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Paper

"Turing mechanisms under pressure": Mesenchymal condensations could be involved in digit patterning of mice.

Henrik Pinholt,*[a] Heura Cardona Blaya,[b] James Harvey Swoger and James Sharpe[b]

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In recent years, some attention has been directed towards the possibility that mechanical forces could play a role in creating the expression patterns observed in vertebrate body parts. By compressing regions of cultured embryonic mesenchymal stem cells from mouse autopods, this study provides a novel way of testing effects of mechanochemical stimulation of the Turing mechanism responsible for digit patterning in mice. The study found important features to be included in these compression systems for mesenchymal cells: Com- pressing a small area of the culture, using transparent pistons, and maintaining a stable environment from vibrations and disturbances. The results suggest that mechanical forces upregulates Sox9 in the boundary between compressed and uncompressed regions, but doesn't seem to change the compressed pattern in comparison to uncompressed cells. However incomplete, these results suggest that both chemical and mechanochemical regulation of Sox9 might be involved in patterning the digits in mice.

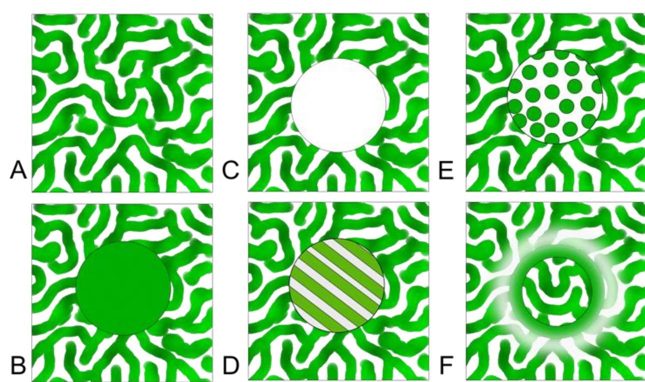


Figure 1: A) In micromass cultures of E11,5 mesenchymal tissue Sox9 is maintained in a stable Turing pattern regulated by Wnt and BMP. B) A compression might upregulate the entire region through mechanically stimulated Sox9 synthesis. C) It could also lead to down regulation through Wnt and BMP signaling. Or maybe the compres- sion changes the Turing mechanism and leads to new patterns like stripe formation (D), dot formation (E) or maybe the interactions happen only in the border zone between the compressed mechanism and the uncompressed (F).

Introduction

The attempt to understand why our body is shaped the way it is, seems to be one of the most challenging questions in the field of developmental biology. An important distinction to make in this regard is the difference between local up- and down regulation of genes, and the spatial patterning of genetic expression to control the morphology of resulting tissue[1]. The study of the latter took a new turn a few years ago, when experiments, conducted by Raspopovic et al., showed how a Turing mechanism[2,3] controls digit patterning in mice: In their model, Bmp and Wnt act under influence of Fgf signaling and Hox13, to position expression of bone-inducing Sox9[4] in digital regions, and the apoptosis inducing Bmp[5] is localized in interdigital mesenchyme with apoptotic cell faiths[6].

This study falls in an increasingly large category of studies investigating the possibility that a Turing mechanism, originally proposed by Alan Turing in 1952, could be driving patterning of some vertebrate body features[7-13]. These studies are different from most standard ways of approaching vertebrate development in that they investigate the global expression patterns of genes, and not local up- and downregulation.

Many great discoveries have been made from looking at the chemical signaling of morphogens[14,15]. However, in recent years, it has been found that One such environmental problem is the fact that mechanical forces can induce genetic expression[16-19]. This fact has led to an increased attention towards the effects of mechanics on patterning the embryo[20-24]. The same applies for digit patterning, and the biochemical pathways used in the Turing mechanism proposed by Raspopovic et al. is not the only way to regulate bone formation. Past studies show that mechanical forces alone, can activate expression of Sox9 and several other chondrogenic factors[25]. The question is therefore if this Sox9 activation pathway is relevant for patterning in vertebrate digit formation. Indeed, in recent years, theories and simulations on more general mechano- chemical regulation of morphology has indicated that mechanochemical pathways could be regulating Turing mechanisms[26,27]. A great deal of work has been put into this computationally[28-31], but to our knowledge, not much experimental work has been done to investigate these mechanochemical effects in relation to Turing mechanisms. This might, in part, be due to the difficulty of working with physical effects when you go from testing local genetic activation or inactivation, to testing global effects of mechanic stimuli to expression patterning.

