Multidimensional microscopy: A suitable technique to follow *in vivo* the interactions between biodegradable biomaterials and cells✝

F. Alvarez[1], R.M. Lozano Puerto[2], B.T. Pérez-Maceda[2], C.A. Grillo[1] and M.A. Fernández Lorenzo[1,3]

[1] Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas, La Plata, Argentina
[2] Cell-Biomaterial Recognition Group. Cellular and Molecular Biology Department. Centro de Investigaciones Biológicas (CIB-CSIC), Madrid, España
[3] Facultad de Ingeniería, Universidad Nacional de La Plata, La Plata, Argentina, mmele@inifta.unlp.edu.ar
✝ In memory of Florencia Alvarez (Flor) whose lovely life, plenty of fortitude and spiritual values, we had the honor of sharing

Biodegradable metallic materials have important applications in medicine for orthopaedic, dental and cardiovascular therapies. These metals interact with the biological medium and are easily corroded due to the presence of chlorides. During degradation micro-debris are released. Macrophages, cells involved in immune response, interact with debris in different complex ways.

Multidimensional microscopy refers to the generation of multiple images of a sample over time. This technique is very appropriate to follow *in vivo* the degradation process of a biodegradable biomaterial and the interactions of the macrophages with the released debris. In this chapter we will describe the use of this technique to study the interaction of Mg microparticles (Mg-MPs with J774 macrophages, a mouse cell line. Formation of H_2 bubbles, changes in the morphology of macrophages due to interactions through their membranes and pseudopodia, duplication and death of cells were detected. Images of multidimensional microscopy show the great capability of this technique to follow biological and physicochemical processes at real time.

Keywords: *in vivo* multidimensional microscopy; biomaterials; cell culture; macrophage

1. Time-lapse multidimensional microscopy

Many cellular and physicochemical processes involve fast transformations which could be followed by 3D time-lapse microscopy (also called 4D microscopy). A great number of reports dedicated to introduce faster, more robust and more sensitive equipment and algorithms for image and time-lapse processing were published in recent years [1-3]. In time-lapse microscopy images are recorded in fixed intervals over extended periods of time. 4D imaging should collect focal planes in time-lapse mode as rapidly as possible, without perturbing the sample by strong illumination or the use of staining. The original stack of 2D images is reconstructed into a 3-D image that changes with time. This type of microscopy offers great functionality for biological, cytomorphological and physicochemical examinations. Temperature stability, minimum exposure of specimens to light (protection from photobleaching) and highly sensitive recording techniques are frequently required to follow these processes. Thus, a climate chamber, cell chamber, CO_2 controller and incubators must be included in the station when biological samples are analyzed. The resulting images of movies can provide direct insights into the behavior of cells including duplication, cell death and migration among other processes [3, 4]. In case of cell motility quantification, automated tracking is always superior to point-and-click systems in imaging the average migration rate of a cell population. Images of multidimensional microscopy (MM) show the great potential of this technique to follow biological and physicochemical processes at real time.

Different abiotic and biological processes have been monitored by MM. Among them this technique has been used to study degradation processes, crystallization and morphological changes of cells during growth and cell death.

2. Use of time-lapse microscopy to follow changes of the inorganic/organic samples with time

In situ time-lapse imaging techniques coupled with image analysis have been used to follow changes in particles and metals over time [5]. Polymorphic transformation of L-glutamic is one of the systems that were characterized *in situ* by MM [6]. Additionally, growth rates in a nucleation process were also obtained [7-10] using this technique. A very accurate description of the particle shape and characteristic lengths could be assigned to the crystals [5] through a non-invasive measurement.

Corrosion processes can also be followed by time-lapse imaging [11]. The preferential attack of certain phases within a Zn-based alloy could be detected at real time. Localized attack evolution on stainless steel could also be followed [12].

3. Biodegradation of biomaterials

There is a growing interest in using biodegradable metals for orthopedic, dental and cardiovascular applications [13-15]. Mg-based materials have advantages over other existing biodegradable materials such as polymers, ceramics or bioactive glasses due to their low density and mechanical properties close to that of human bone [15].

Mg is easily corroded particularly in chloride-containing media like body fluids [16, 17]. On this respect, it has been found a marked influence of corroding Mg on the peri-implant tissue [18]. It has been reported that during service condition *in vivo* they degrade and release debris particles and metal ions [19]. However, a great deal of work is still necessary to evaluate the effect of local concentrations of metal ions, debris material and hydrogen evolution, among other factors, on the biological system to arrive to definite conclusions.

