Review

Recent Microscopy Advances and the Applications to Huntington’s Disease Research

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Abstract. Huntington is a 3144 amino acid protein defined as a scaffold protein with many intracellular locations that suggest functions in these compartments. Expansion of the CAG DNA tract in the huntingtin first exon is the cause of Huntington’s disease. An important tool in understanding the biological functions of huntingtin is molecular imaging at the single-cell level by microscopy and nanoscopy. The evolution of these technologies has accelerated since the Nobel Prize in Chemistry was awarded in 2014 for super-resolution nanoscopy. We are in a new era of light imaging at the single-cell level, not just for protein location, but also for protein conformation and biochemical function. Large-scale microscopy-based screening is also being accelerated by a coincident development of machine-based learning that offers a framework for truly unbiased data acquisition and analysis at very large scales. This review will summarize the newest technologies in light, electron, and atomic force microscopy in the context of unique challenges with huntingtin cell biology and biochemistry.

Keywords: Fluorescence microscopy, super-resolution microscopy, atomic force microscopy, Huntington/HAP40, tissue clearing, live cell imaging

FLUORESCENCE-BASED IMAGING TO VISUALIZE THE TARGET IN HUNTINGTON’S DISEASE

The full-length huntingtin protein is ubiquitously expressed in all cell types in vertebrates, suggesting critical and basic cell functions beyond normal development. Huntingtin is a 3144 amino acid, 340 kDa protein, one of the largest non-transmembrane proteins in the human proteome, and has no known enzymatic activity nor domains that would suggest any catalytic activity. With an overall solenoid shape and over 80 HEAT repeat structures [1], huntingtin is best defined as a scaffold protein with multiple cellular locations, dictated by membrane association and defined localization signals to the endoplasmic reticulum [2], nucleus [3], vesicles [4], and primary cilium [5–9]. It is challenging to image scaffold proteins by immunofluorescence, as conformations of the protein can bury epitopes to antibodies, and antibody interaction may affect the function and location of the protein. As such, any interpretation of huntingtin location with a single antibody has its caveats.

Live cell imaging has been paramount to understand huntingtin functions, as a static snapshot of a large scaffold protein with many cellular loca...
tions. Live cell imaging with full-length huntingtin fluorescent protein fusions have provided mechanistic insights into huntingtin function at fast axonal trafficking vesicles [4], orientation of the mitotic spindle [10], and the role of huntingtin in primary cilia [5, 9]. Live cell imaging provided insights into the stress-dependent movement of huntingtin from endoplasmic reticulum to the nucleus upon oxidative stress [11] which was prescient to the function of huntingtin in DNA damage repair [12], then subsequent export from the nucleus via the N17 master regulatory signal [5].

One solution to this problem is the use of antigen presentation imaging protocols to cause protein unfolding, typically using alcohols or denaturants that fix proteins, revealing conformationally hidden epitopes. However, this method may not allow antibody access to regions complexed with tightly bound proteins. The first stable defined structure of huntingtin by cryo electron microscopy was with the protein alone, or apoprotein [13]. Subsequent analysis of more stable, more highly refined structures indicates that biologically active huntingtin is dimerized with HAP40 [14], a small flat protein sandwiched between the huntingtin amino-terminal HEAT and carboxy-terminal HEAT domains [15]. Huntingtin is not an abundant protein, and levels of huntingtin are intimately controlled by HAP40 levels indicating that huntingtin stoichiometry is also carefully controlled [16]. This is consistent with the characteristics of a scaffold protein, as the overexpression of any scaffold will have deleterious effects by sequestering factors that should only be interacting at higher local effective concentrations achieved by regulated localization and conformation.

The stoichiometry of protein biochemistry is not different from chemistry. In chemistry, the correct stoichiometry defines a reaction and can affect both rates and direction of product and substrate formation. This is important to consider when using overexpression models to define huntingtin protein biochemistry, and this problem is amplified by orders of magnitude with the use of small huntingtin fragments. To address this issue, between 2010–2012, the Truant lab attempted to genetically edit huntingtin in human cell lines to fuse a fluorescent protein at either the amino or carboxyl terminus. The editing was successfully performed using zinc finger or TALE-N technology, but the cells quickly arrested in cell cycle and died, regardless of which terminus was targeted. While the result is negative and the data unpublished, it highlighted the critical nature of huntingtin in the context of cell biology. In the Zeitlin lab, they were able to tag the amino terminus of huntingtin in a mouse model without any negative effects by using small epitope tags as opposed to a 34 kDa fluorescent protein [17]. This suggests that a large fusion of a fluorescent protein at either termini of huntingtin can affect huntingtin function, which is consistent with the known ability of the huntingtin amino terminus to fold back to the more distal regions of the protein [16, 18] and suggests that huntingtin function is likely allosterically regulated by either termini.

