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A Novel High Throughput High Content Analysis Assay for Intermediate Filament Perturbing Drugs

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ABSTRACT

Keratins are predominantly found in epithelial cells and form the intermediate filament (IF) component of the cytoskeleton. Depolymerisation of these filaments causes the cell to collapse and become more plastic. We have previously shown that short chain fatty acids may trigger depolymerisation of keratins through altered protein acetylation. Currently, there is no single functional assay for screening of the cytoskeleton. The aim of this study is to develop a high-throughput assay to quantify IF depolymerisation and to apply as a screen for IF-perturbing nutrients and drugs. Three treatments were used in a proof-of-principle study: the anti-fungal drugs griseofulvin and cordycepin (the former is known tosuppress microtubule growth, the latter induces abnormal mitosis by suppressing microtubule dynamics with concequent impact on IF organisation) and sodium butyrate, a histone deacetylase inhibitor which disrupts IF formation in cancer cells via post-translational modification of keratins.

Methods were optimised for cell fixation using methanol or formalin, permeabilising agents for Keratin (krt) 8 antibody dilution (triton-x100, digitonin and saponin) and blocking of non-specific binding prior to cell staining using BSA, after which High Content Analysis (HCA) was employed to quantify cell staining intensities by comparing co-occurrence of adjacent pixel intensities. Immunocytochemistry was used to identify Krt 8 intermediate filaments. Indicators of depolymerisation include Krt 8 fluorescence intensity, filamentousness or texture intensity, fibre spot count and fibre spot total area. All treatments resulted in significant decreases for texture intensity. Proof of Principle was established using a Z'calculation. Griseofulvin gave values falling between 0.5 and 1 indicating the assay is suitable for high-throughput work.

In conclusion, a HCA assay for intermediate filament integrity has been demonstrated, establishing proof of principle with griseofulvin, and cross-validating with two further treatments assayable using this method.

Keywords: IF depolymerisation assay; Keratin 8; High content analysis; Proof of principle.

Abbreviations

Krt: keratin; **IF**: intermediate filament; **HCA**: high content analysis; **BSA**: bovine serum albumin; **K**: Lysine residue; **RPMI**: Roswell park memorial institute; **PBS**: phosphate buffered saline; **HPACC**: Health protection agency culture collections; **PTMs**: post translational modifications; **HDACi**, histone deacetylase inhibitor; **SCFAs** short chain fatty acids.

1. Introduction

Intermediate filaments (IFs) are found in all cells, with keratins particulary abundant in epithelial cells. They cross-link to microtubules, actin and myosin by accessory proteins such as filaggrin and plectin and bundle into strong arrays[1]. In contrast to microfilaments and microtubules, it is not understood how IFs remodel the cytoskeleton. By identifying keratins and keratin-associated protein mutations in disease, an insight can be provided into how they act. IFs confer rigidity to epithelial cells, characterised by high visco-elasticity and flexibility [2]. functioning to protect epithelial cells from mechanical and non-mechanical stress [3]. Interfering with the dynamics of IFs leads to a reduced resilience of the epi-

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thelia to mechanical stress [4].The highly dynamic keratin network implicates a perpetual cycle of assembly and disassembly commencing with nucleation of keratin particles at the cell periphery into a proto-filament, followed by elongation towards the nucleus to form a stable network. Perinuclear disassembly releases soluble oligers which diffuse through the cytoplasm and are recycled into new protofilaments at the periphery [4]. Intermediate filaments are formed from polymerised coiled dimers of Type I smaller acidic Krt 9 to 20 and Type II larger basic Krt 1 to 8 [5], comprising mainly Krt 8 and Krt 18 in intestinal epithelia). . Keratin regulation occurs through post translational modifications (PTMs). Krt 8 has been shown to be acetylated at five lysine residues: K10, K100, K392, K471 and K482 in HCT116 colon cancer cells [6] . Over-expression of Krt 8 has been implicated in several colorectal pathologies [7]suggesting that keratins may be essential to epithelial homeostasis and altered function may contribute to conditions such as inflammatory bowel disease[6].