The toxicological effects of metals are frequently assayed *in vitro* by adding extracts that were obtained from the metal dissolution in the culture medium without cells. However, under these conditions the effect of local changes of Mg ions concentration and pH, the formation of hydrogen bubbles and the corrosion of Mg debris on the cells cannot be evaluated. *In situ* study of the interaction of cells with debris particles released during degradation should be analysed. MM is a suitable technique to follow these processes.

4. Interactions close to the biomaterial/biological medium interface

According to the information previously reported, as a result of degradation of metallic biomaterials the surrounding tissues are rich in debris particles. Close to orthopedic implants highly vascularized fibrous membrane, macrophages, multinucleated giant cells and fibroblasts can be found [20]. Macrophages are the cells that are mainly involved in phagocytosis and inflammation [21-26]. The debris particles enter into the periprosthetic tissue and macrophages interact with them and, depending on their size, may be phagocytized inducing a cascade of events. Dead cells have been found in the interface membrane. Stea *et al*. [24] found that the apoptotic cells in the interface membrane collected from revision surgery for aseptic loosening of hip joint prostheses were mainly macrophages, and cell death was related with metal debris [23-25].

Several studies have reported micro-nanoparticles/macrophage interactions. The effects of polymethylmethacrylate (PMMA), cobalt chrome molybdenum alloy (CoCr), and titanium-alloy particles on macrophages co-cultured with lymphocytes have been analyzed *in vitro* a showed a dose-dependent response. High doses of PMMA and titanium-alloy particles stimulated increased levels of cytokine release in the macrophage and macrophage/lymphocyte cell cultures while CoCr particles seem to produce toxic effect [25]. In the presence of synthetic diamond nanoparticles significant numbers of late apoptosis labeled cells (propidium iodide + Annexin-V-FITC labeled cells) were observed which could be attributed to nanoparticle-mediated necrosis [27]. On the other hand, large polystyrene beads (3.5 µm) were shown to cause primarily macrophage necrosis, while smaller polystyrene beads (0.5 µm) caused primarily macrophage apoptosis. Overall, it was demonstrated that the response of macrophages to debris depends on the nature, size, stability and concentration of the particles. However, the mechanisms by which macrophages recognize and respond to particles remain unknown [28, 29].

The study of the degradation of biodegradable metals like Mg in a macrophage culture is particularly interesting because debris interact with the biological medium and is rapidly altered and cells interact with these particles that change with time. Thus, MM is a very suitable tool to follow these processes.

Mg interacts with the biological medium and is easily and rapidly corroded. It must be taken into account that during degradation Mg^{+2} ions are released to the biological medium and H_2 bubbles are evolved, the medium turns more alkaline and $Mg(OH)_2$ precipitates. These processes occur according to the following overall reactions [14]:

$$Mg^0 + 2H_2O \leftrightarrow Mg^{+2} + 2OH^- + H_2 \quad [i]$$
$$Mg^{+2} + 2OH^- \leftrightarrow Mg(OH)_2 \quad [ii]$$

During degradation of the Mg-based implant micro-debris are released. Macrophages interact with debris in different complex ways. In this chapter the interactions of Mg-MPs/biological medium and Mg-MPs/macrophages followed by MM are analyzed.

5. Degradation of Mg microparticles

As mentioned above, in time-lapse microscopy images are recorded in fixed intervals over extended periods of time. Thus, the successive images allow us to follow the degradation process of Mg-MPs. In Fig. 1 the consecutive stages of the degradation of three Mg-MPs can be observed.

