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Use of an imaging station for rapid colony counting in radiobiology studies

Tim A.D. Smith*, Gema Cabello, Marco Mingarelli

School of Medicine Medical Sciences and Nutrition, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK

HIGHLIGHTS

- Rapid determination of colony number.
- Strong correlation with conventional counting by 'eye'.
- Objective measurement.

ABSTRACT

Colony counting by eye is time consuming and subjective. Here comparison between the measurements of proliferative growth inhibition in plates of radiation-treated cells by an imaging station correlated highly significantly with counts determined by eye. This would suggest that an imaging station could be a viable alternative for colony counting for doses over 200KBq.

1. Introduction

There have been many approaches to measuring radiosensitivity using both cell-based and molecular approaches (Ferlazzo et al., 2017). Treatment with radiation induces several cell death pathways which contribute to loss of proliferative potential including mitotic and apoptotic cell death and irreversible G1 arrest (senescence) each of which has its own detectable end points. However some of these cell death mechanisms are attenuated in cells overexpressing mutant p53 (Offnutt, 2018; Butera et al., 2018) whilst fibroblasts rarely exhibit apoptosis. It has been concluded that measurement of any of these phenomenon alone is insufficient to reliably inform on cellular radiosensitivity (Ferlazzo et al., 2017).

The clonogenic assay (or colony-formation assay) established by Puck and Markus in 1956 (Puck and Marcus, 1956), measures the net effect of the cell death and other growth inhibitory pathways that result in loss of proliferative potential is considered the 'gold standard' for measuring radiosensitivity (Chua and Rothkamm, 2013). The assay involves plating out cells at low density, treating then incubating the cells for 1–2 weeks then counting colonies of 50 or more cells. A recent review article (Ferlazzo et al., 2017) concluded and that only the clonogenic assay could reliably measure of cellular radiosensitivity. However the procedure of identifying colonies by eye that are 50 cells or greater in size is subjective and time consuming (Cuneo et al., 2019; Dubuc et al., 2019; Yong et al., 2015; Zhou et al., 2018; Nagle et al., 2016). Digital counting procedures have been suggested (Cai et al., 2011) but have not become routine possibly due to complexity and expense (Guzman et al., 2014).

Here we have explored the use of an imaging station, usually utilised in counting bacterial colonies on agar plates, to the counting of tumour colonies. The counting procedure takes less than 1 min per sample compared with about 1 h for colony counting by eye. The imaging station costs less than £4000 so is also economical.

2. Experimental

2.1. Materials and reagents

Chemicals were purchased from Sigma-Aldrich UK unless otherwise stated. Breast cancer MDA-MB-468 cells were obtained from the American Tissue Culture Collection (Teddington UK). $^{177}LuCl_3$ was purchased from Diagnostic Imaging Ltd (Welford UK).

^{177}Lu-labelled EGFR-targeted AuNPs were prepared as described in Cabello et al. (2018).

2.2. Clonogenic assay

A suspension of MDA-MB-468 breast cancer cells was prepared from a confluent flask of cells using trypsin. After addition of medium cells were counted using a haemocytometer and a cell suspension of

* Corresponding author. Translational Radiobiology Group, Oglesby Cancer Research Building, University of Manchester , 555 Wilmslow Road, Manchester, M20 4GJ, UK.

E-mail address: tim.smith@manchester.ac.uk (T.A.D. Smith).

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1500 cells/ml was prepared. Cell suspension (1 ml) was added to 3 cm diameter plastic petri-dishes (Greiner Bio-one, Germany GmBH)) and the cells left overnight at 37 °C. Medium was carefully removed from each well and 1 ml of medium was added either without or with [^{177}Lu] Lu-AuNPs (in the range 200Kbq – 5MBqMBq). The controls and treated cells were randomly assigned (so that the reader was unaware which had been treated). The medium was removed after 4 h and washed by gentle addition and removal of 2 ml of medium. Two ml of medium was then added to each well and the plates incubated at 37 °C. After 11 days the MDA-MB-468 colonies of 50 cells or more were counted manually under a microscope (Nikon Eclipse TS100 inverting microscope). For this purpose a 9 region grid was drawn on each well with a fine tip marker. The size of a 50 cells colony was first checked at 100x magnification after which colonies of 50 cells or more were counted in each of the 9 regions of a grid at 40x magnification. The procedure was repeated for each well at the corresponding field of view for each region.

2.3. Colony staining and imaging station

Manual counting was carried out on colonies consisting of 50 or more cells. Plates were then incubated for a further 24 h to allow colonies of 70–80 cells to develop stained with 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in medium for 2 h then colonies counted on the imaging station after removal of MTT medium. Control and treated plates after incubation with MTT and removal of medium were subject to colony counting on a UVP Colony-Doc-IT imaging station (Analytic-Jena (California USA)) shown in Fig. 1. The imaging station is loaded with Colony-Doc-IT software which can count colonies automatically. Lighting was set to white light. After counting each colony is allocated a number and brief scrutiny of the plate is carried out to check for merged colonies in the editing mode. Initial experiments were carried out to determine colony size that could be detected by the imaging station and found to be between 70 and 80 cells.