HCA enables quantification of staining intensities by use of computational analysis of images captured by an automated fluorescent microscope adapted to read 96 well plates [8]. It is capable of measuring multiple cellular parameters simultaneously on a cell by cell basis and can also be used for semi-quantification of antibody staining profiles. The automation of acquisition and analysis means that the technique is high throughput and multi-parametric. We have previously employed HCA to analyse the effects of short chain fatty acids on Krt 8 acetylation [9] and cytoskeletal structure [8], demonstrating that butyrate-induced acetylation of Krt 8 is associated with reduced polymerisation of IFs [9]. The benefits of HCA include substantial cost savings due to a reduction in the number of hours involved in performing the experiment and smaller amounts of consumables being used. In drug development, HCA streamlines the work load required for validating drugs prior to animal and clinical testing as well as supplementing systems biology [8].

Currently, there exists no keratin screening tool in a single functional assay. The aim of this study is to establish a viable assay for cytoskeletal staining of intermediate filaments, and to establish proof of principle for high throughput development following perturbation with depolymerising agents.

2. Material and Methods

2.1 *General Reagents and Solutions*

Phosphate-buffered saline Dulbecco "A" (PBS) solution was supplied by OXoid, Basingstoke, U.K. RPMI1640 GlutaMAX media was supplemented with 500 units of penicillin/streptomycin and L-Glutamine 2 mM final conc (Gibco Life Technologies, Invitrogen, U.K) and 10 % Foetal Bovine Serum (Biosera, East Sussex, U.K). The cell treatments griseofulvin, cordycepin and sodium butyrate were supplied by Sigma-Aldrich, Poole, Dorset, U.K. Cells were stained with mouse monoclonal antibodies to Krt 8 supplied by abcam, Cambridge, U.K and Alexafluor 555 donkey anti mouse antibody and Hoechst 33342 weresupplied by Invitrogen, U.K.

2.2 *Cell Culture protocol*

MCF-7 cells obtained from HPACC were cultured in RPMI media and incubated at 37 °C, 5% CO_2 in humidified air. Confluency, viability and cell counts (stained with Trypan Blue, Gibco, Life Technologies, U.K,) were assessed using an optical microscope and haemocytometer ("Improved Neubauer" chamber).

2.3 *Protocols for High Content Analysis (HCA):*

Cell Seeding and Treatment

MCF-7 cells were cultured in Black-sided Costar 96-well culture plates (Sigma-Aldrich) at 2.5 x 10^3 cells per 100 µl RPMI media. Plates were incubated at 37 °C for 24 hours to allow cells to adhere, after which media was replaced with fresh media containing either sodium butyrate treatment at 0, 2.5, 5, 10, 15, 20 mM for 16 hours, n = 20 for initial pilot experiment to optimise staining conditions, griseofulvin in at 0, 2, 5, 10, 20, 50, 100, 150 and 200 µM for 48 hours (n = 3), or cordycepin, (0, 2, 5, 10, 15, 30 and 60 µM for 15 and 30 minutes, n = 3),the latter two containing 0.1% DMSO to aid solubilisation with relevant vehicle only controls.

Optimisation of Staining

Preliminary experiments were undertaken with 20 mM Sodium Butyrate to optimise staining, fixing and blocking protocols, as detailed below.*Fixative:* Cells were fixed with either 10 % buffered formalin (Sigma-Aldrich) for 15 minutes at room temperature, or ice cold methanol (Fisher Scientific, Loughborough, U.K) for 5 minutes at minus 20 ° C, after which, they were washed twice with 100 µl PBS before a final addition of 100 µl PBS. Plates were sealed for storage at 4 °C prior to staining.

Blocker: Prior to antibody staining, plates were blocked for non-specific protein binding with either PBS control or 2% BSA (made up in PBS) for 10 minutes to observe differences in background staining intensities as well as differences in texture and fluorescence intensity.