Later in 2017, the Truant and van Roon-Mom labs collaborated to express a camelid intrabody in cells fused to GFP to form a huntingtin chromobody [12]. This reagent labels endogenous huntingtin in live cells, allowing for the visualization of huntingtin translocation across the nuclear pore complex upon stress, as well as localization to DNA damage. Multiple huntingtin monoclonal antibodies subsequently validated these results. This chromobody also has the same turnover rate as endogenous huntingtin and is quantitative as it binds huntingtin with exactly one fluorophore. Efforts to make stable cell lines by selection after stable transfection were unsuccessful, but to date, expression of this chromobody in transgenic mice has not been attempted. Such a model could be an intriguing way to image huntingtin intravitally and could be an important technology in the context of newer methods of fluorescently imaging entire animal organs using the Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ hybridization-compatible Tissue-hYdrogel, or CLARITY protocols [19, 20]. GFP tags avoid many issues of immunofluorescence by providing a truly specific signal, not obscured by conformational changes, and can allow for quantitative imaging as the protein to fluorophore ratio is 1:1. However, a single fluorophore means the signal intensity is low relative to secondary immunofluorescence, where multiple fluorescent antibodies amplify the primary antibody recognition through binding to the Fc fragment of the primary antibody. Addition of a fusion protein at a protein terminus may affect biological function.

With CLARITY, an intact mouse brain tissue can be infused with a hydrogel to preserve its structure and then stripped of lipids. The resulting brain is optically transparent and can be penetrated by reagents for immunofluorescence, permitting striking high-
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resolution 3D imaging of the entire organ. With an increasing interest in Huntington’s disease (HD) phenotypes in peripheral organs, such as the liver or heart, a future project using CLARITY to image entire organs [21, 22] of an HD mouse knockin model could be an important resource for the HD research community. Optical clearing protocols similar to CLARITY have been able to image entire animal embryos in 3D, thus providing a powerful tool in the emerging interest of huntingtin activity and HD phenotypes during early development. CLARITY has been recently used in a mouse gene therapy study in the R6/2 model [23].

As with any immunofluorescence-based imaging, the most critical criterion is the validity of the primary antibody. Several antibodies are used to study huntingtin, but very few were fully validated for specificity and accuracy. Recent comprehensive studies have identified several valid anti-huntingtin antibodies [24]. When in question, simple validation for imaging involves signal competition with the antigenic peptide and loss of signal with huntingtin mRNA silencing, making sure to allow enough time to account for the long >24-hour half-life of huntingtin to reduce levels, which is typically 72 hours or more of repeated siRNA or branaplam treatment [16]. While the comparison to a GFP fusion protein is often used for signal validation, the concern with huntingtin is that any GFP fusion must be expressed at endogenous levels, or overexpression-induced artifactual localization will occur, and transient plasmid expression of a 380-kDa huntingtin-GFP fusion is technically difficult due to the large plasmid size.

With the considerations of the target huntingtin protein in place, the next consideration is the recent advances in imaging modalities and how they can be exploited for imaging the huntingtin protein and automated for use in drug discovery.

SUPER-RESOLUTION FLUORESCENCE MICROSCOPY

To extend resolution, the diffraction-limit can be reduced by increasing the numerical aperture of the objective, as outlined in Abbe’s law. Traditionally, microscopes collect light from only one side of the sample, leaving much of the spherical emitted light wasted. This reduces sensitivity and distorts the shape of the light path in the axial direction, leading to poor z-resolution. A second objective can be used to collect light from the opposite side of the sample, symmetrically focusing light from nearly all directions as done in 4Pi and I5M microscopy, which can reach lateral and axial resolutions of 140 and 100 nm, respectively [25]. However, the modest improvement in lateral resolution, the complex optics needed for the coherent alignment of two light paths, cost, and the severe restrictions on sample thickness and mounting must be considered, and have limited the use of 4Pi and I5M microscopy.