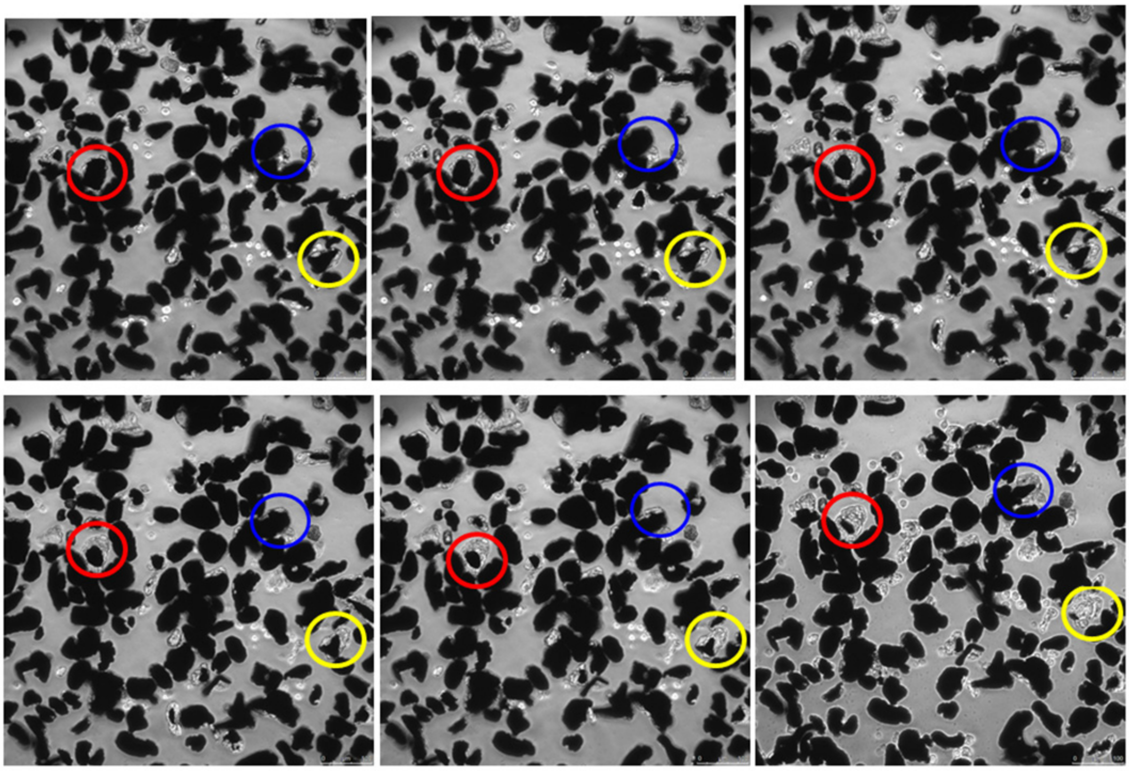

Fig. 1 Sequential stages of the degradation of Mg-MPs (circles). Top left panel corresponds to an initial image and bottom right panel corresponds to a final image. Other panels correspond to intermediate images between initial and final images.

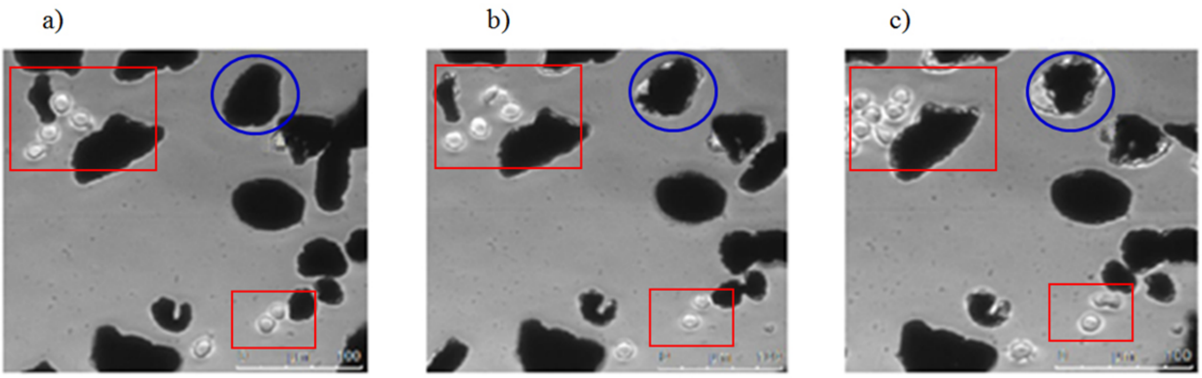

Fig. 2 Time-lapse microscopic images corresponding to a J774 macrophage culture with Mg-MPs (black).

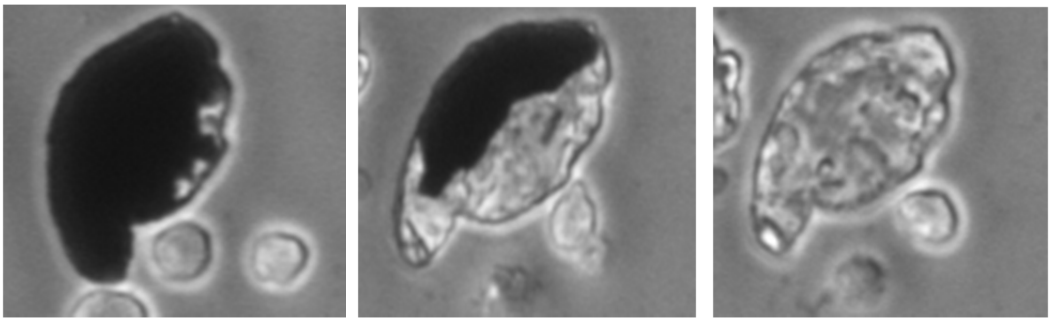

Fig. 3 Sequential stages of a Mg-MP degradation. The formation of $Mg(OH)_2$ layer (light grey) on the surface can be seen.

