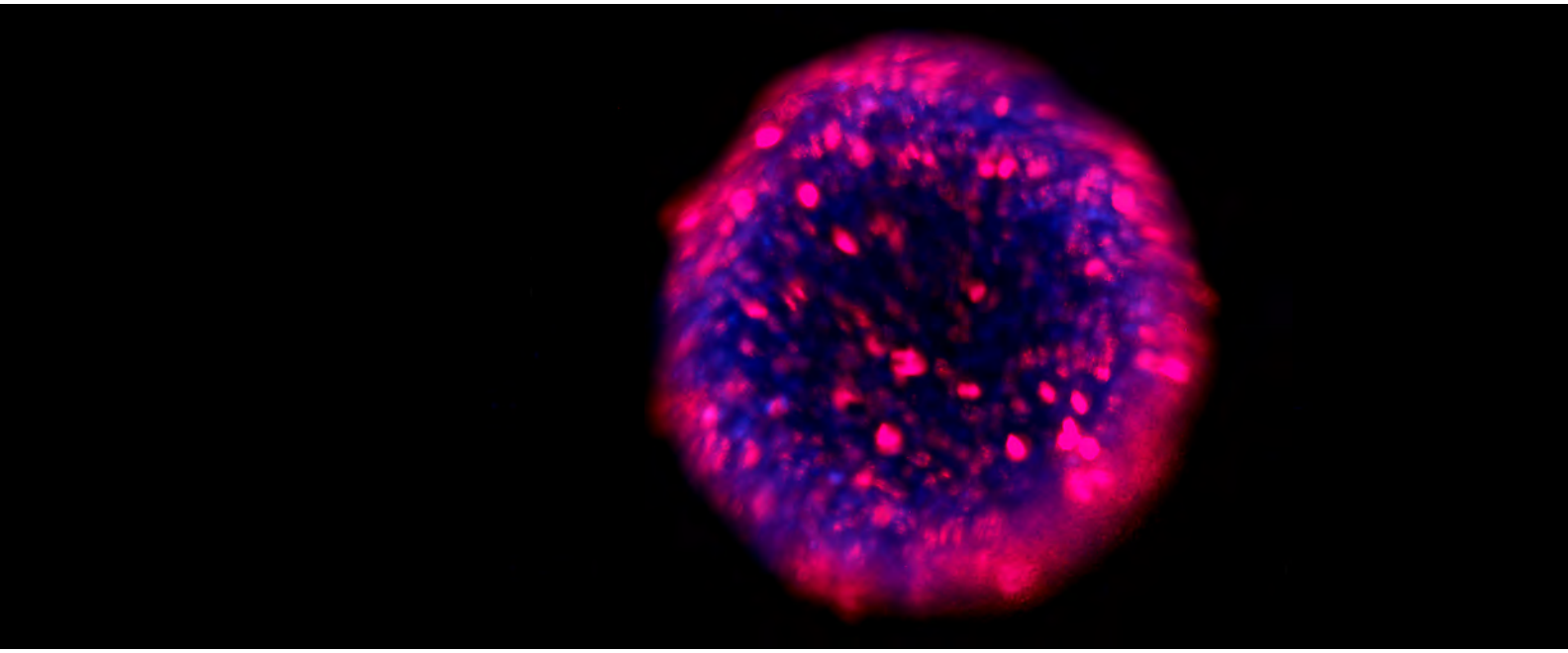

Elucidate the underlying mechanisms of Parkinson's disease and other neurological disorders

Fixed and live cell research tools for neuronal protein and structural analyses.

Parkinson's disease (PD) is a debilitating, chronic, and progressive neurological disease that affects around 10 million people worldwide. PD is accompanied by loss of motor function and, without a curative treatment, care remains palliative. Early diagnosis of PD and similar neurodegenerative diseases is difficult because of their common symptoms and pathophysiology and the lack of understanding of their distinguishing molecular characteristics. Thermo Fisher Scientific is committed to developing research tools for neuroscientists investigating the molecular mechanisms of PD and other neurodegenerative diseases using *in vitro*, *ex vivo*, and *in vivo* experimental systems.