Permeabilisation: To facilitate entry into the cell, the antibody was diluted in a detergent or permeabilising agent according to Cellomics' HCA protocols. Mouse monoclonal Krt 8 antibody was diluted at a final concentration of 0.01 mg/ml in PBS containing 500 µg/ml digitonin, or 1 mg/nl saponin or 0.1% triton X-100. 50 µl of antibody was added to each well and left for 1 hour at room temperature. Wells were washed three times with 100 µl PBS and subsequently stained for 30 minutes at room temperature with 50 µl of 0.005 mg/ml Alexafluor red 555 donkey anti-mouse antibody containing 0.004 mg/ml Hoechst 33342 DNA stain. As before, wells were washed three times with 100 µl PBS, with a

final addition of 100 µl PBS to store the cells in. Plates were sealed and stored at 4 °C prior to image analysis.

HCA Image Analysis and Quantification

Analysis was undertaken using a Cellomics Arrayscan II platform which employs a Compartmental Analysis algorithm for measurement of total cytoplasmic fluorescence intensity (arbitrary units) [8]. A morphology algorithm enables cell texture intensity measurements (arbitrary units) to be determined, based on the co-occurrence of adjacent pixel intensities. This is derived from the probability of pixels with different intensities occurring next to each other. Co-occurence measures how filamentous the cytoskeleton is. A strong healthy protein is visualised as rough filaments whereas a broken protein causes filaments to become 'mushy' with a smooth, diffused appearance,. This non-filamentousness, consistent with cellular depolymerisation, is characterised byuniform staining, providing a low texture intensity measurement .Highly varied intensities indicate structural staining, providing a larger value for this parameter. The contrast measurement reflects the strength of occurences of pixels of differing intensity being adjacent to each other, whereby the greater the difference, the stronger the weighting. Other parameters measured included cell count (per well), fibre spot count and fibre spot total area (arbitrary units). The method for fibre-spot size analysis identifies which pixels belong to spots or fibres by evaluating the change in intensity over space within the object. A background correction is applied automatically to differentiate between spots and fibres, and intra-cellular noise [10].

3. Results and Discussion

Optimisation of Intermediate Filaments Staining for HCA

Both methanol and formalin fixation were used to assess the viability of developing a dual assay for keratin/tubulin or keratin/actin staining. Methanol is traditionally employed in immunofluoresence experiments as the fixative of choice for microtubules and intermediate filaments since cross-linking reagents such as formaldehyde, though good at preserving cell structure, may reduce antigenicity of components [11]. Preliminary experiments of cells treated with sodium butyrateshowed that methanol fixation provided consistently sharper images (Fig 1, panel 2) compared to 10% formalin (Fig 1, panel 1). Additionally, cells treated with increasing concentrations of butyrate and fixed with methanol remained consistent in number compared to those fixed with formalin which showed a reduction in cell count, P <0.005 (Fig 1D, expressed as percent of control population), hence methanol was used as a fixative of choice.

An initial 30 minute blocking step with BSA (2 %) was included to block non-specific binding of the Krt 8 antibody (Fig 1A - 1C, panels 1 and 2). Visual inspection of acquired images showed no major difference between PBS control or BSA blocked cells. No significant difference was observed in texture intensity measurement when comparing unblocked (PBS) cells to 2% BSA blocked cells using a students paired T test (PBS 1.07 ± 0.62 vs 2% BSA 1.09 ± 0.63, P = 0.86, arbitrary units) or Krt 8 mean total fluorescence intensity (PBS $3.6 \times 10^6 \pm 2 \times 10^6$ vs 2% BSA $3.3 \times 10^6 \pm 1.9 \times 10^6$, P = 0.44, arbitrary units). Consequently this step was omitted in future experiments enabling mechanical manipulations to be kept to a minimum, thus ensuring the quality of final images would remain uncompromised.