PATTERNED SUPER-RESOLUTION TECHNIQUES

The second class of super-resolution techniques are considered to be “functional”, as they intermix sub-diffraction-limited information in the illumination pattern and/or extract those features by exploiting the different photophysical states of fluorescent dyes. These techniques fall under the general category.
of Reversible Saturable Optical Linear Fluorescence Transitions (RESOLFT), which in one way or another cause the selective suppression and detection of fluorescent molecules within a sub-diffraction-limited space to achieve super-resolution.

In principle, the simplest implementation of RESOLFT involves the overlay of a long-wavelength, doughnut-shaped illumination pattern over a focused, short-wavelength excitation beam. Thus, if molecules are excited by the lower-wavelength focused light beam, then the molecules that reside within the doughnut-shaped light will undergo stimulated emission at a wavelength longer than that of the conventional fluorescence process. With sufficient light intensity, the fluorescence emission of all the molecules within the doughnut will be suppressed due to saturated stimulated emission. This limits fluorescence to molecules that only reside within the center of the doughnut, effectively reducing the size of fluorescence emission to below the diffraction-limit. This is the working principle of Stimulated Emission Depletion (STED) Microscopy, which raster scans the illumination pattern over the sample to yield images with a lateral resolution of 30 nm [26]. If a pinhole is inserted, out-of-focus light can be blocked to achieve confocality and an axial resolution of 400 nm. Alternatively, STED can be combined with a 4Pi setup to reach a z-resolution as low as 30 nm [27]. Again, limitations of this system include very high cost and a high level of technical expertise to maintain alignment.

Structured Illumination Microscopy (SIM) is another patterned-illumination technique using two interfering, phase-shifted light sources to create a sinusoidal illumination pattern. Exciting a fluorescent structure with this pattern results in a moiré effect, bringing the high-frequency spatial information present in the sample to the low-frequency range that can be detected by the microscope [28]. Acquiring a set of images where the pattern is shifted in one direction by changing the phase of the light source, and in another direction by a rotating mask, allows the reconstruction of images with 100-nm resolution. This technique can also utilize the principles of RESOLFT, where high-intensity lasers can close the diffraction-limited gap between the illumination bands by saturating the emission of dyes in areas where there is constructive interference. This method, known as Saturated SIM (SSIM), can produce images with a resolution of 50 nm [28]. Unlike most super-resolution techniques, SIM has the advantage that it can use all of the classic fluorophores or fluorescent proteins without special imaging buffers, but requires additional components to be added to a widefield microscope and offers a modest two- to four-fold improvement in resolution.

The first use of SIM to image a fragment of huntingtin was in 2014, followed by endogenous full huntingtin in 2017 [12, 29]. This level of resolution allowed for the definition of huntingtin fragment aggregate morphology not possible by classic microscopy resolutions, as well as resolution of endogenous huntingtin within nuclear speckles. Nuclear speckles, or interchromatin granule clusters, are nuclear domains enriched in pre-mRNA splicing factors located in the interchromatin regions of the nucleoplasm, and can change morphology and composition upon stress. These sub-nuclear structures are not membrane-bound but use liquid-liquid phase separation to maintain a highly effective local concentration adjacent to chromatin [30]. By classic microscopy, they appear as singular puncta in the nucleus, but SIM reveals these structures to be heterogeneous protein droplets, with sub-speckle localization of proteins like huntingtin.

Recent advances in SIM devices have made them more affordable, paired with faster computer algorithms and hardware that allows for image reconstruction in real time.