Figure 1 (above). Tubulin Tracker Deep Red as a marker of neurosphere maturation. Neural stem cell (NSC)-derived neurospheres were cultured using Gibco™ Neurobasal™ Plus Medium (Cat. No. A3582901) with Gibco™ CultureOne™ Supplement (Cat. No. A3320201) in Thermo Scientific™ Nunclon™ Sphera™ 96 U-Well Microplates. The neurospheres were then stained with Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (Cat. No. R37605) and Invitrogen™ Tubulin Tracker™ Deep Red (Cat. No. T34077) to assess neurosphere maturation. Image was generated using an Invitrogen™ EVOS™ FL Auto 2 Imaging System with a 20x objective and an Invitrogen™ EVOS™ Cy®5 Light Cube.

In general the etiology of PD and many other neurodegenerative disorders, including Alzheimer's disease (AD), is poorly understood. The loss of motor function associated with PD has led researchers to investigate the degeneration of dopaminergic neurons in the substantia nigra [1,2]. Key molecular mechanisms implicated in this neurodegeneration can be broadly classified into categories that include mitochondrial dysfunction, genetic mutations, and defects in protein clearance pathways that lead to protein aggregation, formation of neuronal inclusion bodies, and ultimately neuronal deterioration [2-5].

Historically, diagnosis has relied upon the identification of protein aggregates (e.g., Lewy bodies, in the case of PD) that occur in the later stages of the disease. Neuronal inclusion body formation, defined by the presence of these insoluble protein aggregates, is responsible for the breakdown of neuronal microtubules, a causative event of neuronal diseases [1-5]. Two proteins commonly identified in neurodegenerative diseases—also referred to as proteinopathies due to protein misfolding—are tau and synuclein [5]. The proteinopathy of PD is primarily attributed to alpha-synuclein, the main structural component of Lewy bodies in nerve cells, which are a defining characteristic of not only PD but also Lewy body dementia (LBD) and other disorders. Although tau aggregation and the formation of amyloid plaques have been more extensively described in AD, tau has also been linked with alpha-synuclein as an interdependent partner in the progression of PD [2-6].

Although the protein–protein interactions that influence formation of inclusion bodies and plaques have been studied at the molecular level, the underlying causes of the degenerative pathophysiology are still unknown. Obviously, there is a need for more appropriate models and reagents to elucidate the principal mechanisms and indicators for the onset and progression of PD. In the first half of this article, we give an overview of some key proteins involved in PD and other neurodegenerative diseases, and the Invitrogen™ primary antibodies available →

Table 1. Recommended antibodies for detecting heat shock proteins (HSPs), from Thermo Fisher Scientific.

Target protein	Cat. No.
HSP40 (polyclonal)	PA5-17382
HSP60 (clone 4B9/89 or clone 2E1/53)	MA3-012, MA3-013
HSP60 (polyclonal)	PA5-34760
HSP70 (clone 4G4)	MA3-009
HSP70 (clone 2A4)	MA3-008
HSP90 alpha (clone 3B6)	MA3-010
HSP90 beta (clone H9010)	37-9400
Phospho-HSP27 (Ser15, polyclonal)	PA1-018
Phospho-HSP27 (Ser85, polyclonal)	PA1-005