Intracellular epitopes require permeabilising to allow the

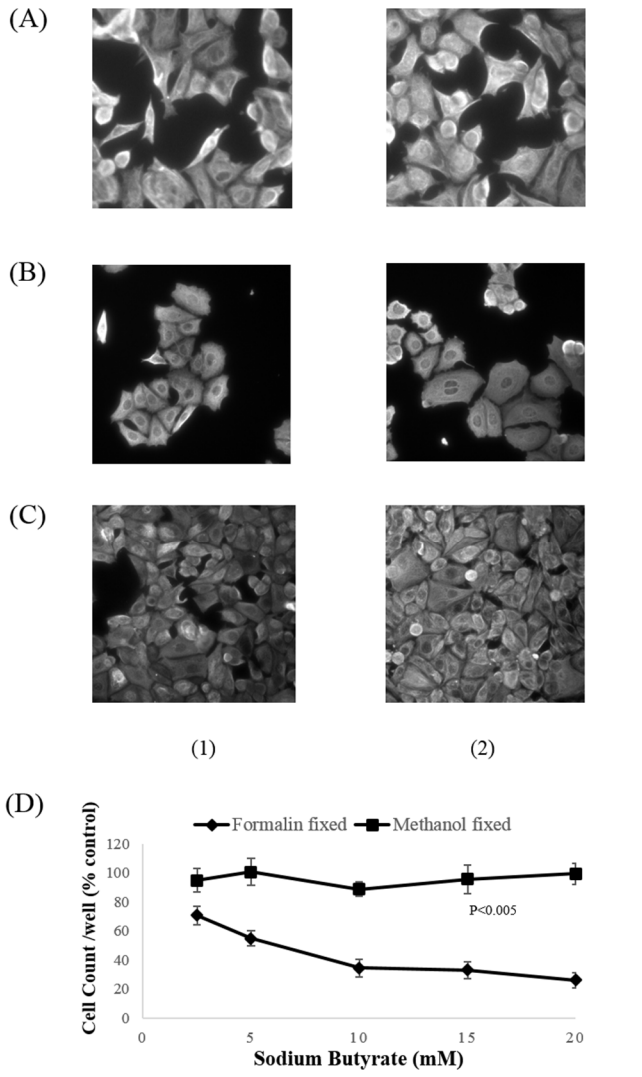


Figure 1. *A comparison of fixation methods on MCF-7 cells* (x20 magnification) treated with 20 mM sodium butyrate using (1) 10% formalin, (2) methanol. (A) No blocker, Krt 8 antibody diluted with PBS (B) No blocker, Krt 8 antibody diluted with 1mg/ml saponin, (C) 2% BSA blocker, Krt 8 antibody diluted with 1mg/ml saponin and (D) Effect of formalin (10%) vs methanol fixation on MCF-7 cell count per well expressed as a percent of control (n = 20, mean ± SEM) following sodium butyrate treatment (0 – 20 mM)

antibody to access the inside of the cell to detect the protein of interest. Low concentrations of Triton X-100 has been shown to remove some cortical fluorescence from mitotic cells [12], where detergent-resistant material was identified as non-filamentous keratin aggregates. Drake et al [8] and Khan et al [9] diluted antibodies with a digitonin permeabilising solution in accordance with Cellomics' HCA internal protocols. In preliminary experiments using sodium butyrate, cells stained with Krt 8 antibody at a concentration of 0.01 mg/ml diluted in PBS (Fig 1A) or 0.1 % Triton X-100 did not stain as well as those using the milder membrane solubilisers digitonin (500 μg/ml) and saponin (1 mg/ml), the latter two thought to produce pores sufficiently large enough for the antibody to pass through without dissolving the plasma membrane. On closer visual inspection, saponin showed better uniform staining of cellular architecture and was used in subsequent experiments (Fig 1B, 1C).

Assay validation with intermediate filament disrupting agents.