WIDEFIELD FUNCTIONAL SUPER-RESOLUTION TECHNIQUES

The RESOLFT imaging techniques mentioned so far require advanced modifications to the optical setup of a conventional fluorescence microscope to produce the illumination patterns needed for achieving super-resolution. These patterns suppress fluorescence in a sub-diffraction-limited manner through fluorescence saturation or stimulated emission. However, fluorescence molecules can reside in other “dark”, or non-emissive states even when illuminated. RESOLFT techniques that exploit these photophysical properties of dyes fall within the category of Ground State Depletion (GSD) microscopy. The working principle of GSD microscopy ultimately relies on achieving a sparse population of emitting fluorophores such that each emission is isolated within a diffraction-limited area. Recording single-molecule emission events on a sensitive camera, often an electron multiplying charge coupled device (EMCCD), would allow for the localization of each emitting molecule in a wide-field setup with
nanometer precision. If the emitting molecules can enter a dark state, and a different subset of fluorophores is activated, then repeating the process of activation, localization, and deactivation for random fluorophore populations (blinking) over thousands of recorded frames allows the localization of the fluorescent molecules comprising the labeled structure. Thus, combining all the single-molecule localizations allows the reconstruction of an image with a lateral resolution of 20–50 nm [31]. This technique can be expanded to the axial dimension if the point spread function (PSF) is deformed in a z-dependent manner. The point spread function is simply understood as the way light paths are defined in a sample when light is focused on a single plane. In the most simple form, a PSF is two cones of light with tips touching at the focal point but the geometry in real terms can vary due to many parameters of the imaging modality and sample preparation. An astigmatic lens can elongate the PSF in the horizontal and vertical direction depending on whether the molecule lies below or above the focal plane. By calculating the extent and direction of the PSF ellipticity, the z-position of each molecule can be determined to a resolution of approximately 50 nm if it is within 1000 nm of the focal plane [32]. This method was used to reveal a fundamentally new structure of actin rings within neuronal processes, which were not observed in over 100 years of classic microscopy [32].

Photoblinking, where fluorophores undergo rapid fluctuation between their emissive and dark states, can be accomplished in various ways. A population inversion from the “on” to the “off” state can be achieved by combining high-intensity illumination with imaging buffers containing reducing agents and an oxygen-scavenging system. The most popular super-resolution technique, STochastic Optical Reconstruction Microscopy (STORM), utilizes these principles to yield stochastically blinking fluorophores that allow the localization of isolated, single-molecule emissions [9]. In the original implementation of STORM, photoblinking was achieved through the stochastic interaction of activator-reporter dye pairs. Other GSD techniques achieve photoblinking through different pathways, such as photoactive dyes and proteins that can be activated or deactivated by specific wavelengths of light, as done in Photoactivated Localization Microscopy (PALM or fPALM), or binding-activated fluorescence, as done in Points Accumulation for Imaging in Nanoscale Topography (PAINT), originally known as Binding-Activated Localization Microscopy (BALM) [33–36]. One way or another, these techniques all exploit the dark and emissive states of fluorophores to achieve photoblinking and utilize single-molecule localization algorithms to reconstruct super-resolution images with a 10-fold enhancement in resolution compared to the starting diffraction-limited image. The difficulties with STORM are the stringent requirements of fluorophores and imaging buffers, but hardware costs are relatively low due to the use of a conventional widefield microscope.

SUPRAMOLECULAR STRUCTURE AND GROWTH KINETICS OF AMYLOID AGGREGATES

The growth of fibrillar systems is highly relevant in the field of neurodegenerative diseases to observe protein inclusions within the brain. The aggregation and eventual fibrillar growth of disordered proteins, such as α-synuclein, Tau and amyloid-beta (Aβ), and huntingtin fragments, have been intensively studied in Parkinson’s disease, Alzheimer’s disease, and HD for over 25 years. Due to the diffraction-limited sizes of the highly-toxic protein oligomers and the relatively short length of their fibrillar aggregates, these structures are usually visualized by electron microscopy. However, their growth has been monitored using ensemble-averaged methods such as surface plasmon resonance, quartz crystal microbalance, and bulk fluorescence assays [37–39]. With the recent development of super-resolution fluorescence microscopy, these structures and their kinetic assembly can now be resolved and examined on a single-fibril basis with minimal perturbation from sample preparation. This allowed the evaluation of true population heterogeneity, an important aspect of amyloid fibrils, as they exhibit polymorphic character with manifold growth processes [40]. Identification and characterization of subpopulations are important for the observation of intermediate states or anomalies that can act as a seed during the emergence of pathological behavior.