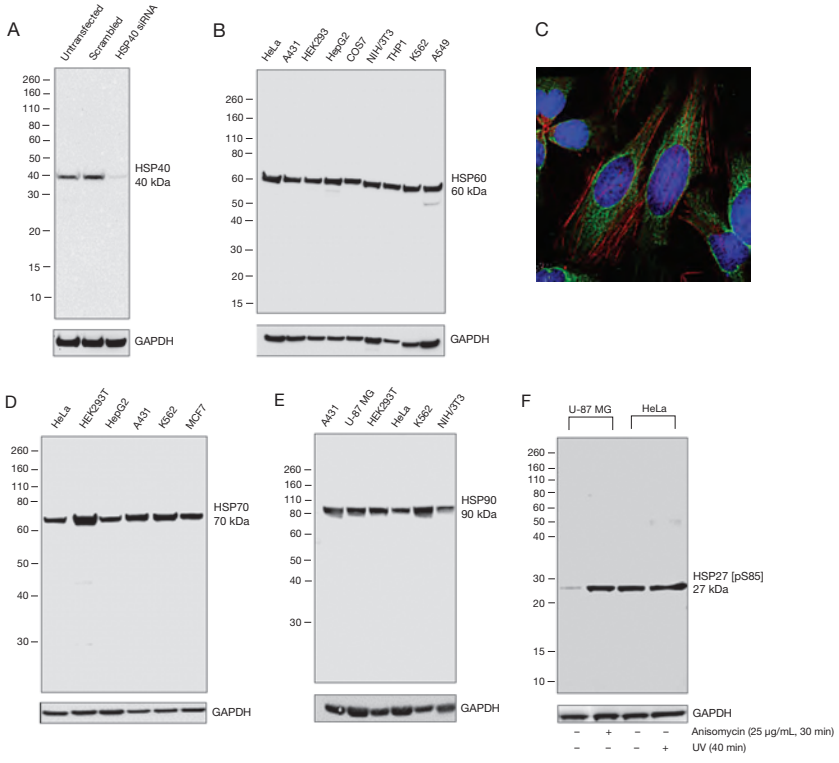

Figure 2. Assessment of heat shock protein (HSP) antibody specificity. (A) Western blot analysis of HSP40 antibody (Cat. No. PA5-17382) using RNAi. **(B)** Western blot analysis across multiple cell lines and **(C)** immunofluorescence analysis in HeLa cells using HSP60 antibody (clone 2E1/53, Cat. No. MA3-013). For the immunofluorescence analysis, HeLa cells were labeled with HSP60 antibody (green) and counterstained with rhodamine phalloidin (red, Cat. No. R415) and DAPI (blue, Cat. No. D1306). **(D)** Western blot analysis of HSP70 (clone 2A4, Cat. No. MA3-008) and **(E)** HSP90 (clone 3B6, Cat. No. MA3-010) antibodies across multiple cell lines. **(F)** Western blot analysis of phospho-HSP27 (Ser85) antibody (Cat. No. PA1-005) in cells subjected to anisomycin or UV, both known to activate HSPs. For the western blot analyses, primary antibodies were probed with Invitrogen™ Goat Anti–Rabbit IgG or Goat Anti–Mouse IgG Superclonal™ Recombinant Secondary Antibody, HRP (Cat. No. A27036 or Cat. No. A28177), detected using the Thermo Scientific™ Pierce™ ECL Western Blotting Substrate (Cat. No. 32106), and imaged using the Invitrogen™ iBright™ FL1000 Imaging System.

to study these targets (Figures 2–5, Tables 1–3). In the second half, we highlight recently developed fluorescent tubulin-selective probes that can be used in live neurons to further reveal the relationships between neuronal protein aggregation and microtubules, as well as the possible roles of this association in neuronal disease.

Antibody detection of molecular chaperones in PD

Several proteins have emerged as key players in signaling pathways associated with various stages of PD, including early onset, and late-stage progression. Cellular defense mechanisms inherent to neurons play a critical role in preventing protein aggregation and maintaining cellular homeostasis. Molecular chaperones, also known as heat shock proteins (HSPs), have gained attention in PD research for their protective nature against misfolded proteins, preventing their aggregation.

HSPs are constitutively expressed or inducibly regulated in a number of different cell types, and disruption in their function can quickly lead to the accumulation of misfolded proteins and eventually cell death. These proteins, therefore, are being investigated as a means both to understand PD disease biology and to study their potential role in disease treatment. The functions of several HSPs (including HSP27, HSP40, HSP60, HSP70, and HSP90) that are localized to axons or synapses and associated with alpha-synuclein are found to be compromised in PD [7-9]. Figure 2 shows the validation* data for representative Invitrogen™ HSP antibodies, and Table 1 provides a list of antibodies against several members of the HSP family.

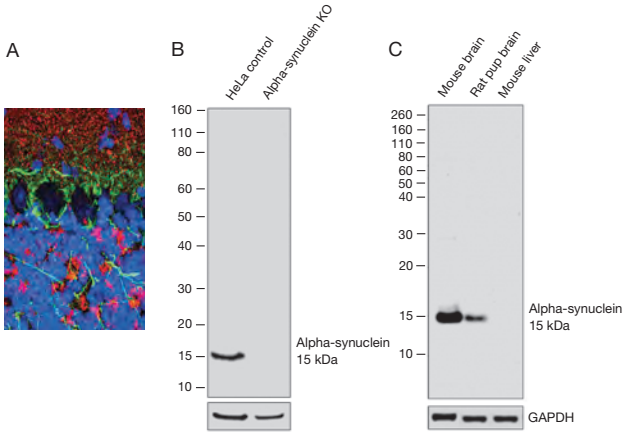

Figure 3. Assessment of alpha-synuclein antibody specificity. (A) Immunofluorescence analysis in mouse brain tissue using alpha-synuclein polyclonal antibody (red, Cat. No. PA5-17239) and a neurofilament-L monoclonal antibody (green); cells were counterstained with DAPI (blue). Western blot analysis of alpha-synuclein antibody using **(B)** CRISPR knockout (KO) of alpha-synuclein in HeLa cells and **(C)** relative expression of alpha-synuclein in brain and liver. Western blot detection was performed as described in Figure 2.