All cytoskeletal components are co-ordinated – no part acts alone. Drugs affecting actin, tubulin, or both may also affect the intermediate filament organising system. Interference of intermediate filament dynamics in human disease and transgenic mice leads to reduced resilience of the epithelia to mechanical stress [4]. Gordon et al [13] demonstrated disruption of microtubules, but not actin, could lead to intermediate filament reorganisation. Effects of the cytostatic drugs cytochalasin B, D and β lumicochicine and vinblastine on microtubules and microfilaments showed an induction of keratin rearrangement in Hela cells [14]. SW13 cells treated with latrunculin B (destroys actin) and nocozadole (depolymerises microtubules) completely blocked keratin motility, implying that this function relies both on intact actin microfilaments and microtubules [15].

Following an extensive knowledgebase review, griseofulvin was identified as a potent trigger of intermediate filament breakdown. A metabolic product of *Penicillium griseofulvium*, griseofulvin is absorbed by the GI tract into body fluids and tissues eventually reaching keratinised skin structures [16]. Griseofulvin-damaged mouse livers incorporated with ^{32}P orthophosphate showed alterations in keratin filament architecture where a shift towards acidic isoforms of Type I keratins was thought to be due to hyperphosphorylation [17], demonstrating the role PTMs play in IF homeostasis *in vivo*. This C-mitotic anti-fungal drug induces G2-M arrest / apoptosis in several cell lines, selectively killing cancer cells yet sparing healthy cells [18]. MCF-7 cells treated with 15 - 90 μM griseofulvin for 24 – 48 hours stabilised microtubule dynamics by reducing the length and rate of growing and shortening phases [19]. For this reason, griseofulvin was chosen as an IF perturbing tool in the current studies to evaluate its effect on cytoskeletal depolymerisation.

The adenosine analogue Cordycepin, deriving from culture filtrates of *Cordyceps militaris* and *Aspergillus nidulans*, was the first naturally occurring nucleotide (3'-deoxyadenosine) to be isolated [20]. Zeive et al [21] demonstrated rapid collapse of intermediate filaments in keratinocytes treated with cordycepin where microtubules were depolymerised to small stumpy asters. MCF-7 cells treated with cordycepin suggested the initial effect on cell cycle was due to changes in polyadenylate polymerase activity prior to apoptosis [22]. OEC-M1 cells treated with cordycepin and stained with annexin V for early apoptosis appeared rounded up after 3 hours but were still adherent to the matrix (longer treatments of up to 48h reflected an apoptotic trend thought to be cordycepin inducing G2/M cell arrest [23]). Low doses have been shown to decrease cell proliferation whereas high doses affect cell adhesion and indirectly reduce protein synthesis [24]. Inhibition of MCF-7 cell proliferation by cordycepin has been attributed to autophagy rather than apoptosis [25]. As a result of these findings, cordycepin was identified as a potential candidate for IF disruption in this proof of principle study.

The short chain fatty acid sodium butyrate was chosen for preliminary experiments. The most biologically potent of the SCFAs, it has previously been shown to strongly effect cellular depolymerisation prior to apoptosis [8], making it an ideal candidate for optisation of staining protocols. It is naturally produced in the colon by fermentation of dietary fibre and is a fuel for colonocytes. Colon carcinoma cell proliferation is inhibited via early G1 phase arrest at concentrations of 1 -5 mM sodium butyrate with no impact on cell viability (proliferation is stimulated in normal colonic epithelium) [26] and induces apoptosis *in vitro*. Hela cells incubated with 1 – 5mM butyrate increased histone acetylation by inhibiting histone deacetylase, paralleling changes seen in H3 phosphorylation [27]. Multiple sites of acetylation in Krt 8 and Krt 18 have been identified by our group [8] in HCT116 and CaCo2 cells, where butyrate-induced acetylation of Krt 8 was associated with breakdown of the cytoskeleton via reduced polymerisation of IFs [9]. Immuno blots of intermediate filaments isolated from butyrate treated HCT116 cells treated further confirm Krt 8 is acetylated, thus conveying the HDACi status of butyrate as a post translational modifier of cytokeratins [6].

