconnecting cell pairs. *In vitro* imaging has shown that the length of TNTs displays large variation, differing between cell lines (Table 1). TNTs connecting T cells, for example, were reported to have an average length of 22 μm ,⁷ whereas in PC12 cells, the length was found to be much less.¹ The TNT lengths can vary as the connected cells migrate and the distances between them change, indicating that TNT length can be dynamically regulated. In addition, some cells show a negative correlation between the TNT lifetime and the cell migration speed.⁷ TNTs break when the intercellular gap becomes too large. Therefore, statistical analysis of TNT length will provide information about the effective distance for TNT formation, and also the threshold distance for TNT-dependent cell-to-cell communication.

Measuring the diameter of TNTs using light microscopy cannot be done with adequate accuracy due to the resolution limit. So far, electron microscopy is still the best method for diameter measurements. Transmission electron microscopy analysis has revealed that TNTs have a diameter in the range of 50–200 nm in PC12 cells and 180–380 nm in T cells (Table 1).^{1,7} However, to preserve and search for intact TNTs in series of sample slices is laborious. An alternative solution is to measure the diameter of TNTs using scanning electron microscopy.^{1,11} Confocal microscopy has shown that some TNTs reach thicknesses of over 700 nm, which could be due to incorporation of additional components inside the TNTs, such as microtubules.³ It should also be noted that multiple thin TNTs could stick together to form what looks like a single, thick TNT (unpublished data). Since cells after division sometimes form transient thin intercellular connections containing a midbody ring, a double labeling can help to distinguish TNTs from incompletely divided cells.

TNTs are not empty membrane tubes, but filled with cytoskeletal filaments (Table 1). F-actin is found in most TNTs, spanning uniformly along their entire length,¹ and is thus an important labeling target in TNT-imaging. F-actin also plays a crucial role in the formation of TNTs, as shown in experiments where incubation with F-actin depolymerization drugs, such as

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Table 1. The diversity of TNTs

Cell type	Length	Thickness	Cytoskeleton	Membrane detection	Refs
PC12	Avg. 6 μm *	50 - 200 nm	actin, no microtubules	WGA-staining, SEM	1
HEK293	N/A	< 500 nm	actin, no microtubules	GFP-PrPwt-transfection	41
Jurkat T cells	Avg. 22 μm , max 100 μm	< 380 nm	actin, no microtubules	DiD-staining, TEM	7
ARPE-19	Avg. 44 μm , max 120 μm	50 - 300 nm	actin, no microtubules	DIC, WGA-staining, SEM	11
NRK	Max 70 μm	N/A	actin, no microtubules	DIC, WGA-staining, SEM	2,12
HeLa	Avg. 17.7 μm , max 40 μm	N/A	actin, no microtubules	WGA-staining	35
Cardiac myoblast H9c2 cell	Max 100 μm	< 1000 nm (AFM)	actin and microtubules	DIC, DiD-staining	42
Human lung carcinoma A549	Max 105 μm	400 - 1500 nm	actin and microtubules	Brightfield	43
Human monocyte-derived macrophages	N/A	700 nm	actin, microtubules **	Brightfield	3
Primary neurons and astrocytes	Avg. 7.1 μm	N/A	microtubules, actin ***	DIC	9

WGA, wheat germ agglutinin; DiD, Vybrant® DiD cell labeling solution; SEM, scanning electron microscopy; TEM, transmission electron microscopy; DIC, differential interference contrast; AFM, atomic force microscopy. * Unpublished data; ** All nanotubes contained actin, and a subgroup also contained microtubules; *** All nanotubes contained microtubules, and a subgroup also contained actin (65%).

cytochalasin B, inhibit TNT formation.¹⁵ In addition, evidence show that various cellular components are transported inside TNTs in the speed range of F-actin-associated myosin-motors.⁸ Besides F-actin, microtubules are also detected in TNTs in a few cell lines, such as immune cells,³ between primary neurons and astrocytes,⁹ and in HUVEC cells during cancer-induced angiogenesis.¹⁶ Why and how microtubules are present in some TNTs remains to be investigated. As with F-actin and myosin, microtubules could serve as tracks for transport of cargo via a kinesin/dynein-mechanism. Furthermore, microtubule-filaments have shown a bending stiffness many orders of magnitude higher than that of actin-filaments.¹⁷ Thus, incorporation of microtubules could provide a high degree of rigidity and longer lifetime to the TNT.