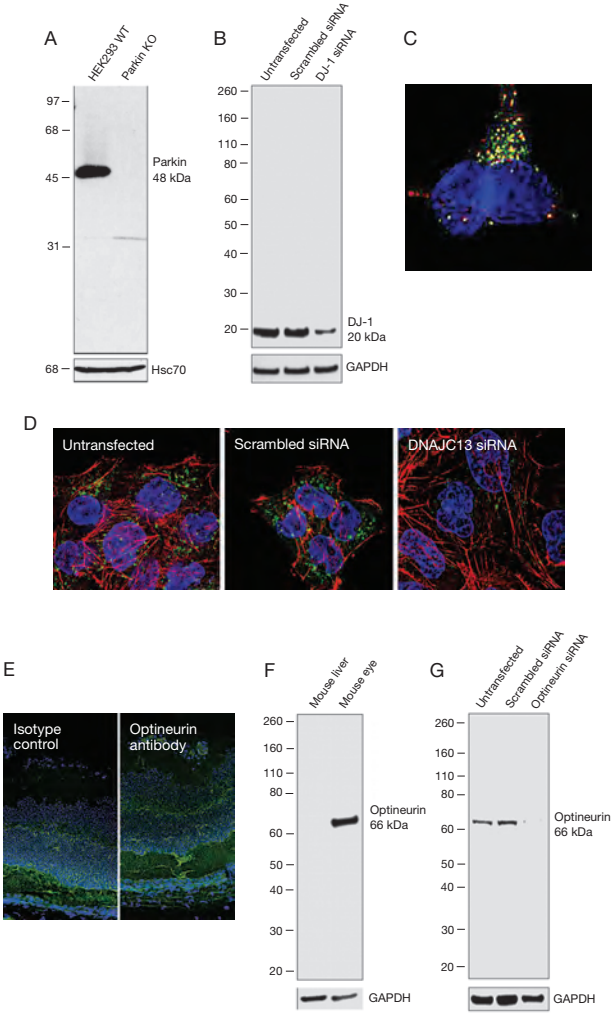

Figure 4. Assessment of antibody specificity. Western blot analysis of **(A)** parkin (clone 21H24L9, Cat. No. 702785) and **(B)** DJ-1 (Cat. No. PA5-13404) antibodies to show specificity using CRISPR knockout (KO) and RNAi, respectively. **(C)** Immunofluorescence analysis in HepG2 cells labeled with LIMP2 antibody (green; clone 22H6L14, Cat. No. 703037) and Invitrogen™ LysoTracker™ Red DND-99 (red, Cat. No. L7528); cells were counterstained with DAPI (blue). **(D)** Immunofluorescence analysis of DNAJC13 antibody (green; clone 11H13L5, Cat. No. 702773) in A431 cells using RNAi; cells were counterstained with rhodamine phalloidin (red) and DAPI (blue). **(E)** Immunofluorescence analysis of optineurin in mouse retina using an isotype control (green) or optineurin recombinant polyclonal antibody (green, Cat. No. 711879); cells were counterstained with DAPI (blue). Western blot analysis of the optineurin antibody in **(F)** liver and eye lysates and **(G)** optineurin siRNA–silenced NIH/3T3 cells. Western blot detection was performed as described in Figure 2.

