Unlabeled image analysis-based cell viability assay with intracellular movement monitoring

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ABSTRACT

The need for technologies to monitor cell health is increasing with advancements in the field of cell therapy and regenerative medicine. In this study, we demonstrated unlabeled optical metabolic imaging of cultured living cells. This imaging technique is based on motion vector analysis with a block-matching algorithm to compare sequential time-lapse images. Motion vector analysis evaluates the movement of intracellular granules observed with a phase-contrast microscope. We demonstrated that the motion speed of intracellular movement reflects adenosine triphosphate (ATP)-dependent intracellular trafficking in cells. We also confirmed that intracellular motion speed is correlated with the ATP concentrations of the cells. This assay can measure cellular viability at a single-cell level without requiring any reagents.

METHOD SUMMARY

This imaging technique is based on motion vector analysis with a block-matching algorithm to compare sequential time-lapse images. Each image was divided into small square blocks, then, the current block was matched to the corresponding block at the same coordinates in the previous image. Motion vectors were calculated for every 4×4 pixels in the image of 2448×2050 pixels.

KEYWORDS:

adenosine triphosphate • cell viability • cellular motion • cellular spheroid • computer-assisted image analysis • drug screening • intracellular trafficking • lysosome • organelle • unlabeled assay

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Cell viability and cytotoxicity tests are widely used for phenotypic evaluations of cells, for example, quality control of cell growth and maintenance, measurement of cytotoxic activities of substances, and functional evaluations of cytotoxic immune cells [1–4]. These tests are based on different cell functions, such as cell metabolic activity, membrane permeability and nucleotide uptake activity [5]; in particular, cell viability assays based on metabolic activity provide quantitative evaluations on the basis of enzymatic activities. These assays include ones that utilize different classes of colorimetric tetrazolium reagents, use resazurin reduction and enzyme substrates that generate a fluorescent signal, or measure ATP by luminescence [6–9]. However, it is difficult to quantify viability or cytotoxicity at a single-cell level because these assays require cell lysis or detect the substances released from cells [10].

Recently, heterogeneous cell cultures have been used in the fields of anticancer drug screening and regenerative medicine [11,12], and intracellular marker-staining dyes provide single-cell level detection of viable or dead cells in heterogeneous cell cultures [13]. However, these staining intensities depend on the accumulation of the reagent in the individual cells [14]. Unlabeled live-cell imaging technologies solve these problems and also enable continuous monitoring of cell viability. Walsh *et al.* reported an unlabeled optical imaging method based on the autofluorescence intensity and lifetime of reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) in order to measure the cytotoxicity of chemical agents on primary tumor organoids [15].

In this study, we proposed a phase-contrast microscopy-based imaging technique to monitor cell viability or compound cytotoxicity. The technique involves high-resolution motion vector analysis based on a block-matching algorithm to compare sequential time-lapse

images, enabling theoretical detection of movement at sub-micrometer resolution. Cells transport intracellular granules and consume energy in the process, leading to changes in morphology of the cells and their organelles. Our technique can measure the movement of phase-contrast-visible intracellular granules, consequently enabling the monitoring of cell viability.

MATERIALS & METHODS

Cell preparation

Monolayer cultured human osteosarcoma U2OS cells, human colon carcinoma Caco-2 cells and human hepatoma HepG2 cells (JCRB Cell Bank, Osaka, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin. In order to label lysosomes or mitochondria in live cells, we incubated the cells with Hanks' Balanced Salt Solution (HBSS) (+) without phenol red (Wako Pure Chemical Industries, Ltd, Osaka, Japan) supplemented with 100 nm LysoTracker Red DND-99 (Life Technologies, CA, USA) for 1 h.

Video imaging

We obtained phase-contrast and fluorescent images using a Cell Motion Imaging System SI8000R (Sony, Tokyo, Japan). We also recorded movie images for the movement of intracellular granules as sequential phase-contrast or fluorescent images with a 20× objective lens at a frame rate of 5 fps (total frames, 50), a resolution of 2448×2050 pixels and a depth of 8 bits.