Images of a J774 macrophages culture in the presence of Mg-MPs are shown in Fig. 2. The degradation process of a Mg-MP (circle) can be seen at higher magnification than in Fig. 1. Modifications at the borders of Mg-MPs can be detected in Figs. 2b and 2c (particle in blue circle). Changes in the location of particles can also be detected. A more detailed sequence of the degradation of a Mg-MP can be seen in Fig. 3. The light grey deposits could be associated to $Mg(OH)_2$ formation according to equations [i] and [ii].

In Fig. 4 the assays with different concentrations of Mg- MP are shown with the aim of comparison. The images on the top correspond to the initial stage of the culture and those of the bottom after 24 h. Formation of $Mg(OH)_2$ can be detected on the border of some Mg-MPs. Cell division can also be observed (circles). The rectangle (Fig 4c) indicates that the location of cells and particles changed probably due to the turbulence exerted by hydrogen bubbles, as a result of Mg-MP corrosion process.

a) b) c)

1000 µg/ml Mg-MP 1500 µg/ml Mg-MP 2000 µg/ml Mg-MP

T0

T 24

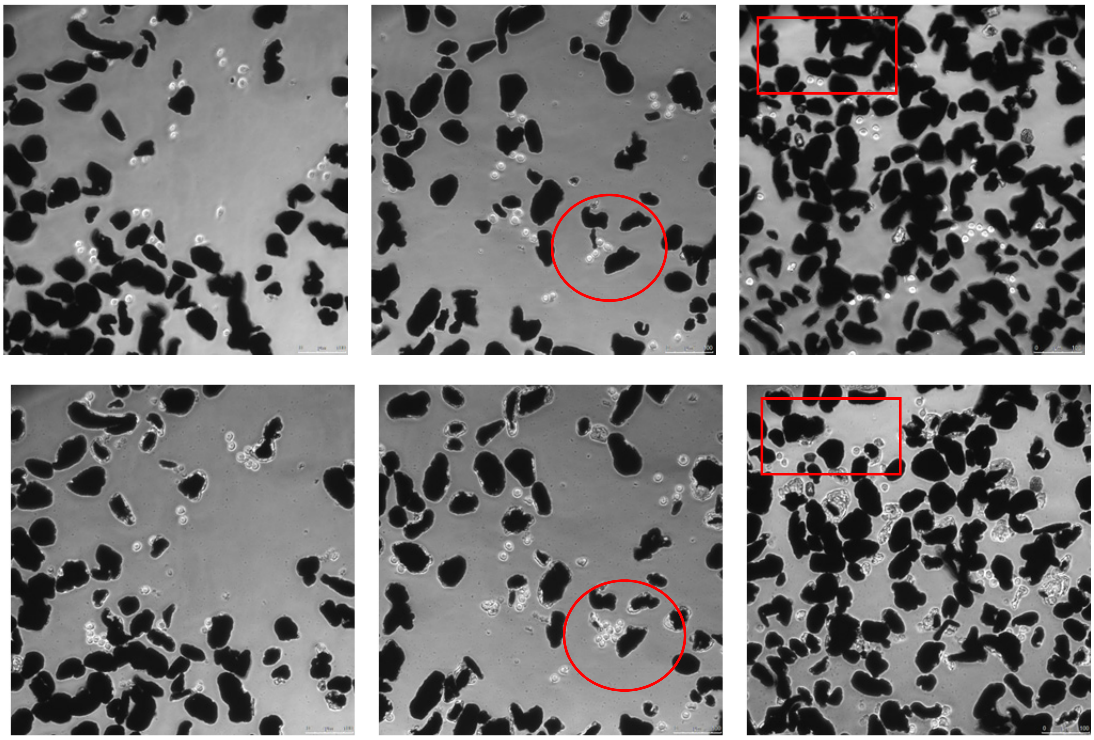

Fig. 4 Time-lapse microscopic images corresponding to a J774 macrophage culture with different concentrations of Mg-MPs (black).