3. Results

Cells were treated with 200KBq to 5MBq (Marcatili et al., 2016) producing a range of cell survival between 80% and 5% as determined by colony formation. Colonies of 50 cells or more were counted by eye then the plates incubated for a further day to ensure sufficient colonies of 70–80 cells or more that could be detected by the Imaging station. Results from each counting method, expressed as a percentage of colonies on untreated plates, were subject to regression analysis. The results shown in Fig. 2 demonstrate a strong correlation ($r = -0.89$, $p < 0.001$, $n = 30$) between the two methods. Results shown in Fig. 3 suggest that cell survival effects can only be detected by the imager using a dose of 200KBq as doses of 50 and 100KBq did not produce a detectable effect on cell survival, when determined using the imager.

Fig. 1. The imaging station ColonyDoc-It™.

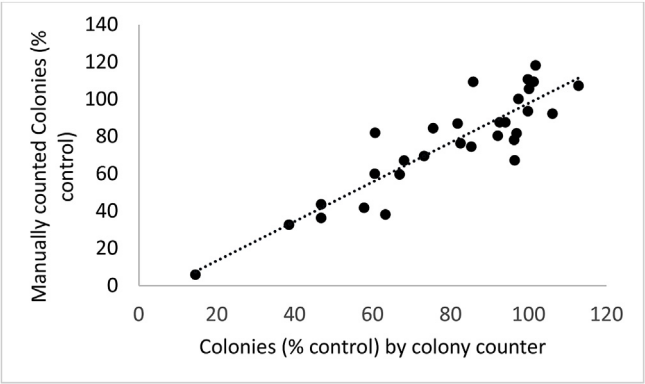

Fig. 2. A scatter plot of colony formation (% of untreated cells) by MDA-MB-468 breast tumour cells incubated with 200KBq - 5MBq of 177Lu AuNPs targeted to the epidermal growth factor receptor counted by eye (x-axis) and by an imaging station (y-axis) (correlation co-efficient, $r = 0.89$, $p < 0.001$).

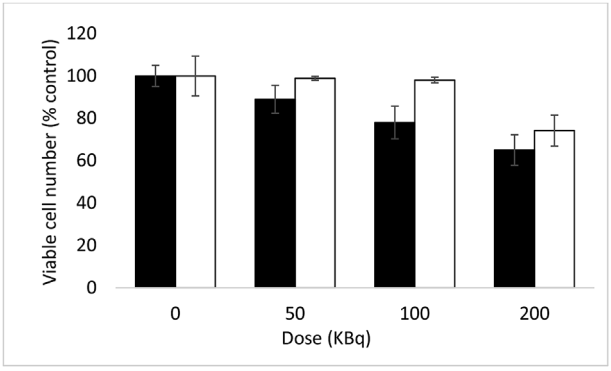

Fig. 3. Cell survival of MDA-MB-468 breast tumour cells incubated with 50–200KBq of 177Lu AUNPs targeted to the epidermal growth factor receptor counted by eye (manually) (solid) and by an imaging station (empty bars).

4. Discussion

The clonogenic assay is the most reliable method for determining radiosensitivity but is considered time consuming (Ferlazzo et al., 2017; Chua and Rothkamm, 2013). However setting up the assay and treating cells requires only about 2 h. The treated cells are then incubated for 1–2 weeks depending on the growth rate of the cells which require only medium changes every 2 or 3 days. The most time-demanding part of the assay is determining and counting colonies, by eye, that are greater than a pre-determined cell number, usually 50. We have found that this takes between 30 and 60 min per plate so restricting the number of samples per day to about 8 - the procedure is also mentally demanding for the operator requiring periodic breaks.

It was found that the sensitivity of the method was insufficient to detect the effect of very low radiation levels where treatment only decreased cell survival by a few percent. However the treatment effect with 200KBq (which equates to a dose of 0.5Gy) or more was readily detectable using the imaging station. Low dose hypersensitivity occurs at 0.5Gy (Enns et al., 2004) so it's effect on cell survival will be detectable using this device. Other studies using digital devices have used doses of 2Gy (Cai et al., 2011). Targeted radiotherapy studies treat cells with dose ranges from 1 MBq or more to produce > 20% decreases in cell survival (Yook et al., 2015; Cai et al., 2016). When counting by eye colonies of 50 cells or greater were counted. The imaging station was able to detect colonies of 70–80 cells or more. MDA-MB-468 cells are about 12–15 μm in diameter (Veb et al., 2017). This corresponds well

with the manufacturer's quoted detectable colony diameter of 100 μm.

Although the imager could not detect cell kill using activities of 50 or 100KBq this equates to 0.125 and 0.25Gy respectively most studies use doses of 2Gy or more. The rapid data acquisition that the colony counter facilitates, requiring less than 1 min per sample including sample changing, increases procedure efficiency, makes the assay more objective and greatly increases the potential number of samples that can be used per experiment.

Disclosure

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apradiso.2019.06.028.

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