Proof of Concept as a high-throughput assay

Assay efficacy was established and validated by using the proof of principle Z' calculation to acertainwhether the response was sufficient enough to warrant further investigation. Acceptable Z' values fall between 0.5 and 1.0, with perfect assay values approaching 1.0 [28].

MCF-7 cells treated with 100 μM griseofulvin for 48 hours (n = 3) show altered morphology, becoming multi-nucleated when compared to controls (Fig 2A $_{(1)}$ and $_{(2)}$). This is reflected in the measurements of filamentousness (Fig 2A $_{(5\text{-}7)}$ arbitrary units) where significant decreases in texture intensity (4.4 ± 0.47 vs 2.2 ± 0.03, $P<0.05$), fibre spot count (16.4 ± 0.64 vs 10.7 ± 2.1, $P<0.01$), fibre spot total area (1368 ± 51 vs

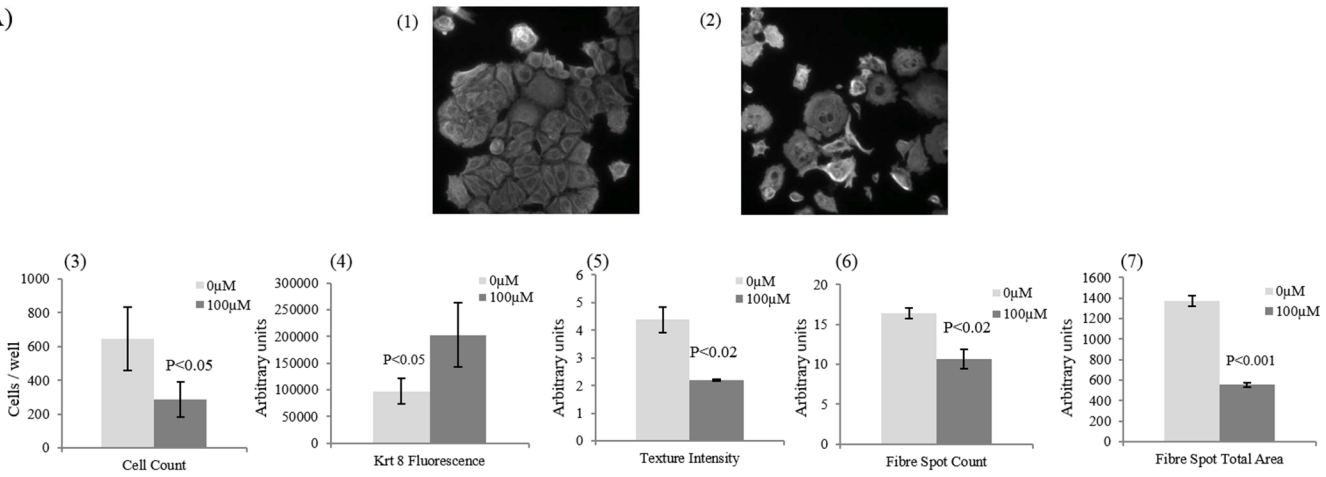
555 ± 23, P=0.0005) as well as cell count per well were observed (646 ± 186 vs 288 ± 103, P<0.05, Fig 2A $_{(3)}$) A significant increase in Krt 8 mean total fluorescence intensity (202632 ± 60337 vs 97262 ± 23343, P<0.05, arbitrary units, Fig 2A $_{(4)}$) was observed when comparing treated cells to controls. Acceptable Z'values (Fig 2B) were obtained for fibre spot count (0.95) and fibre spot total area (0.73), demonstrating suitability for high throughput development (Fig 2B).

Two further treatments affecting intermediate filaments; sodium butyrate and cordycepin, are assayable using the described methods.