Super-resolution fluorescence microscopy allowed the in vitro visualization of individual 90-nm huntingtin fragment globular species and their time-dependent aggregation to form 100-nm wide fibrils with near 1-μm lengths that grew longer with increased incubation times [40]. These globular and fibrillar species of over-expressed huntingtin fragments were previously defined, with respect to
protein phosphorylation, using SIM [29]. SIM also allowed the observation of full endogenous huntingtin within the nucleus under stress conditions and resolution of huntingtin at nuclear speckles and sub-speckle regions and as validation of the entire signaling and salvaging pathway of CK2 -mediated signaling of huntingtin in DNA damage repair which would not be possible at classic resolution [41]. Topographical studies by near-field scanning optical microscopy [42], which uses the properties of evanescent waves to overcome the resolution limit, displayed high structural heterogeneity across β2-microglobulin fibrils, an observation consistent with the polymorphic nature of amyloids [43]. Super-resolution imaging of amyloids has also been carried out in vivo, where intracellular uptake of both Aβ oligomers and fibrils was seen using STORM with structural lengths ranging from 0.3 to 2 μm [44]. Using STED, 3D images of tau aggregates acquired from 50-μm brain sections were resolved to 70- to 80-nm wide puncta [45].

Super-resolution imaging of amyloid fibrils has also been demonstrated with BALM using fluorescent probes that are activated upon their specific binding to the surface of amyloids. This can be advantageous due to reduced background signal from freely-diffusing labeled monomers, the elimination of possible interferences from labeled monomers, and the flexibility in avoiding the use of reducing buffers that may interfere with fibrillar growth. BALM imaging of α-synuclein fibrils was accomplished using the polyelectrolyte probe pentamer-formyl thiophene acetic acid and NIAD-4 [46, 47]. The latter study achieved impressive cross-sectional measurements of 14.3 nm due to the high photon count of the fluorophore and the effective labeling density achieved by BALM. This enabled the observation of a 44-nm periodic labeling pattern, which was indicative of a twisted ribbon-like structure previously observed by atomic force nanoscopy.

SUPER-RESOLUTION IMAGING OF CHROMATIN AND DNA FIBRILS

The role of DNA repair factors as modifiers of HD [37–39, 48–50] incites the use of microscopy techniques to study chromatin and DNA. Investigations of ultrastructural organizations through super-resolution fluorescence microscopy have also been done on DNA fibrils both in vivo and in vitro. Typically, this involved DNA labeling with intercalating dye YOYO-1, or the less cytotoxic and weaker-interacting YO-PRO and PicoGreen dyes. Super-resolution through STORM using these dyes has been demonstrated in a few in vitro studies that achieved labeling densities every 15 nm with resolutions of ~30 nm. Using the same dyes, BALM achieved 1-nm labeling efficiencies and a remarkable resolution of 14 nm [51–53]. These studies were expanded to in vivo environments, enabling the observation of nanoscale ischemia-induced changes in chromatin condensation within the whole mouse and human nuclei [54]. Function-specific labeling allowed for mapping the distribution of centromere-associated histone proteins, along with the visualization of 70-nm filament-like structures composed of 35-nm sub-filaments, empty cavities, and unstructured chromatin [54].

MICROSCOPY AND DNA DAMAGE ASSAYS

Microscopy has evolved from the use of imaging to the development of functional assays exploiting the fine focus and micrometer level of mechanical control now typical on modern microscopes. The DNA damage laser irradiation assay focuses a 405-nm laser on a stripe or point across chromatin to broadly induce DNA damage by severing DNA or increasing local reactive oxygen radicals [55]. While historically, the lasers and optics required for this assay were highly specialized and costly, modern 405-nm light emitting diode pumped LED laser sources have reduced these costs by orders of magnitude. This assay has been shown effective in imaging endogenous full-length huntingtin recruitment to sites of DNA damage, even in live cells using a huntingtin chromobody [12]. It was further used to locate huntingtin, the nucleotide salvager APRT, and CK2 kinase to DNA damage stripes to understand the mechanism of the N6-furfuryladenine cytokine, which could signal the restoration of mutant huntingtin hypo-phosphorylation [41].

Below 50-nm resolution, the use of biophotonic and biophysics has exploited the phenomenon of Förster Resonance Energy Transfer (FRET) as a molecular ruler to measure spatial relations within 10 nm. By fusing fluorophores on amino- and carboxyl- termini of huntingtin exon1 fragments and measuring FRET levels with different CAG lengths,
the reduced flexibility of mutant huntingtin could be seen at an inflection point of 37 CAG repeats [18]. A derivative of this assay was recently used to screen for compounds that could restore the flexibility of mutant polyglutamine expanded huntingtin by researchers at Sanofi [56].