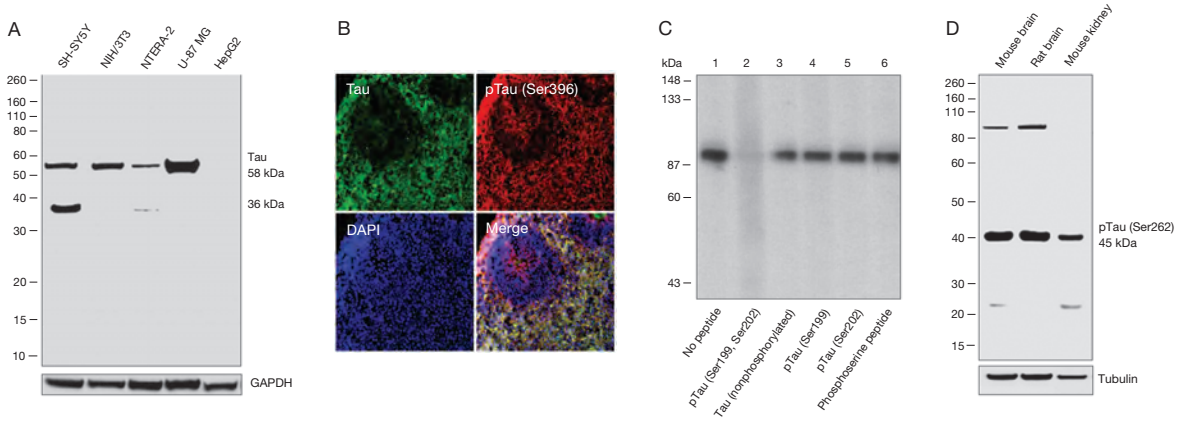

Figure 5. Assessment of tau antibody specificity. (A) Western blot analysis of tau antibody (clone TAU-5, Cat. No. MA5-12808) across multiple cell lines. **(B)** Immunofluorescence analysis in human iPSC-derived forebrain organoids at day 40 using tau antibody (green; clone TAU-5, Cat. No. AHB0042) and phospho-tau (Ser396) (red, Cat. No. 44-752G), and counterstained with DAPI (blue); data used with permission from Zhexing Wen, Emory University. **(C)** Western blot analysis of the specificity of phospho-tau (Ser199, Ser202) antibody (Cat. No. 44-768G) binding in the presence of the specific phosphopeptide target (lane 2), the corresponding nonphosphorylated peptide (lane 3), and other phosphoserine-containing peptides (lanes 4–6); signal loss was only seen with the specific peptide. **(D)** Western blot analysis of phospho-tau (Ser262) antibody (Cat. No. 44-750G) in mouse brain, rat brain, and mouse kidney lysates. For the western blot analyses, primary antibodies were probed with Invitrogen™ Goat Anti–Rabbit IgG or Goat Anti–Mouse IgG Secondary Antibody, HRP (Cat. No. G21234 or Cat. No. 62-6520), detected using the Thermo Scientific™ Pierce™ ECL Western Blotting Substrate (Cat. No. 32106), and imaged using the Invitrogen™ iBright™ FL1000 Imaging System.

Antibody detection of aggregation markers in PD

The accumulation of alpha-synuclein, one of the main structural components of Lewy bodies, is a hallmark of the latter stages of PD [10]. Although its specific role in the neuronal degeneration process is not fully understood, studies have suggested that it is involved in aggregation of misfolded protein that eventually converges upon dopaminergic neurons in the form of Lewy bodies [6,10,11]. Figure 3 demonstrates validation data for the Invitrogen™ alpha-synuclein antibody, which was tested using different parameters to ensure its specificity.

A growing number of genes associated with PD are related to mitochondrial function and oxidative stress, emphasizing the role of mitochondria in neuronal degeneration and disease progression [12]. Similarly, impairment of lysosome-mediated protein degradation via autophagy or endocytosis directly impacts the ability of neurons to clear alpha-synuclein aggregates in Lewy bodies [13,14].

Table 2. Recommended antibodies for Parkinson's disease research, from Thermo Fisher Scientific.

Target protein	Protein function	Cat. No.
Ataxin3	Protein clearance	711823
DENND5A	Vesicle-mediated transport and RAB-activating guanine nucleotide exchange factor (GEF)	702789
DJ-1 (Park7)	Molecular chaperone	PA5-13404
DNAJC13 (RME-8)	Membrane trafficking	702773
DNML1	Clathrin-mediated endocytosis	702782
LIMP2	Lysosomal membrane protein	702770
Mitofusin-2 (MFN2)	Mitochondrial maintenance	711803
Optineurin	Membrane trafficking	711879
Alpha-synuclein	Regulation of neurotransmitter release	PA5-17239
TMEM230	Synaptic vesicle recycling	702792
VAMP1	Vesicular transport	702787

Table 3. Recommended tau antibodies, from Thermo Fisher Scientific.