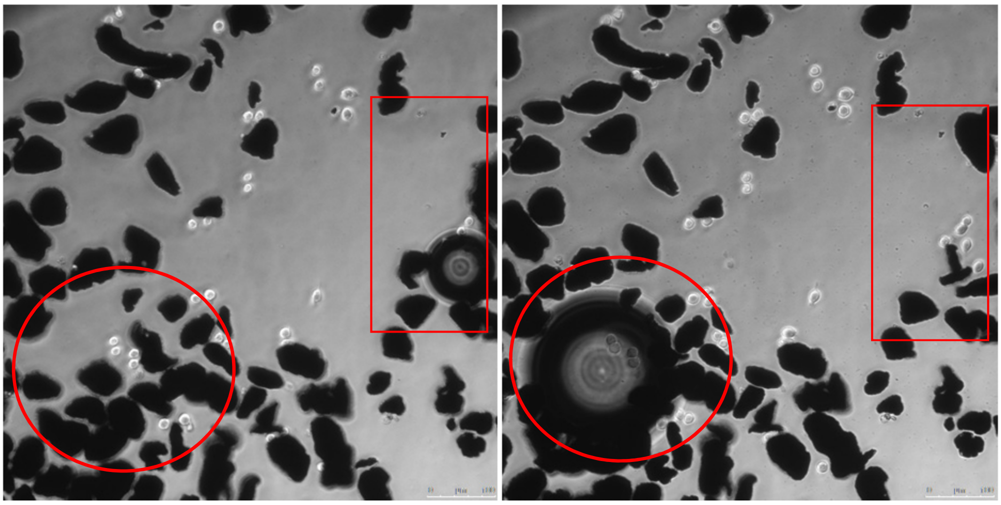

Fig. 5 Formation of hydrogen bubbles in J774 macrophage culture in presence of 1000 µg/ml of Mg-MPs. Figure (a) (rectangle) shows a bubble that is absent in the following image (b) because it was detached from the surface, and Fig. (a) circle shows a region in which a bubble was subsequently formed (b).

According to equation [i] corrosion of Mg-MPs leads to H_2 bubble development. H_2 bubbles formation was detected in the culture with Mg-MPs from the beginning to at least six hours after. Accordingly, Di Virgilio *et al*. [16] noted by light microscopy, H_2-bubbles after addition of Mg-MPs of similar size on osteoblastic cells. In Fig. 5 the generation of a bubble *in vivo* is shown. The bubble is detected in Fig. 5b but is absent in Fig. 5a (circle). Additionally, Fig. 5a shows a bubble on the left that is absent in the next image (Fig. 5b, rectangle). It can also be detected that the development of these bubbles produces high turbulence in the vicinity. The location of some particles and cells changed due to the turbulence generated during bubble detachment (Fig. 4c, rectangle). Noteworthy, 1000 µg/ml was the optimal higher concentration for these assays, due to the large amount of bubbles released at higher concentrations of Mg-MPs that hindered the observation.

6. Macrophage duplication in the absence and in the presence of Mg-MPs

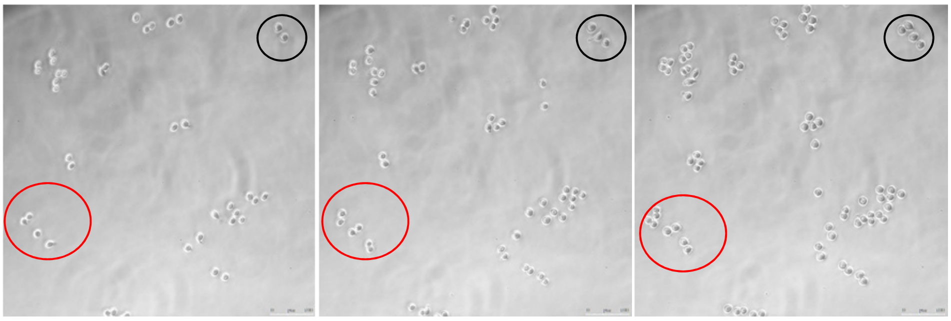

Fig. 6 Duplication of macrophages J774 in the absence of Mg-MPs.

In situ imaging of duplication of macrophages can be seen in Fig. 6. The number of cells significantly increased during the culture period (24h). In the absence of Mg-MPs no cell death could be detected in the images and the cells seem to be healthy.