Following 16 hours treatment with 20 mM sodium butyrate (n = 20), cells showed similar morphology, with contrast between the cytoplasm and the nuclear staining more pronounced when compared to untreated cells. 20 mM butyrate (Fig 3A $_{(3-5)}$) significantly reduced texture intensity (3.06 ± 0.38 vs 2.41 ± 0.39, P< 0.001), fibre spot count (5.78 ±0.85 vs 5.23 ± 0.6, P<0.02) and fibre spot total area (144.8 ± 11.8 vs 134.9 ± 8.3, P<0.005, all arbitrary units, paired students T test). A non-significant reduction in cell count per well was observed when comparing control to treated cells (670 ± 171 vs 627 ± 50, Fig 3A $_{(1)}$) emphasising the suitability of butyrate as a depolymerising agent without causing a disruption in cell number. As with griseofulvin, an increase in mean total Krt 8 fluorescence intensity was observed, though this was not significant (161983 ± 51982 vs 165148 ± 42567, arbitrary units, Fig 3A $_{(2)}$). Higher concentrations may be required to cause sufficient depolymerisation prior to cell death.

Visual inspection of cells treated for 15 minutes with 60 µM cordycepin (n = 3) confirmed that keratin fibres do retract to form a peri-nuclear ring, as observed by its effects on microtubules (Zieve et al [4]). This may explain the significant decrease in mean total Krt 8 fluorescence intensity (arbitrary units, Fig 3B $_{(2)}$) from 385698 ± 9862 vs 353452 ± 3297, P<0.05, in contrast to the increase observed with griseofulvin, P<0.05, and sodium butyrate (not significant). As with griseofulvin and sodium butyrate, texture intensity was also significantly decreased (Fig 3B $_{(3)}$, 2.39 ± 0.18 vs 1.57 ± 0.07, P<0.05, arbitrary units) indicating depolymerisation and a loss of filamentouness when compared to controls, though no significant differences were observed for measurements of fibre spot count (Fig 3B $_{(4)}$, 10.39 ± 1.15 vs 11.23 ± 1.98), fibre spot total area (Fig 3B $_{(5)}$, 733 ± 13 vs 653 ± 70, arbitrary units) and cell count per well (Fig 3B $_{(1)}$, 124 ± 12 vs 145 ± 60) when comparing controls to treated cells. This could possibly be attributed to the shorter treatment time with cordycepin (15 minutes) compared to griseofulvin (48

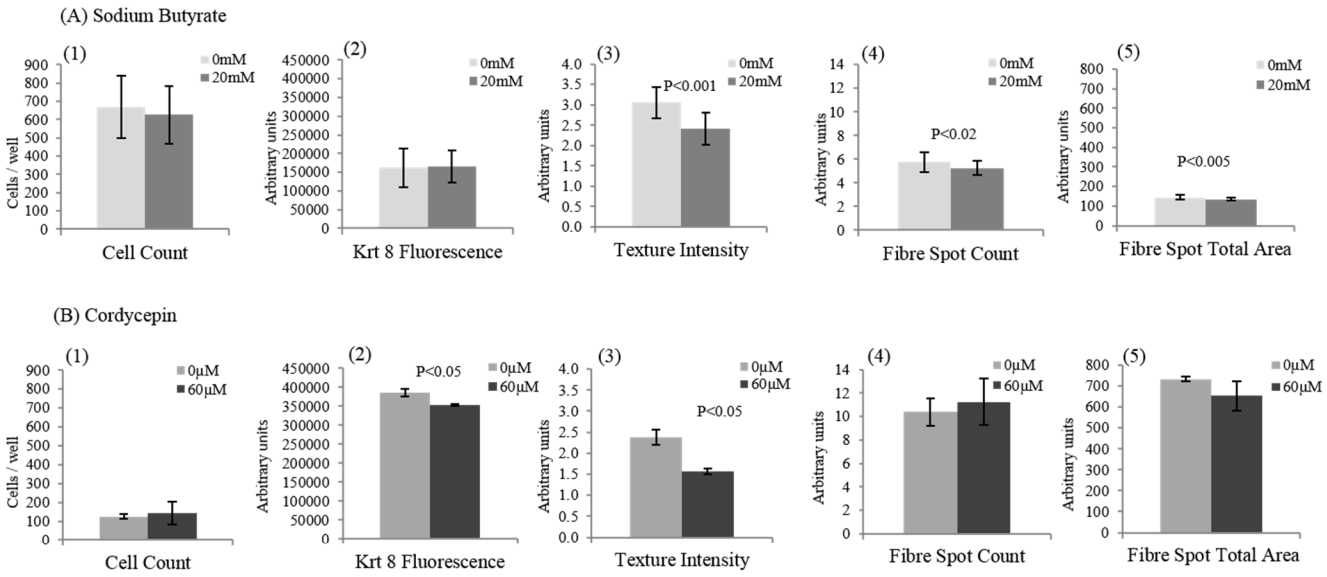
(A)