Target protein	Cat. No.
Tau (clone HT7)	MN1000, MN1000B (biotin conjugate)
Tau (clone TAU-5)	AHB0042, MA5-12808, MA5-12805 (biotin conjugate)
Tau (clone T46)	13-6400
Phospho-tau (Ser199, Ser202)	44-768G
Phospho-tau (Ser202, Thr205) (clone AT8)	MN1020, MN1020B (biotin conjugate)
Phospho-tau (Thr231) (clone AT180)	MN1040
Phospho-tau (Thr212, Ser214) (clone AT100)	MN1060

Several proteins involved in normal mitochondrial or lysosomal functioning, such as parkin, LIMP2, and PINK1 [15], are found to be mutated in PD. As a result, affected cells are subjected to increased oxidative stress and eventually cell death. Additionally, mutations in proteins →

such as DNAJC13 (required for receptor-mediated endocytosis) and optineurin (important for maintenance of the Golgi complex and membrane trafficking) have been identified as contributing to PD pathology [16,17]. In Figure 4, we showcase representative validation data for antibodies against several of these targets that can be used across applications, including immunofluorescence and western blotting. Table 2 provides a list of antibodies against a variety of important PD targets.

The link between alpha-synuclein and tau in PD

Tau, a member of the microtubule-associated protein family (MAPT), is associated with microtubule stabilization, membrane binding, and axonal transport [17-20]. Under normal physiological conditions, tau is soluble and unfolded; however, under pathological conditions in PD or AD, misfolding of tau leads to insoluble neurofibrillary tangles (NFTs)—a pathological effect called a tauopathy [4-6,21]. In such pathology, hyperphosphorylation of tau causes protein aggregation and accumulation, which in turn damages neurons and disrupts axonal transport [17,18,21]. Although alpha-synuclein dysregulation is a possible cause of PD, it has recently been reported that both alpha-synuclein and tau are present in the same Lewy body aggregate in many postmortem brains with PD and dementia [4-6]. Consequently, clinicians and researchers alike are focusing on the link between tau and alpha-synuclein in PD at the molecular level [4-6,10]. Invitrogen primary antibodies against total tau as well as several important phosphorylated forms have been published and highly cited in publications. Figure 5 shows representative validation data for some of these antibodies, and Table 3 provides a list of antibodies against these important PD targets.

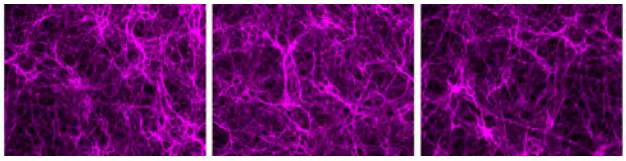

Figure 6. Live rat cortical neurons labeled with Tubulin Tracker Deep Red. Gibco™ Primary Rat Cortex Neurons (Cat. No. A1084001) were prepared and grown using the Gibco™ B-27™ Plus Neuronal Culture System (Cat. No. A3653401) and Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061) for 14 days. Cells were then stained with Invitrogen™ Tubulin Tracker™ Deep Red (Ex/Em = 652/669 nm, Cat. No. T34077) and imaged in Invitrogen™ Live Cell Imaging Solution (Cat. No. A14291DJ) on an Invitrogen™ EVOS™ FL Auto 2 Imaging System with a 20x objective and an Invitrogen™ EVOS™ Cy®5 Light Cube.

Relationship between tau and the microtubule cytoskeleton

Recently, tau aggregation and spreading into naive neurons has been shown to occur through tunneling nanotubes (TNTs) [8,18,20,21]. There are different types of TNTs that are thought to provide cell-to-cell communication, and one type of TNT is composed of actin and microtubules [22]. Microtubules are highly dynamic polymers of tubulin that are important for neuronal outgrowth, morphology, and migration,