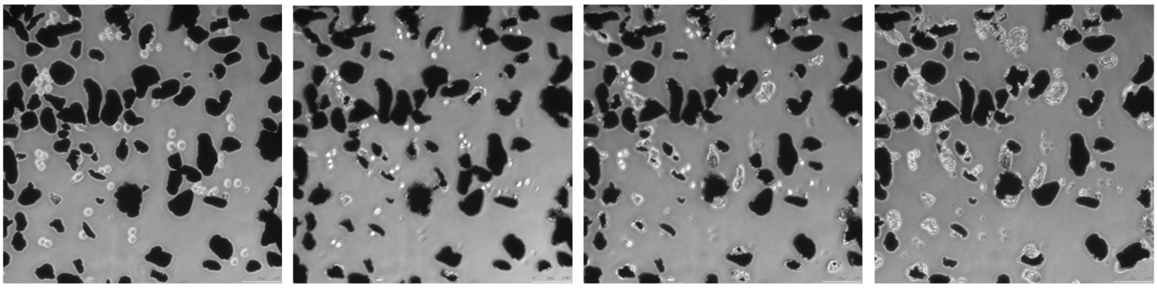

Fig. 7 Duplication of macrophages J774 in presence of 1500 µg/ml of Mg-MPs.

In the presence of Mg-MPs (Fig. 7) cells are affected by the alterations of the environment and duplication rate is slower than in the absence of Mg-MPs (Fig. 6). A high number of dead cells together with others that do not seem to be vigorous can be detected and simultaneously Mg-MPs are degrading in the vicinity (Fig. 7). Similarly, cells of Fig. 4c are strongly affected by the presence of high Mg-MPs concentration. This deleterious effect is less significant when the concentration of particles in macrophage cultures is lower.

7. Interactions macrophage/Mg-MPs

It is well known that filopodia are abundant in macrophages because they function as sensors of the local environment, particularly associated to their role during phagocytosis [30]. Yue *et al*. [29] found that particle size affects the cellular response in macrophages. Thus, the internalization of particles of diameters higher than 1.9 µm was very poor. In the case of cells/Mg-MPs system particles were large (average diameter 58.9 ± 20.7 µm (SD)) and no internalization was observed. In fact, only interactions between filopodia and Mg-MPs (1000 µg/ml) could be distinguished. In Fig. 8 panel two macrophages cells in the Mg-MP vicinity were observed. Later, one of these cells showed the formation and elongation of filopodia which extended towards the particle surface (Fig. 8 b). Interesting, as a consequence of this interaction Mg-MP was rotated (Fig. 8 c).

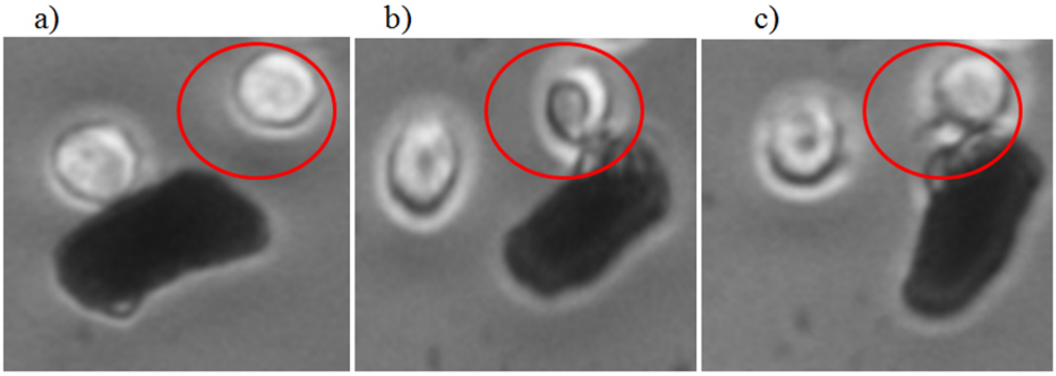

Fig. 8 J774 macrophages showing filopodia/Mg-MP interactions.

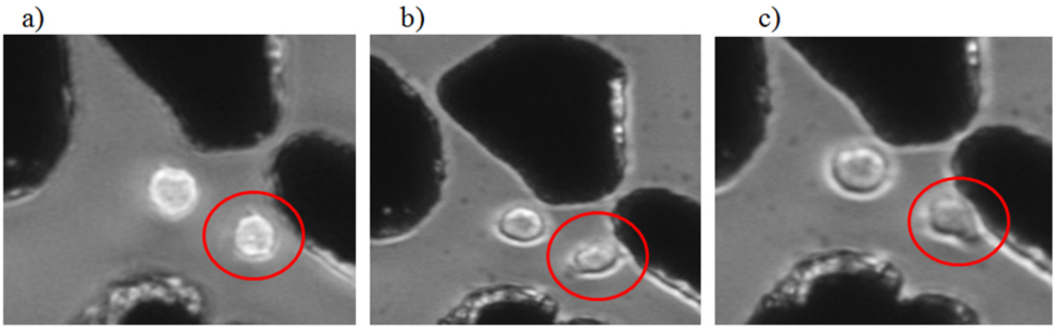

Fig. 9 J774 macrophages showing membrane/Mg- MP interactions.