Pharmacological experiment

The cells were seeded in a 48-well multiplate (Eppendorf, Hamburg, Germany) and a 24-well microspace (200- μ m square) multiplate Elplasia (Kuraray, Tokyo, Japan) and maintained separately in culture media for 1–2 days or 7–10 days, respectively. The culture media were replaced with DMEM supplemented with 10% FBS and 0.01–100 μ M doxorubicin hydrochloride (Wako) or HBSS (+) without phenol red

supplemented with 2 μ M paclitaxel (Wako), 2 μ M nocodazole (Sigma-Aldrich, MO, USA), 0.1–100 μ M ciliobrevin D (Merck Millipore, Darmstadt, Germany), 0.01–100 μ M CCCP (Immunochemistry Technologies, MN, USA), 2 or 10 μ M oligomycin A (Sigma-Aldrich), or 4–100 mM 2-deoxyglucose (2DG; Wako). After video imaging, we measured intracellular ATP concentration using CellTiter-Glo (Promega, WI, USA) and ATP standard reagent (Wako).

Motion vector analysis

Motion vectors of the movement of intracellular granules were calculated with the Cell Motion Imaging System SI8000R. Briefly, each frame was divided into square blocks of 16×16 pixels (Figure 1, red squares). Then, the current pixel block was matched to the corresponding block at the same coordinates in the previous frame within a square window of 24 pixels [16,17]. Motion vectors were calculated for every 4×4 pixels in the frame of 2448×2050 pixels. The average spatial speed was calculated by dividing the average motion vector length in the whole field of view or the region of interest with every five-frame interval. Finally, the average spatiotemporal speed was calculated over 50 frames.

Statistical analysis

Data were presented as means $\pm$ standard deviation. A two-tailed paired Student's *t*-test was performed to analyze changes in the spatiotemporal motion speed compared with those in the control.

RESULTS & DISCUSSION

Imaging-based motion vector analysis

Phase-contrast imaging of the U2OS cell line with a $20 \times$ objective lens revealed granular intracellular structures (Figure 1A). The relatively larger granules were circumscribed by blocks of $3.6 \times 3.6 \mu\text{m}$ in our block-matching algorithm, as shown in the phase-contrast images (Figure 1, red squares). Comparing frames 1 and 5 in sequential images taken at 5 fps, a block in frame 1 moved to a block in frame 5 (red, solid-line square); its equivalent position of frame 1 is displayed as a yellow, dashed-line square in frame 5. When we calculated the spatial distribution of motion speed using motion vector analysis (displayed as a color-map image), the region surrounding the nucleus, where relatively larger granules were present,

Figure 1. Image-based motion vector analysis of intracellular granules. (A) Sequential phase-contrast microscopic images of U2OS cells obtained with a $20 \times$ objective lens at 5 fps (total frames, 50), a resolution of 2448×2050 pixels and a depth of 8 bits (left panel: whole field of view; middle and right panels: frames 1 and 5 of digital magnification of the region outlined by the white square). A square block (red, solid-line square in frame 5) containing 16×16 pixels in the current image is matched to the corresponding block in the previous image (red, solid-line square in frame 1). The yellow, dashed-line square represents the position of the block in frame 1. (B) Color-map image obtained from the average motion speed during 50 frames from (A). (C) Phase-contrast image (left panel) and color-map image (right panel) of Caco-2 cells. (D) Phase-contrast image (left panel) and color-map image (right panel) of HepG2 cells. (E) Overlay image of phase-contrast image and fluorescent image using LysoTracker (red) of U2OS cells (left panel: whole field of view; right panel: digital magnification of the region outlined by the white square). Scale bar (white line) represents $10 \mu\text{m}$.

exhibited higher speeds (Figure 1B). Applying motion vector analysis to Caco-2 and HepG2 cells produced similar results (Figure 1C, 1D). These results suggested that motion vector analysis can detect the movement of intracellular granules. To identify the kinds of granules observed with phase-contrast microscopy, we confirmed co-localization with various fluorescent dyes for labeling

specific organelles. As a result, LysoTracker, a fluorescent dye for labeling acidic organelles, is co-localized with relatively larger granules observed by phase-contrast microscopy (Figure 1E). These results strongly suggest that motion vector analysis with phase-contrast imaging detects the movement of acidic organelles, including lysosomes.