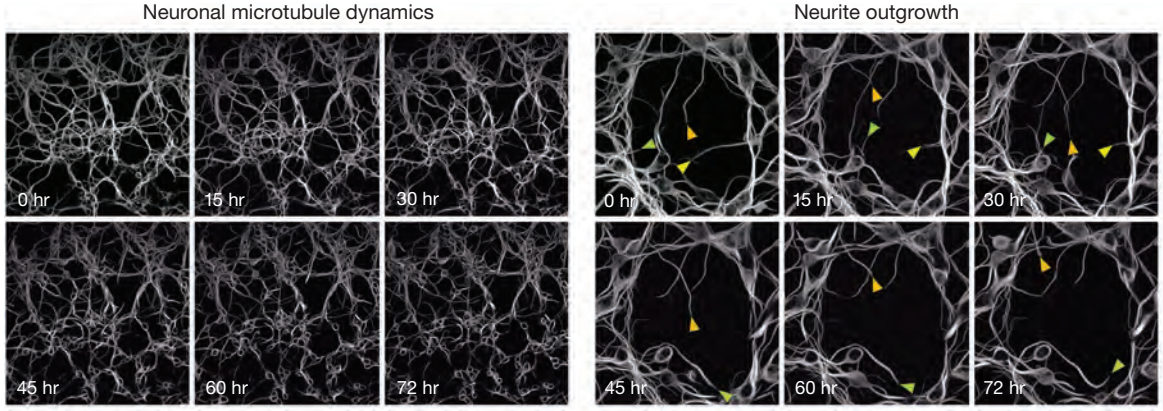

Figure 7. Tubulin Tracker Deep Red for extended time-lapse imaging in primary neurons. Gibco™ Primary Mouse Hippocampal Neurons (Cat. No. A15587) were cultured for 14 days *in vitro* using the Gibco™ B-27™ Plus Neuronal Culture System (Cat. No. A3653401) and Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061) and then imaged across 72 hr at 5 min intervals. Left panel shows no changes in neuronal dynamic assembly, even after 72 hr of staining in Invitrogen™ Tubulin Tracker™ Deep Red (Cat. No. T34077). Right panel shows a 4-fold higher magnification of the neurons to monitor potential changes in neuronal outgrowth with dye treatment; arrows denote outgrowth activity, with each color tracking an individual neuron over time. Tubulin Tracker Deep Red was added to the culture at the beginning of the experiment; no wash step was required throughout the study. The labeled neurons were imaged in a live-cell chamber on a confocal laser-scanning microscope.

and there is growing evidence that their dysfunction is related to PD [21,23,24]. The relationships between alpha-synuclein and tau, as well as between tau and the microtubule cytoskeleton, present an opportunity to better characterize the mechanisms involved in protein aggregate formation in PD.

Tracking the microtubule cytoskeleton in live neurons

To visualize dynamic microtubules in live neuronal cultures, we offer the tubulin-selective fluorescent probes Invitrogen™ Tubulin Tracker™ Deep Red and Tubulin Tracker™ Green. These are cell-permeant fluorescent dyes that bind to polymerized tubulin within the microtubule. The Tubulin Tracker Green reagent (Ex/Em = 494/522 nm) has been used for the study of microtubule dynamics in Parkinson's disease–related research [18]. Neuronal staining using the recently introduced Tubulin Tracker Deep Red reagent (Ex/Em = 652/669 nm) is shown in Figures 6–8.

Time-lapse imaging in neurons

To test and demonstrate the low cytotoxicity and phototoxicity of Tubulin Tracker Deep Red, mouse embryonic hippocampal neurons were labeled with the tubulin-selective fluorescent reagent and microtubule formation in neuronal cultures was monitored over the course of 72 hours (at 5-minute intervals) using extended time-lapse imaging (Figure 7). Normal levels of dynamic assembly, outgrowth, and retraction of neurites were observed throughout the entire time course, with no detachment from the growth substrate or indication of other cytotoxic phenotypes, even under aggressive imaging conditions. Given the relative sensitivity of primary neuronal cultures, these data clearly demonstrate the advantages of long-wavelength excitation in live cells, as well as the low cytotoxicity of Tubulin Tracker Deep Red in primary neuronal cultures.

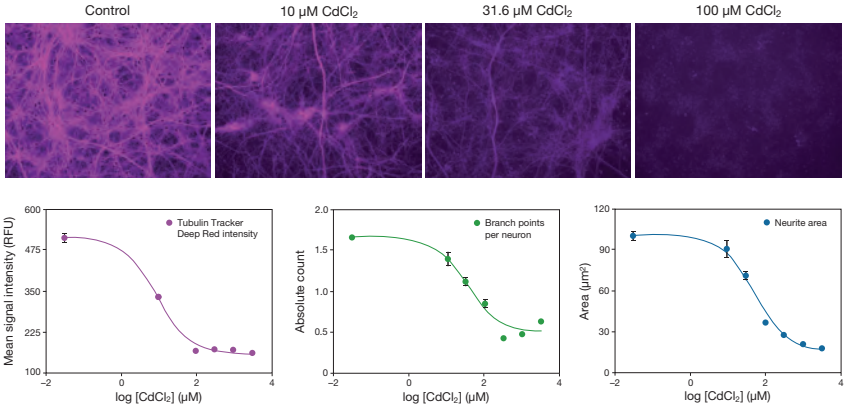

Figure 8. Pharmacological screening of neurite outgrowth with Tubulin Tracker Deep Red. Gibco™ Primary Rat Cortex Neurons (Cat. No. A1084001) were cultured for 14 days *in vitro* using the Gibco™ B-27™ Plus Neuronal Culture System (Cat. No. A3653401) and Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061). Cells were then treated with different concentrations of cadmium chloride ($CdCl_2$) for 18 hr and stained using Invitrogen™ Tubulin Tracker™ Deep Red (Cat. No. T34077). Neurite morphology was assessed using the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform.