(B)

Treatment	Statistical Test	Cell Count	Krt 8 Fluorescence	Texture Intensity	Fibre Spot Count	Fibre Spot Total Area
Griseofulvin 0 v 100µM n = 3	Z-Prime	-1.42	-1.38	0.32	0.95*	0.73*
	Paired T-Test	0.024†	0.046†	0.015†	0.012†	0.0005†

Figure 2. *Assay Development and proof of Principle.* (A) Effect of Griseofulvin (n =3) on indicators of intermediate filament staining (arbitrary units). (1) PBS control 48h, x20 magnification, (2) 100µM Griseofulvin 48h, x20 magnification, (3) Cell count per well, (4) Mean total Krt 8 Fluorescence, (5) Texture Intensity, (6) Fibre spot count and (7) Fibre spot total area. (B) Statistical analysis of 0µM vs 100µM treatment using a Z prime calculation and paired students T-test. *Z prime values between 0.5 and 1 indicate suitability for high throughput development. †P values <0.05 are statistically significant.



Treatment	Statistical Test	Cell Count	Krt 8 Flourescence	Texture Intensity	Fibre Spot Count	Fibre Spot Total Area
Sodium Butyrate (n=20)	Paired T-Test	0.2	0.8	0.001*	0.0156*	0.00336*
Cordycepin (n=3)	Paired T-Test	0.57	0.04*	0.025*	0.64	0.16

Figure 3. *Application of assay to demonstrate proof of principle.* A1 - A5; Sodium Butyrate, 16h, n = 20 and B1 – B5; Cordycepin, 15min, n = 3. Measurements for cell count per well (A1) and (B1) showed no significant difference when comparing control PBS treated cells to respective treatment. Significant decreases for indicators of intermediate filament staining (arbitrary units) were observed in butyrate treated cells for (A3) Texture intensity, (A4) Fibre spot count and (A5) Fibre spot total area, P<0.001, P<0.02 and P<0.005 respectively. Cells treated with cordycepin showed a significant decrease (P<0.05) in mean total Krt 8 fluorescence (B2) and texture intensity (B3).

hours) and sodium butyrate (16 hours) leading to a lower overall cell population by the end of the experiment (higher cordycepin doses around 200μM have been found to affect cell adhesion [26]).

The results indicate that a functional assay for the direct assessment of IF-perturbing drugs has been developed, optimised by the directed and informed combination of existing technologies. Keratins are emerging as potentially important targets in colorectal pathologies [7] and therefore may become the target for development of new therapeutics. As such, assays need to be in place for such applications. General cytoskeletal biology indicates that the various cytoskeletons are interlinked and collapse of one may impact upon another [4, 14, 15, 16]. This assay has the potential to be combined with a chemical stain (phalloidin) or antibody from another species to form a duplex or triplex assay for integrity of the microtubular, microfilamentous and IF cytoskeletons and thereby determine hierarchicality of impact of chemotherapeutics at the cellular level.

Acknowledgements

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