STEP	PROBLEM	POSSIBLE REASON	POSSIBLE SOLUTION
Step 3	Severe cell death is observed even for wild-type cells.	Culture manipulation; Culture condition	There are several steps during cell lifting, collection and resuspension that require to be performed very gently and slowly, as listed in the procedure. Pay attention to those steps. In addition, when testing new SC-derived neuronal models, start with more protective and previously reported culture conditions to ensure survival of wild-type neurons, and then remove protection factor to see if the wild-type neurons are fine, whereas disease neurons show degeneration.
Step 3	Severe cell death of disease neurons is observed under protective conditions even before loading the plates into IncuCyte.	Culture manipulation; Culture condition; Sickness of disease neurons	See above for culture problems. Otherwise, it is likely due to the sickness of disease neurons. Try more protective culture conditions by adding more protective factors, etc. In addition, start the transfection and degeneration experiments sooner so that the neurons do not get extremely old and sick.
Step 6-8	Known modifiers fail to show expected effects on the neurodegeneration phenotype.	Modifier problem; Cell model problem; Imaging problem	Check each individual image to ensure that the images are taken correctly. Quality controls are needed for both the model and the modifier, to ensure that the phenotype observed is faithfully depicting the disease relevant neuronal death, and the modifiers are functional at the molecular levels in the particular cell models tested.
Step 4-5	siRNA transfection causes severe cell death on its own.	siRNA toxicity; Interferon response	Test several different non-targeting control siRNA to ensure it is not due to the toxicity of the specific one utilized. In our protocol, the final siRNA concentration is about 125 nM, and we did not detect any cell death caused by siRNA transfection, i.e. no survival difference is observed between non-transfected and non-targeting siRNA transfected samples[6]. However, if interferon response does happen and causes severe cell death in certain models, lower siRNA concentrations could be tested, and the interferon inhibitor B18 could be added without affecting the knock-down efficiency.
Step 4-5	siRNA transfection fails to achieve enough knock-down.	siRNA sequence problem; siRNA transfection problem	Confirm the knock-down efficiency of the siRNAs in HEK293T or other easy-to-transfect cell lines. Ensure to use reverse transfection as described in this protocol, which give ~2 times knock-down compared to forward transfection protocols. In addition, use the lipofectamine RNAiMax, as it is the only one that gave efficient knock-down among a number of different ones that we have tested. While the siRNA transfection protocol worked fine for both ESC- and iPSC-derived neurons in our hands, further optimization might be needed for other SC-derived neurons, possibly due to the difference in differentiation protocol, culture condition, etc. The major factors are the

			cell density and the siRNA concentration. Test several combinations with different values of these two factors to obtain the best knock-down condition.
Step 5	Focus issues caused by air bubbles on the surface of the media or at the bottom of the well.	Pipetting technique problem	Air bubbles should be prevented or removed prior to imaging in IncuCyte. This can be achieved by optimizing pipetting technique to prevent bubbles at the bottom of the well or by gently blowing over each well to dispel the bubbles on the media surface. This technique is performed using an empty sterile wash bottle with the inner straw removed. Gently blow over each well by squeezing the bottle keeping the tip of the wash bottle approximately 5 cm from the media surface.
Step 8	Confluence values generated do not seem to match visual estimations for some of the images.	Algorithm problem	For fluorescent images, the object confluence values are almost always correct. If required the user can export the images for simple re-analysis using other software such as ImageJ. For phase contrast images the Confluence v1.5 algorithm may appear incorrect for a small number of the images. This can occur if the cell peripheries are low contrast and difficult to resolve from background. Increasing the number of images per well, the scanning frequency and number of replicates per condition are potential methods to address this issue as they reduce the influence of outliers on the data series.
Step 6	Images appear blurry.	Vibration of the plates	The incubator that contains IncuCyte must be located on a safe, sturdy surface away from sources of vibration. The IncuCyte gantry should not touch the walls of the incubator. If possible, scans should be scheduled at times when the incubator will not be accessed, as carless opening and closing of the incubator door during a scan can cause image blurring.
Step 6	IncuCyte imaged the condensation present on the underside of the plate instead of the cells.	Condensation	After placing a microplate in the humidified environment of an incubator it is common for condensation to appear on the inside lid and underside of the vessel. Allow time for the vessel to equilibrate to the temperature of the incubator before commencing the first scan (approximately 15- 20 minutes).