Neurite outgrowth assays for pharmacological screening

The benefits of microtubule specificity and absence of cytotoxicity in live primary neuronal cultures prompted us to investigate the use of Tubulin Tracker Deep Red as a novel tool for pharmacological screening in neurons. Figure 8 shows a dose-response study in live rat cortical neurons. Upon treatment with cadmium chloride ($CdCl_2$), we quantified the dose-dependent toxicity in primary neurons by measuring Tubulin Tracker Deep Red intensity branch points per neuron, and neurite area using image segmentation software found on the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform. The results mirrored the known cytotoxic profile of $CdCl_2$ in neuronal cultures, providing proof-of-concept that Tubulin Tracker Deep Red can be utilized for a readout of these stereotypical neuronal traits to aid in the screening of new drugs aimed at a variety of neuronal degenerative diseases, including PD.

3D models for determining neurosphere maturation

3D models have emerged as effective systems for bridging traditional monolayer cell-based assays and *in vivo* animal experiments, and their adoption is increasing in many different research areas. Gibco™ Neurobasal™ Medium combined with Tubulin Tracker Deep Red was used to establish 3D neurospheres, a useful approach for neuronal disease modeling. To demonstrate the specificity of Tubulin Tracker Deep Red for microtubules in neural stem cell (NSC)-derived preparations, the total neuronal content of 3D neurospheres cultured using different growth conditions was assessed by measuring the overall intensity of labeling with Tubulin Tracker Deep Red (Figure 1). When comparing traditional Neurobasal Medium to Neurobasal Plus Medium in the presence and absence of Gibco™ CultureOne™ Supplement, clear differences in microtubule content across the four growth conditions were observed (data not shown). We found →

that cultures in Neurobasal Plus Medium were far better at promoting neuronal growth for applications in 3D neuronal models.

Research tools for the study of PD and other neurological diseases

Although the number of genes and proteins implicated in PD continues to grow, a majority of them are proving to be a challenge to study given their low abundance and a lack of quality reagents to investigate their functions. Invitrogen antibodies are tested in relevant models and applications, and their specificity is confirmed using siRNA- and CRISPR-mediated gene silencing, relative expression, and cell treatment. Learn more about our expanded antibody validation methods at **thermofisher.com/antibodyvalidation**, and search our complete portfolio of primary and secondary antibodies for flow cytometry, immunofluorescence, western blotting, ELISAs, and other applications at **thermofisher.com/antibodies**.

Tubulin Tracker reagents have been used to visualize microtubules in a wide variety of live cell types, and Tubulin Tracker Deep Red has been demonstrated to label 3D neurospheres. Tubulin Tracker Deep Red can be multiplexed with common blue, green, orange, and near-IR fluorescent reagents and enables the study of microtubule dynamics with extended time-lapse imaging, including pharmacological effects on microtubule networks in live neuronal cultures. Learn more at **thermofisher.com/tubulintracker**. ■

*The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.

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Product	Quantity	Cat. No.
Antibodies for Parkinson's disease research: see Tables 1, 2, and 3.		
Cell culture tools		
Primary Rat Cortex Neurons	1 x 10^6 cells 4 x 10^6 cells	A1084001 A1084002
Primary Mouse Hippocampal Neurons	1 x 10^6 cells	A15587
Neurobasal™ Medium	500 mL	21103049
B-27™ Plus Neuronal Culture System	1 system	A3653401
GlutaMAX™ Supplement	100 mL 20 x 100 mL	35050061 35050079
CultureOne™ Supplement (100X)	5 mL	A3320201
Cell imaging tools		
CellInsight™ CX5 High-Content Screening (HCS) Platform	1 each	CX51110
CellInsight™ CX7 LZR High-Content Analysis (HCA) Platform	1 each	CX7A1110LZR
EVOS™ Light Cube, Cy®5	1 each	AMEP4656
EVOS™ M7000 Imaging System	1 system	AMF7000
Microtubule labeling tools		
Tubulin Tracker™ Deep Red	60 slides 300 slides	T34077 T34076
Tubulin Tracker™ Green (Oregon Green™ 488 Taxol, Bis-Acetate)	60 slides 300 slides	T34078 T34075
Tubulin Tracker™ Variety Pack	2 x 60 slides	T34079