In Fig. 9 panel a, two macrophages cells and a Mg-MP were close but not in contact. Subsequently one of the cells extended its filopodium (Fig. 9 panel b). Later, the cell contacted the particle through their plasmatic membrane (Fig. 9 panel c). It seems that the corrosion products on the surface (grey layer) did not affect these interactions.

8. Macrophage duplication and cell death

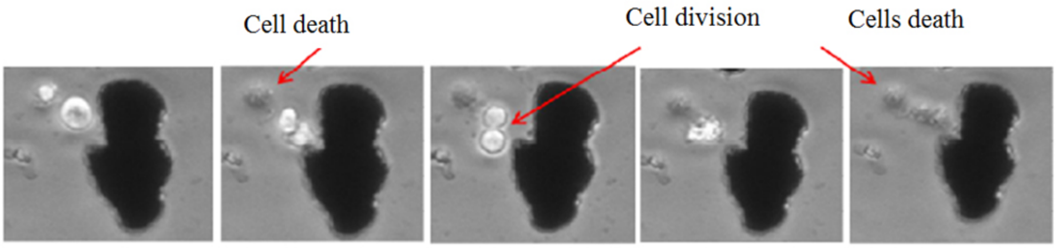

Fig. 10 Cell division and death of macrophages cultured in the presence of a Mg-MP.

MM technique is particularly useful to follow cell duplication at real time. Sequences of duplication processes can also be observed in Fig. 2 (rectangles) and in Fig. 4 (circles). Interestingly, Fig 10 panel shows that immediately after the division, macrophages located close to the Mg-MP displayed morphological changes that developed in cell death.

9. Conclusions

It was demonstrated that MM shows great capacity to follow biological and physicochemical processes at real time by collecting images that are recorded in fixed intervals over extended periods of time. During these periods the control of different cell culture parameters such as temperature, CO_2 pressure and 95 % humidity during the assay is required to achieve physiological relevant data. The resulting images of movies can provide direct insights into the behavior of cells including division, cell death and migration, among other processes. The images corresponding to interactions of J774 macrophages with Mg-MPs were particularly analyzed as an example of the potential use of the MM. Changes in the growth rate and morphology of macrophages due to interactions through their membranes and pseudopodia with Mg-MPs were detected *in vivo* at real time and, simultaneously, the formation of corrosion products and evolution of H_2 bubbles as a consequence of the degradation of Mg-MPs could be followed. The great functionality of MM during biological, cytomorphological and physicochemical assays was shown.