**EXPANSION MICROSCOPY**

Most of the methods highlighted here require considerable equipment investments and higher-level technical support. These might be fundamental limitations at some institutions. A newer method flips the imaging problem around 180 degrees: instead of using technology to see small things with higher resolution, make the small things bigger for use with standard microscopes. The Boyden Lab at MIT first defined expansion microscopy as a method in which chemical groups are added to all proteins and nucleic acids in cells, then expanded using a polymer reaction [57]. Cells are fixed and stained by classic immunofluorescence, then linked to a gel matrix polymer. The cell contents are digested, and finally, the gel matrix is polymerized to expand in three dimensions equally. The net result is the expansion of the target cell or tissue, essentially providing super-resolution data using a standard microscope. This method has been used successfully in neuronal imaging [58]. Super-resolution imaging of expanded samples can produce images with enough resolution to resolve the nuclear pore complex in yeast [59].

**CORRELATIVE LIGHT-ELECTRON MICROSCOPY (CLEM)**

One way to exceed the resolution limits of light microscopy is to use electron microscopy. However, restrictions of specific and multicomponent labeling in electron microscopy limits the visualization of specific proteins and their interactions within the cell matrix. To combine the best of both worlds, microscopy manufacturers have developed systems to transfer samples between fluorescent and electron microscopes, using embedded fiducial markers to register the images [60]. This directly combines the specificity of fluorescence microscopy with the ultrastructural information offered by electron microscopy on a single-particle basis. CLEM has been recently successfully applied to imaging huntingtin exon1 protein inclusions triggered by overexpression [61].

**MICROSCOPY AND DRUG SCREENING**

The advances in computer technology, high sensitivity cameras, and robotics have been synergized with advances in automated image analysis algorithms. This was first accomplished in HD by the Finkbeiner lab, using automated microscopy to watch huntingtin exon1 aggregation and correlate aggregation dynamics with neuronal survival [62, 63]. This has progressed the use of microscopy for qualitative observations to those that can be quantified, essentially using the pixel array of a digital image as a data set of points with location, pattern, and intensity, which can be multiplexed across multiple fluorophores. Robotic microscopes using near-infrared lasers can now perform precise autofocusing and micrometer-level stage control in XYZ on stages holding 96- or 384-well plates compatible with automated liquid handlers. Methods like FRET can now be automated for high content analysis and have been successful at finding small molecule inhibitors of huntingtin exon1 aggregation, by assaying at the nanometer scale [64]. This has allowed the development of high-throughput screening platforms capable of screening thousands to millions of compounds, and the potential generation of petabytes of data. This leads to a scoring or quantification problem, which is impossible to solve by human observation. Solutions to large-scale data analysis also solve other problems associated with human observation.

One classic limitation of microscopy has been the introduction of investigator bias. This is an unconscious bias to acquire images perceived to depict the desired effect. Another problem with using humans to obtain images is that human eyes vary greatly in their ability to distinguish signal intensity across the light spectrum, with no ability in the ultraviolet and infrared light ranges. Human observation is notoriously inconsistent and biased, which has been a chronic problem in pathology assays by microscopy. This also leads to a problem of reproducibility, because no two investigators see images in the same way. Indeed, pigeons have been observed to have more accurate image identification abilities than humans [65].

Pioneers like Robert F. Murphy at Carnegie Mellon have been developing methods and algorithms towards a goal of computer or machine-based sorting [66]. By scoring images using algorithms, we now can use the full range of pixel data in 16-bit images of 65,536 levels, equally across all light wavelengths, and in a
multiplex manner. On spectral imaging devices, this can mean up to 32 different channels and is far beyond the dynamic range of human eyes. Using algorithms, we can develop scripts to sort images based on the desired outcomes by training software to make step-wise quantification to define images as either positive or negative control. This is referred to as supervised machine learning. A powerful open-source example of supervised machine learning was developed at MIT Broad Institute as CellProfiler [67] (CellProfiler.org) and is highly supported by the imaging community. Using CellProfiler, scripts can be generated to sort and quantify images, allowing for large numbers of observations for high statistical power. One of the first applications of CellProfiler in HD was to quantify a huntingtin mediated stress response involving actin dynamics [68]. Typically any quantification studies include the scripts developed, so that any other lab can reproduce observations in the same manner.

