(i.e., intracellular reductive potential or ATP levels), which in turn can serve as a surrogate of cell number or proliferation. Any of these assays might be compromised if knockdown of a particular gene directly interferes with the parameters addressed in the readout system. One can, for example, imagine that knockdown of a gene involved in ATP metabolism results in a reduced signal even though cell number per se might not have been affected. Therefore, the type of readout has to be carefully adapted to the biological question asked.

The most accurate way to determine cell number and other phenotypic changes upon knockdown of a target is by high-content screening (HCS). Usually cells are labeled with markers, which can be detected by fluorescence microscopy. Theses markers can be, for example, fluorescent dyes that stain DNA or fluorophore-labeled antibodies directed against any given target. Several different markers that fluoresce at different wavelengths can be used at once to address several biological processes simultaneously within the same well by using different detection channels. This information can be complemented by phase contrast or transmission light microscopy to yield complex information on the cellular level. Usually cells that adhere to the bottom of multi-well plates are investigated by HCS, but it is also possible to record several Z-stacks (optical cuts through the Z-dimension) with the aim to capture additional information.

Also several 3D screening approaches using HCS have been reported in the literature. Wenzel et al., for example, developed a 384-well-based screening platform in which they grow multicellular tumor spheroids (one spheroid per well), which allows them to reproduce several parameters of the tumor environment, such as oxygen and nutrient gradients and the development of dormant tumor regions (Wenzel et al. 2014, Fig. 4). In this report a compound library was screened, but it is easily conceivable that a similar assay setup could also be used in an RNAi screen. In order for the shRNAs to penetrate all cells in 3D multicellular structure, such as spheroids, cells are usually first seeded and transduced in a 2D monolayer. Inducible shRNAs are best suited to address the phenotypic effect gene knockdown has in 3D cell culture as one can induce knockdown after spheroid formation is completed. Using constitutive shRNAs, one would rather address the question whose knockdown prevents tumor spheroid formation.

Large-scale arrayed screening approaches require costly automated robotics to handle sufficient throughput of plates and to enable readout especially when HCS is used. In stark contrast, pooled shRNA screenings do not require robotic setup to perform the actual screening and can be done in any standard laboratory in which infectious viruses can be handled. In a pooled approach, cells are simply infected by a library of shRNAs in large cell culture flasks or dishes. Another advantage of pooled formats is that cells can undergo several doublings as they are not restricted by the limited space in 96- or 384-well plate cavities before the experiment is read out. This is of importance when the phenotype upon gene knockdown needs some time to develop. In general pooled screens are read out by monitoring the amount of shRNA integrated in the genomic DNA of cells at the start of the experiment and at the end of the experiment. This is usually done by next-generation sequencing of