References

[1] Collins TJ. ImageJ for microscopy. BioTechniques 2007;43:25-30.
[2] Held M, Schmitz MHA, Fischer B, Walter T, Neumann B, Olma MH, et al. CellCognition: Time-resolved phenotype annotation in high-throughput live cell imaging. Nature Methods 2010;7:747-54.
[3] Huth J, Buchholz M, Kraus JM, Mølhave K, Gradinaru C, Wichert GV, et al. TimeLapseAnalyzer: Multi-target analysis for live-cell imaging and time-lapse microscopy. Computer Methods and Programs in Biomedicine 2011;104:227-34.
[4] Baradez M-O, Marshall D. The Use of Multidimensional Image-Based Analysis to Accurately Monitor Cell Growth in 3D Bioreactor Culture. PLoS ONE 2011;6:e26104.
[5] Schorsch S, Vetter T, Mazzotti M. Measuring multidimensional particle size distributions during crystallization. Chemical Engineering Science 2012;77:130-42.
[6] De Calderon Anda J, Wang XZ, Roberts KJ. Multi-scale segmentation image analysis for the in-process monitoring of particle shape with batch crystallisers. Chemical Engineering Science 2005;60:1053-65.
[7] Simon LL, Abbou Oucherif K, Nagy ZK, Hungerbuhler K. Bulk video imaging based multivariate image analysis, process control chart and acoustic signal assisted nucleation detection. Chemical Engineering Science 2010;65:4983-95.
[8] Simon LL, Nagy ZK, Hungerbuhler K. Endoscopy-based in situ bulk video imaging of batch crystallization processes. Organic Process Research and Development 2009;13:1254-61.
[9] Simon LL, Nagy ZK, Hungerbuhler K. Comparison of external bulk video imaging with focused beam reflectance measurement and ultra-violet visible spectroscopy for metastable zone identification in food and pharmaceutical crystallization processes. Chemical Engineering Science 2009;64:3344-51.
[10] Wang XZ, Calderon De Anda J, Roberts KJ. Real-time measurement of the growth rates of individual crystal facets using imaging and image analysis: A feasibility study on needle-shaped crystals of L-glutamic acid. Chemical Engineering Research and Design 2007;85:921-7.
[11] Sullivan J, Mehraban S, Elvins J. In situ monitoring of the microstructural corrosion mechanisms of zinc-magnesium-aluminium alloys using time lapse microscopy. Corrosion Science 2011;53:2208-15.
[12] Williford RE, Windisch Jr CF, Jones RH. In situ observations of the early stages of localized corrosion in Type 304 SS using the electrochemical atomic force microscope. Materials Science and Engineering A 2000;288:54-60.
[13] Witte F. The history of biodegradable magnesium implants: A review. Acta Biomaterialia 2010;6:1680-92.
[14] Witte F, Hort N, Vogt C, Cohen S, Kainer KU, Willumeit R, et al. Degradable biomaterials based on magnesium corrosion. Current Opinion in Solid State and Materials Science 2008;12:63-72.
[15] Zheng YF, Gu XN, Witte F. Biodegradable metals. Materials Science and Engineering R: Reports 2014;77:1-34.
[16] Di Virgilio A.L. RM, Arnal P.M, Fernández Lorenzo M. Biocompatibility of magnesium particles evaluated by in vitro cytotoxicity and genotoxicity assays. *J Biomed Mat Res B* 2011.
[17] Kirkland NT, Lespagnol J, Birbilis N, Staiger MP. A survey of bio-corrosion rates of magnesium alloys. Corrosion Science 2010;52:287-91.
[18] Witte F, Kaese V, Haferkamp H, Switzer E, Meyer-Lindenberg A, Wirth CJ, et al. In vivo corrosion of four magnesium alloys and the associated bone response. Biomaterials 2005;26:3557-63.
[19] Gu XN, Xie XH, Li N, Zheng YF, Qin L. In vitro and in vivo studies on a Mg–Sr binary alloy system developed as a new kind of biodegradable metal. Acta Biomaterialia 2012;8:2360-74.
[20] Yang F, Wu W, Cao L, Huang Y, Zhu Z, Tang T, et al. Pathways of macrophage apoptosis within the interface membrane in aseptic loosening of prostheses. Biomaterials 2011;32:9159-67.
[21] Catelas I, Petit A, Zukor DJ, Marchand R, Yahia L, Huk OL. Induction of macrophage apoptosis by ceramic and polyethylene particles in vitro. Biomaterials 1999;20:625-30.
[22] Huk OL, Zukor DJ, Ralston W, Lisbona A, Petit A. Apoptosis in interface membranes of aseptically loose total hip arthroplasty. Journal of Materials Science: Materials in Medicine 2001;12:653-8.
[23] Landgraeber S, von Knoch M, Löer F, Wegner A, Tsokos M, Hußmann B, et al. Extrinsic and intrinsic pathways of apoptosis in aseptic loosening after total hip replacement. Biomaterials 2008;29:3444-50.
[24] Stea S, Visentin M, Granchi D, Cenni E, Ciapetti G, Sudanese A, et al. Apoptosis in peri-implant tissue. Biomaterials 2000;21:1393-8.
[25] Trindade MC, Lind M, Sun D, Schurman DJ, Goodman SB, Lane Smith R. In vitro reaction to orthopaedic biomaterials by macrophages and lymphocytes isolated from patients undergoing revision surgery. Biomaterials 2001;22:253-9.
[26] Zhang XS, Revell PA. In situ localization of apoptotic changes in the interface membrane of aseptically loosened orthopaedic implants. Journal of Materials Science: Materials in Medicine 1999;10:879-83.
[27] Thomas V, Halloran BA, Ambalavanan N, Catledge SA, Vohra YK. In vitro studies on the effect of particle size on macrophage responses to nanodiamond wear debris. Acta Biomaterialia 2012;8:1939-47.
[28] Pearl JI, Ma T, Irani AR, Huang Z, Robinson WH, Smith RL, et al. Role of the Toll-like receptor pathway in the recognition of orthopedic implant wear-debris particles. Biomaterials 2011;32:5535-42.
[29] Yue H, Wei W, Yue Z, Lv P, Wang L, Ma G, et al. Particle size affects the cellular response in macrophages. European Journal of Pharmaceutical Sciences 2010;41:650-7.
[30] Kress H, Stelzer EHK, Holzer D, Buss F, Griffiths G, Rohrbach A. Filopodia act as phagocytic tentacles and pull with discrete steps and a load-dependent velocity. Proceedings of the National Academy of Sciences of the United States of America 2007;104:11633-8.