For compound screening or high content analysis, sometimes the experiment can benefit from no investigator bias at all, by simply asking which compounds perturb a system in any way possible. For this approach, machine supervision would introduce bias, so optional methods of unsupervised sorting can be utilized. This typically involves sorting the digital data by Principal Component Analysis (PCA) in 3D space, where various measures, or “textures”, are applied to the entire dataset, only measuring which textures give the most variance. The top three texture variances are then plotted in 3D PCA space, where the data points farthest from the central mass of data points have the strongest effect, yet not knowing what that effect is. An open-sourced software resource to do this is Phenoripper [69] (Phenoripper.org).

In 2018, we published a study that mated robotic microscopy for unsupervised data acquisition to unsupervised machine sorting for a protocol with no possible investigator bias [41]. We asked what compounds from a natural products library could affect the phosphorylation of huntingtin in any way at serines 13 and 16. By secondary assays, tertiary assays, and animal model dosing, we defined N6-furfuryladenine (N6FFA) as a signaling cytokine that could restore mutant huntingtin phosphorylation and correct phenotypes in the YAC128 mouse. Upon human observation of the images from the primary screening data, we could not detect any differences in cells treated with N6FFA, yet the algorithms detected the effect and subsequent assays confirmed the effect. Similarly, high throughput screening of neurons is difficult, because of the complex morphologies, but can be applied if using more complex neural net mediated sorting [70], and even without labeling using in silico labeling [71].

Open-source software like CellProfiler and ImageJ/Fiji is now being incorporated into complex logistics strings to execute multi-platform analyses ranging from image correction to quantification to spreadsheet annotation to statistical analysis. This can all be automated in an object-oriented manner using KNIME software [72]. KNIME is an Open Source platform for data mining developed at the University of Konstanz (https://www.knime.org). The software can be applied for many different types of analysis, including image analysis. KNIME provides a wide set of functionalities for tasks such as input/output, data processing, statistics, data mining, and visualization. KNIME is an application based on the Eclipse platform, thus utilizing the Eclipse plug-in concept. This automates the process of taking raw data from the microscope, completing statistical analyses, and providing interactive Java-based graphs, which saves weeks of time for investigators performing tedious tasks between software. Practically, this allows phenotypic quantification of HD cell lines like TruHD [73], or iPSC-derived HD neurons [74] with millions of observations, thus providing massive statistical power and inherent reproducibility due to automation.

**Atomic Force Microscopy**

The final modality discussed is Atomic Force Microscopy (AFM), which is a misnomer, as it isn’t optical or microscopy as observations are on the nanometer scale. AFM involves the dragging of a nanoprobe “needle” across a surface of a flat substrate, such as mica. As the probe moves across the surface, it will deflect based on the topology of the sample, and this deflection in the order of nanometers is measured by a laser line reflected off the back of the probe. This results in resolutions of a few nanometers.

There is a history of the use of AFM in HD but limited to the study of small polyglutamine expanded protein fragment aggregates starting in 2005 [75] and later [76–78]. However, with the recent availability of recombinant human huntingtin/HAP40 at purity levels high enough for cryo electron microscopy [79], the use of AFM to study full huntingtin could be revisited, especially in the context of DNA repair,
for which AFM has been a powerful and successful tool [80].

CONCLUSIONS

While the technology development in microscopy (or more accurately, nanoscopy), and imaging hardware and software is rapidly evolving, the model system must be considered, and the system must be as accurate as possible to human disease. HD has a mean allele size of 43 CAG in heterozygotes and is caused by a mutation within the full 350 kDa protein, expressed at low levels in all cells. However, few models acknowledge these aspects of HD. Imaging technology will not make systems more accurate to human disease. Thus, a holistic approach needs to be considered to respect the accuracy of single-cell observations when attempting to advance studies of HD. We now see mutant huntingtin effects in the very early stages of differentiation [81] and development in humans [21], with effects at fundamental aspects of cell biology [10]. As we focus on the prodomal stage of HD to prevent disease onset, this accuracy is paramount to both understanding huntingtin functions and which of these functions can be targeted for disease therapies.

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CONFLICT OF INTEREST

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