Figure 2. Dose-dependent effect of doxorubicin on intracellular motion. (A–C) Spatiotemporal average motion speed calculated from sequential phase-contrast images of (A) U2OS, (B) Caco-2 and (C) HepG2 cells 20 h after doxorubicin treatment and obtained with a 20 × objective lens at 5 fps. Values are expressed as means ± SD (n = 4). (D) Color-map image obtained from average motion speeds during 50 frames from (A). Scale bar (white line) represents 50 μm. (E & F) Intracellular ATP concentration in (E) Caco-2 and (F) HepG2 cells 1 h after image capture of (B) and (C) as determined by CellTiter-Glo. Values are expressed as means ± SD (n = 4). (G) Correlation between (B) and (E). (H) Correlation between (C) and (F).

(Figure 3C & D). These results suggest that the motion being detected is indeed microtubule dependent.

ATP catabolism-dependent evaluation of motion vector analysis

Motor protein-driven intracellular transport on microtubules is an ATP-dependent process; therefore, we assessed the ATP-concentration dependence of the motion vector analysis. First, we examined the effects of carbonyl cyanide m-chlorophenylhydrazone (CCCP), which collapses the mitochondrial proton gradient and leads to ATP production inhibition, on intracellular motion speed. We observed that 1 h after CCCP treatment, the average motion speed in U2OS and Caco-2 cells decreased in a dose-dependent manner (Figure 4A & D). Cellular ATP concentration also showed a dose-dependent decrease after CCCP treatment (Figure 4B & E). We also observed a strong correlation between motion speed and ATP concentration in U2OS and Caco-2 cells (Figure 4E & F). The mitochondrial H⁺–ATP synthase inhibitor oligomycin A and the glycolysis inhibitor 2DG reduced intracellular motion speed, indicating its dependence on ATP production (Figure 4G & H). We applied motion vector analysis to sequential fluorescent images obtained while using the organelle-labeling dyes and compared them to the phase-contrast images. There was a significant correlation between the average motion speed calculated by phase-contrast imaging and fluorescence imaging of LysoTracker in U2OS cells treated with CCCP (Figure 5C). Compared with LysoTracker-labeled lysosome, Hoechst-labeled nucleus displayed lower correlation with phase-contrast imaging (Figure 5F). The movement of nucleus may partly contribute to the motion calculation by phase-contrast imaging.

3D cell spheroid culture application

In the past decade, 3D cell culture models that can mimic *in vivo* tissues in a better manner have been developed to improve predictions of drug responses and efficacy *ex vivo* [21–25]. However, monitoring cellular responses to drugs in 3D cultures is challenging. We hypothesized that unlabeled monitoring with motion vector analysis would be suitable for real-time, dynamic drug pharmacokinetic studies. In order to assess

► Cytotoxicity assay with motion vector analysis

Next, we applied motion vector analysis with phase-contrast imaging to a cytotoxicity assay using an anticancer agent. We found that 20 h after treatment with the antineoplastic anthracycline doxorubicin, the average motion speeds in U2OS, Caco-2 and HepG2 cells decreased in a dose-dependent manner (Figure 2A–D). In order to compare the results with a conventional cell viability assay, we lysed the cells after video imaging and performed a luminogenic ATP assay. ATP concentration also showed a dose-dependent decrease with doxorubicin treatment (Figure 2E & F), and there was a strong correlation between motion speeds and ATP concentrations in Caco-2 and HepG2 cells (Figure 2G & H).

Mechanism of movement of intracellular granules

Next, we attempted to analyze whether the intracellular granules whose movements could be detected with motion vector analysis were transported on microtubules. Nocodazole, which induces depolymerization of microtubules, and thereby inhibits intracellular transport, significantly decreased the movement of phase-contrast-imaged intracellular granules. However, paclitaxel, which stabilizes microtubule polymers, had a comparatively non-significant effect (Figure 3A & B). Intracellular transport on microtubules is driven by the motor proteins kinesin and dynein, whose functions depend on ATPase activity [18–20]. Ciliobrevin D, a dynein ATPase inhibitor, decreased the movement of intracellular granules in a dose-dependent manner

Figure 3. Effect of a microtubule or motor-protein inhibitor on intracellular motion. (A) Average spatiotemporal motion speed calculated from sequential phase-contrast images of U2OS cells 1 h after treatment with 2 μM paclitaxel or 2 μM nocodazole, obtained with a 20 × objective lens at 5 fps. Values are expressed as means ± SD (n = 3, **p < 0.01 compared to the control). (B) Color-map image obtained from average motion speeds during 50 frames from (A). (C) Average spatiotemporal motion speed calculated from sequential phase-contrast images of U2OS cells 2 h after 0.1–100 μM ciliobrevin D treatment, obtained with a 20 × objective lens at 5 fps. Values are expressed as means ± SD (n = 4, **p < 0.01 compared to 0 μM). (D) Color-map image obtained from average motion speeds during 50 frames from (C). Scale bar (white line) represents 50 μm.