Transmembrane proteins and membrane-binding proteins, such as N-cadherin and Myosin X, are considered necessary in the recognition and attachment to target cells during TNT formation (unpublished data).^{18,19} In addition, membrane proteins are also important in mediating TNT function. For example, interposed gap junctions on the TNT/cell-contact site allow transmission of depolarization signals.¹² Moreover, the accumulation of MHC class I chain-related protein A (MICA) at the tip of nanotubes of natural killer cells can induce immune responses in target cells.²⁰ In T cells, the transfer of endogenous FasL from effector cells can result in apoptosis in the receiving cell.²¹ Finally, certain membrane components could accumulate passively along TNTs, thus becoming a potential marker for the imaging of TNT-like structures. In support of this theory, experiments with liposomes have shown that both specific proteins and lipids can be sorted spontaneously into artificial nanotubes.^{22,23}

TNT-Like Structures

In addition to the TNTs described above, some extracellular protrusions can be considered “TNT-like,” as they share some of the characteristics of TNTs (Table 2). Besides filopodia, cytoneme is arguably characterized the best. Cytonemes are

long and thin protrusions containing F-actin found in the *Drosophila* imaginal disc. They facilitate peripheral uptake and subsequent transport of extracellular signaling molecules toward the cell body.²⁴ It is not clear if these protrusions attach to other cells like TNTs do, or if they just act as periscope-like sensors. Another TNT-like structure was observed in cultured B cells upon antibody-opsinization.²⁵ These protrusions, called streamers, form within two minutes of antibody exposure. The streamers also contain F-actin, and do not form when incubated with the F-actin depolymerization drug cytochalasin D.^{25,26} However, the streamers, like cytonemes, do not necessarily attach to other cells. When they do make contact to other cells, evidence suggests that this confers a protective effect to complement-mediated cell lysis.²⁶ In 2011, a new kind of thin extracellular protrusions, called a nanopodium, was reported.²⁷ Nanopodia emanate from endothelial cells expressing the tetraspanin-like protein TM4SF1, which is also found in puncta along the protrusions. Being relatively deficient in F-actin, and also not necessarily attached to other cells, their function in intercellular communication is uncertain. Although the morphology of the structures discussed above resembles TNTs closely, they usually do not interconnect cells. However, it is reasonable to believe that the cell employs much of the same machinery to form these structures, and some could possibly be TNT-precursors under certain conditions since TNTs can derive from filopodia.¹⁹

TNTs in Tissue

The search for TNTs in tissue is important not only to establish their presence in multicellular organisms, but also as a key step to understand their physiological functions. The development of fluorescent protein tags and advanced confocal microscopy have facilitated TNT identification in vivo by enabling the labeling of specific proteins only present in a certain subpopulation of cells. In chick embryos, TNT-like structures were successfully identified between neural crest

cells after this specific cell population had been labeled with fluorescent fusion proteins.^{28,29} There are similar findings from gastrulation-stage embryos,³⁰ and in immune cells in the adult mouse cornea.^{31,32} TNTs between neural crest cells have been shown to mediate the transfer of cellular material, indicating that TNTs play a functional role also *in vivo*.²⁸ To date, most of the reports on TNTs *in vivo* are studies on embryos, which might hint at a role of TNTs during the development of multicellular organisms.³³