Figure 4. Effects of ATP-catabolism inhibitors on intracellular motion. (A & D) Average spatiotemporal motion speed calculated from sequential phase-contrast images of (A) U2OS and (D) Caco-2 cells 1 h after CCCP treatment, obtained with a 20 × objective lens at 5 fps. Values are expressed as means ± SD (n = 3). (B & E) Intracellular ATP concentrations in (B) U2OS and (E) Caco-2 cells 1 h after image capture of (A) and (D) determined using CellTiter-Glo. Values are expressed as means ± SD (n = 3). (F) Correlation between (A) and (B). (F) Correlation between (C) and (D). (G & H) Average spatiotemporal motion speed calculated in U2OS cells (G) 1 h after oligomycin A treatment and (H) 2 h after 2DG treatment, obtained with a 20 × objective lens at 5 fps. Values are expressed as means ± SD (n = 3, **p < 0.01 compared to 0 μM). CCCP: Carbonyl cyanide m-chlorophenylhydrazone.

the feasibility of kinetic determination of cytotoxic activities in 3D cultured cells using motion vector analysis, we created and cultured cell spheroids by using a microspace

and low-attachment culture plate. We applied motion vector analysis with phase-contrast imaging to cultured U2OS and Caco-2 cell spheroids at 0, 12, 24 and 36 h after doxorubi-

bicin treatment and observed that the average motion speed in both spheroid types decreased in a time- and a dose-dependent manner (Figure 6A–C). In order to evaluate the heterogeneous response among cells within the doxorubicin-treated cell spheroids, we divided the area within the cell spheroids into 64 × 64 pixel square blocks (with a total of 16 × 16 = 256 blocks). Then, we calculated the average motion speed and expressed the spatial median and distribution with a box-and-whisker plot (Figure 6D). This plot and the motion speed color map display the heterogeneous and spatially distributed response within the doxorubicin-treated cell spheroids (Figure 6D & E).

In this study, we introduced a robust methodology to create an unlabeled ▶

Figure 5. Correlation of dose-dependent change in motion between phase-contrast and organelle-labeled fluorescent images. (A & B) Average spatiotemporal motion speed calculated from (A) sequential phase-contrast and (B) fluorescent images of LysoTracker-stained U2OS cells 1 h after CCCP treatment, obtained with a 20 × objective lens at 5 fps. Values are expressed as means ± SD (n = 5). (C) Correlation between (A) and (B). (D & E) Average spatiotemporal motion speed calculated from (D) sequential phase-contrast and (E) fluorescent images of Hoechst 33342-stained U2OS cells 1 h after CCCP treatment. Values are expressed as means ± SD (n = 8). (F) Correlation between (D) and (E). CCCP: Carbonyl cyanide m-chlorophenylhydrazone; LT: LysoTracker.

analysis measures internal ATP by using endogenous enzymes (motor proteins). A technique with unlabeled and noninvasive cell viability measurement with motion vector analysis by using conventional phase-contrast microscopy is a powerful tool to monitor cellular viability within a population showing change in composition. Because the measurement of motion vector analysis is also influenced by other factors, such as cytoskeletal stability and arrangement, further validation for specific applications is required.

FUTURE PERSPECTIVE

This unlabeled image analysis-based assay will largely contribute to the study of membrane vesicle trafficking, and is a powerful tool for anticancer drug screening or monitoring cell viabilities for the field of cell therapy or regenerative medicine.

AUTHOR CONTRIBUTIONS

KN: substantial contributions to the design of the work; and the acquisition, analysis and interpretation of data for the work; TK: agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy of any part of the work are appropriately investigated and resolved.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

All authors are employed by Sony Imaging Products & Solutions Inc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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ETHICAL DISCLOSURE

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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► cell viability assay. This imaging-based assay overcomes the technical limitations of standard colorimetric or luminescent assays that require cell lysis to detect markers released from cells. The assay is sensitive to the cytotoxicity of chemical agents on the cells, achieves high resolution to allow the analysis of heterogeneity in 2D or 3D cell cultures, and detects endogenous ATP-dependent activities in unstained or untransfected living cells in real-time, along with longitudinal monitoring of individual cells and cell spheroid viability.

In the case of the cell spheroid culture application for anticancer drug screening, spheroid size, along with the cell number and density, affects the nature of the pathophysiological gradient formed by the diffusion gradient and the pharmacokinetics and pharmacodynamics of drug penetration. This in turn contributes to cell-based assay variability [26]. Our imaging technique using motion vector analysis could quantitate the spatiotemporal distribution of cell viability within cell spheroids, indicating that this method could evaluate

the diffusion gradient and the pharmacokinetics and pharmacodynamics of drug penetration.

In the fields of cell therapy and regenerative medicine, cell quality characteristics directly influence the safety and efficacy of the product or treatment. In a cell culture, growth rate and viability are often indirectly assessed by measuring the average metabolic activity in the culture, which can be affected by a changing cellular population composition or changing culture conditions [2]. As a detection principle, colorimetric tetrazolium reagents measure an internal metabolic enzyme's activity using an exogenous substrate, and the ATP bioluminescent assay measures internal ATP using an exogenous enzyme [6,7,10]. In contrast, imaging-based techniques enable direct monitoring of endogenous enzyme activities with endogenous substrate at the single-cell level. Autofluorescence imaging has been reported to measure the redox state of internal NADH or reduced FAD [15] by using time-resolved fluorescence microscopy. The data presented here suggest that motion vector

REFERENCES

Papers of special note have been highlighted as: • of interest

- Kepp O, Galluzzi L, Lipinski M, Yuan J, Kroemer G. Cell death assays for drug discovery. *Nat. Rev.* 10(3), 221–237 (2011).
- Unlabeled optical imaging method based on the autofluorescence intensity and lifetime of NADH and FAD.
- Lipsitz YY, Timmins NE, Zandstra PW. Quality cell therapy manufacturing by design. *Nat. Biotechnol.* 34(4), 393–400 (2016).
- Motion vector analysis.
- Mery B, Guy JB, Vallard A *et al.* *In vitro* cell death determination for drug discovery: a landscape review of real issues. *J. Cell Death* 10, 1179670717691251 (2017).
- Motion vector analysis.
- Whiteside TL. Immune monitoring of clinical trials with biotherapies. *Adv. Clin. Chem.* 45, 75–97 (2008).
- 3D cell culture models have been developed to improve predictions of drug responses.
- Adan A, Kiraz Y, Baran Y. Cell proliferation and cytotoxicity assays. *Curr. Pharma. Biotechnol.* 17(14), 1213–1221 (2016).
- 3D cell culture models have been developed to improve predictions of drug responses.
- Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol. Ann. Rev.* 11, 127–152 (2005).
- 3D cell culture models have been developed to improve predictions of drug responses.
- Daniukov Iu G, Mil'man LS. [Simplified photometric apparatus for enzymatic determination of ATP by using luciferin–luciferase system]. *Voprosy Meditsinskoi Khimii* 2(5), 548–554 (1966).
- Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K. A combined assay of cell viability and *in vitro* cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol. Pharma. Bull.* 19(11), 1518–1520 (1996).
- Levitz SM, Diamond RD. A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. *J. Infect. Dis.* 152(5), 938–945 (1985).
- Riss TL, Moravec RA, Niles AL *et al.* *Cell Viability Assays*. Sitampalam GS, Coussens NP, Brimacombe K *et al.* (Eds). Bethesda, MD, USA (2004).
- Fatehullah A, Tan SH, Barker N. Organoids as an *in vitro* model of human development and disease. *Nature Cell Biol.* 18(3), 246–254 (2016).
- Vela I, Chen Y. Prostate cancer organoids: a potential new tool for testing drug sensitivity. *Exp. Rev. Anticancer Ther.* 15(3), 261–263 (2015).
- Kramer CE, Singh A, Helfrich S *et al.* Non-invasive microbial metabolic activity sensing at single cell level by perfusion of calcein acetoxymethyl ester. *PLoS One* 10(10), e0141768 (2015).
- Hollo Z, Homolya L, Davis CW, Sarkadi B. Calcein accumulation as a fluorometric functional assay of the multidrug transporter. *Biochim. Biophys. Acta* 1191(2), 384–388 (1994).
- Walsh AJ, Cook RS, Sanders ME *et al.* Quantitative optical imaging of primary tumor organoid metabolism predicts drug response in breast cancer. *Cancer Res.* 74(18), 5184–5194 (2014).
- Hayakawa T, Kunihiro T, Ando T *et al.* Image-based evaluation of contraction-relaxation kinetics of human-induced pluripotent stem cell-derived cardiomyocytes: correlation and complementarity with extracellular electrophysiology. *J. Mol. Cell. Cardiol.* 77, 178–191 (2014).
- Hayakawa T, Kunihiro T, Dowaki S *et al.* Noninvasive evaluation of contractile behavior of cardiomyocyte monolayers based on motion vector analysis. *Tissue Engin.* 18(1), 21–32 (2012).
- Mishra P, Chan DC. Metabolic regulation of mitochondrial dynamics. *J. Cell Biol.* 212(4), 379–387 (2016).
- Stewart AG, Laming EM, Sobti M, Stock D. Rotary ATPases – dynamic molecular machines. *Curr. Opin. Structural Biol.* 25, 40–48 (2014).
- Yount AL, Zong H, Walczak CE. Regulatory mechanisms that control mitotic kinesins. *Exp. Cell Res.* 334(1), 70–77 (2015).
- Celli JP, Rizvi I, Blanden AR *et al.* An imaging-based platform for high-content, quantitative evaluation of therapeutic response in 3D tumour models. *Sci. Rep.* 4, 3751 (2014).
- Derda R, Laromaine A, Mammoto A *et al.* Paper-supported 3D cell culture for tissue-based bioassays. *Proc. Natl Acad. Sci. USA* 106(44), 18457–18462 (2009).