Although the TNTs in tissue share many morphological features with TNTs *in vitro*, there are still dissimilarities that can be ascribed to the complex microenvironment *in vivo*. For instance, the TNTs observed *in vivo* are often not straight. Contorted TNTs have been found both in healthy tissue, such as in the mouse cornea,³² as well as in tumor tissue.⁶ This effect could possibly be due to obstacles, such as other cells and dense extracellular matrix, preventing the protrusion from connecting at the shortest distance between the two cells.⁷ Indeed, when there are no obstacles between the cells, such as between the rims of the neural folds during neural tube closure, straight TNT-like structures can be found *in vivo*.³⁴ In addition, extracellular matrix could in principle provide structural stability and protection from external forces when it is surrounding the TNTs. In a recent paper elaborating on TNTs in the cornea, the lifetime was measured to be more than 90 min.³² A study on primary T cells inside an artificial 3D matrix revealed long-lived TNTs with curved morphologies, in accordance with *in vivo* studies.⁷ Due to the difficulty of imaging TNTs *in vivo*, details about the composition and structure of TNTs in complex tissue environments remain to be investigated. Certainly, such research will expand our knowledge about TNTs, and may also give information about their function and the mechanism of formation in tissue.

Diversity in the Formation and Function of TNTs

The heterogeneous morphology and composition of TNTs suggests that TNTs may form in different ways. According to live imaging of cultured cells, the formation of TNTs occurs by filopodial interplay (“making contact”) or cell dislodgement (“keeping contact”).³³ Recent research on the molecular level has revealed that the unconventional motor protein myosin X, usually associated with filopodia, promotes TNT formation by interacting with several transmembrane proteins, supporting the notion that TNTs can derive from filopodia (unpublished data).¹⁹ Moreover, the M-sec protein has been demonstrated to be an important regulator of TNT formation.³⁵ It was also involved in p53 and MHC class III protein LST1 induced TNT formation.^{36,37} However, recent observation that p53 is not a master protein for TNT formation in every cell type, and the fact that TNTs have been observed between cells that do not express M-sec, suggest that different mechanisms of TNT formation may exist in different cell types.^{19,38} Therefore, there may be a certain level of one-to-one relationship between the diversity of TNTs and the molecular determinants of their formation.

Table 2. TNTs and TNT-like structures

Name	Thickness	Actin	Structural connectivity	Above substratum	Hypothesized functional role	Refs
TNTs	50–200 nm	Yes	Yes	Yes	Yes	1
Filopodia	100–300 nm	Yes	No	Yes/No	Yes	44
Cytoneme	200 nm	Yes	Unknown	N/A, Tissue only	Yes	24, 45
Streamers	“very thin”	Yes	No	N/A	Yes	26
Nanopodia	100–300 nm	No	No	No	No	27

Several studies support the notion of a correlation between the structure and the function of TNTs. An interesting example is the TNT-dependent propagation of calcium-fluxes between cells, for which at least three ways have been described in different cell lines. First, TNT-mediated electrical coupling can transfer depolarization from one cell to another via interposed gap junctions, and thus elicit a depolarization-dependent calcium uptake in the connected cell through voltage-gate channels.^{10,12} Second, $\text{Ins}(1,4,5)\text{P}_3$ receptors bound to the endoplasmic reticulum inside TNTs actively propagate intercellular calcium signals along TNTs via calcium-induced calcium release.³⁹ Lastly, calcium itself can diffuse directly through TNTs, thereby rising calcium level in the receiving cell.^{11,40} Obviously, in mediating this calcium flux, the TNTs need to fulfill certain structural requirements. In the first model, the presence of gap junctions on the contact site between the TNT and the target cell is necessary to transfer electrical depolarization. In the two latter models, a TNT with a larger luminal diameter is required to allow the entry of endoplasmic reticulum or efficient diffusion of calcium from one cell to another. In addition, motor proteins might be necessary to mediate the entry of endoplasmic reticulum into TNTs. Thus, the structural and compositional properties of the TNTs determine the possibilities and limitations of their functions that can be performed.

Future research on TNTs will inevitably increase our knowledge about the morphological and structural diversity of TNTs during embryo development, tissue homeostasis, and pathological processes. However, to resolve the molecular mechanisms underlying the diversity, as well as to depict the genetic, cellular and species-specific variation, represents a great challenge. In addition, to determine the relationship between the diversity and the different functions of the TNTs, particular at the tissue level, is an important task in this research field.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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