Figure 6. Cytotoxicity assay in spheroid culture. (A & C) Average spatiotemporal motion speed calculated from sequential phase-contrast images of doxorubicin-treated (A) U2OS and (C) HepG2 cell spheroids cultured in a microspace well plate (Elplasia), obtained with a 20 × objective lens at 5 fps. Values are expressed as means ± SD (n = 8–14 spheroids and 13–24 spheroids, respectively; *p < 0.05, **p < 0.01 compared to 0 μM). (B) Phase-contrast and color-map images obtained from average motion speeds during 50 frames from (A). (D) Spatial variation of the calculated motion speed in HepG2 cells treated with 100 μM doxorubicin. The region of interest divided into 256 blocks (16 × 16 blocks, 64 × 64 pixels each) was set inside a single spheroid (white, dashed-line square), and average spatiotemporal motion speeds in each block were calculated. (E) Phase-contrast and color-map images obtained from average motion speeds during 50 frames from (D). Scale bar (white line) represents 50 μm.

- Friedrich J, Seidel C, Ebner R, Kunz-Schughart LA. Spheroid-based drug screen: considerations and practical approach. *Nat. Protoc.* 4(3), 309–324 (2009).
- Horning JL, Sahoo SK, Vijayaraghavalu S *et al.* 3-D tumor model for *in vitro* evaluation of anticancer drugs. *Mol. Pharm.* 5(5), 849–862 (2008).
- Monjaret F, Fernandes M, Duchemin-Pelletier E, Argento A, Degot S, Young J. Fully automated one-step production of functional 3D tumor spheroids for high-content screening. *J. Lab. Automation* 21(2), 268–280 (2016).
- Tannock IF, Lee CM, Tunggal JK, Cowan DS, Egorin MJ. Limited penetration of anticancer drugs through tumor tissue: a potential cause of resistance of solid tumors to chemotherapy. *Clin. Cancer Res.* 8(3), 878–884 (2002).

EXECUTIVE SUMMARY

- We present and discuss a new image analysis-based cell viability assay that monitors intracellular movement and requires no labeling.
- We applied this technique to cytotoxic evaluation of anticancer agents with monolayer or 3D spheroid cultured cells.
- Furthermore, we demonstrated that the technique evaluates the movement of intracellular granules, whose motion speed reflects ATP-dependent intracellular trafficking in the cells.