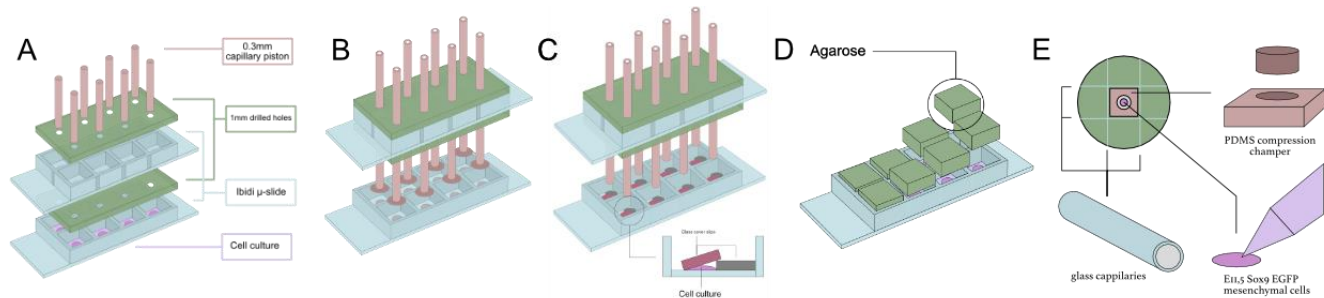


Figure 2: Compression chamber designs. A) The first compression system used metal capillary pistons to compress the cells. B) The second device was made by gluing glass cover slips on 1,5mm glass capillaries. C) A variation of this was done by placing the glass cover slips on the culture and then pushing it down with glass capillaries. The two glasses were laid on top of each other, producing a gradient pressure. D) Hydrostatic pressure was created by casting blocks of agarose gels in µ-slides (Ibidi), and put- ting weights on top. E) We also tried using compression champers cut from blocks of PDMS placed in an Ibidi µ-dish to create hydrostatic pressure.

Here we present an attempt to address this issue. However incomplete, this study seeks to provide a fresh perspective to the topic of testing mechanochemical effects on Turing mechanisms, and provides evidence to support that these mechanochemical interactions could perhaps be involved in the Turing mechanism operating in mouse digit formation.

Digit formation in a petri dish

Raspopovic et al. showed that E11.5 mesenchymal cells in micromass culture express Sox9 in a Turing pattern. This pattern is created in the same fashion as in the digits and could therefore be used as a way of studying the digit patterning process in vitro. By culturing mesenchymal stem cells, we had a way of investigating the global effects of mechanical stimuli on the patterning process rather than the local up- or downregulation of genes. By introducing a compressive force to the culture, we could directly observe how the mechanical forces changed the pattern (fig 1). If the compression just up- or downregulated Sox9, the mechanical forces would not play a role in the spatial patterning of the mechanism (fig 1A-C), but if the pattern, on the other hand, changed un- der compression (fig 1D-F), the mesenchymal condensations during bone formation might play a role in the Turing mechanism. This idea provided the basis for the experimental work in this article.

Materials and methods

Compression chamber design

The mesenchymal condensations in digit formation was simulated using physical compression devices (fig 2). The systems were fabricated by drilling holes through two lids and a µ-slide from Ibidi. These where then glued together using Araldite Cristal. The holes where 1mm in diameter for the capillary pistons (fig 2A) and 1,6mm for the 1,5mm glass capillaries used in the other two devices (fig 2B-C). The capillary pistons were put directly on the culture, and for the glass capillary devices, a 5mm circular glass cover slip was either glued on the capillary (fig 2B) or placed directly on the culture (fig 2C). Weights were put on the top of the pistons and were made by attaching Alpino Plastilina to the top of the pistons.

Micromass culture & imaging

Sox9-EGFP transgenic mice embryos were removed and micro dissected in ice cold PBS to re- move limbs and hindlimbs. E11.5 mice were used for the capillary piston devices, and E12.5 mice were used for the glass slide devices. Autopods were micro dissected, and then digested in 0,5% Trypsin EDTA. The ectoderm was removed, and cells where filtered in a 35 µm cell strainer cap and resuspended in PBS. Cells where plated as10µL drops with a cell concentration of $2.5 \cdot 10^7$cells/mL in 8 well µ-slides (Ibidi). Cells were allowed to attach for one hour at 37°C, 5% CO2 before adding DMEM/F12 with L-Glut +10%FBS +1% Penicillin/Streptomycin to each well. Hereafter the compression was introduced. Cells where incubated at 37°C, 5% CO2 for 24 hours before imaging results.

Following incubation, medium was removed, and cells were stained with DAPI for 15 minutes, samples were washed with PBS and fixed in Performaldehyde for 30 minutes and then washed in PBS. Results were viewed on a Leica TCS SP5 for closeup images, and mosaics where done on a Zeiss Cell Observer HS with 5x objective. Stitching was done using MosaicJ[32], and all other editing was done using Fiji.

Results

Device design

The metal pistons had a smaller surface area than the culture (fig 3). This allowed for an internal control in each well, since the compressed region was next to an uncompressed Turing pattern (fig 2A). The cells adhered to the bottom of the pistons, and the culture was ripped when pistons were removed. This stopped us from viewing the compressed pattern under the piston (fig 3B-C). The results therefore did not, shed light on the behavior of the system under compression since the compressed region was not visible.

This problem was solved partly by using glass cover slips. Gluing the coverslips to the capillaries killed the cells (results not shown), but by placing the cover slips on the cell culture, you could see the compressed region (fig 4). The problem was lack of stability. Since the glass slides were not attached, but merely laid on top, there was no way of knowing if the slides moved, and the system needed greater stability to function properly (fig 4A). It did, however, prove to be a system that easily allowed for visualization and compression of the sample. DAPI staining did not diffuse properly, but bright-field images prove cell density to be uniform underneath the glass, and Turing patterns was could be found underneath the slides (fig 4B).

Pattern changes

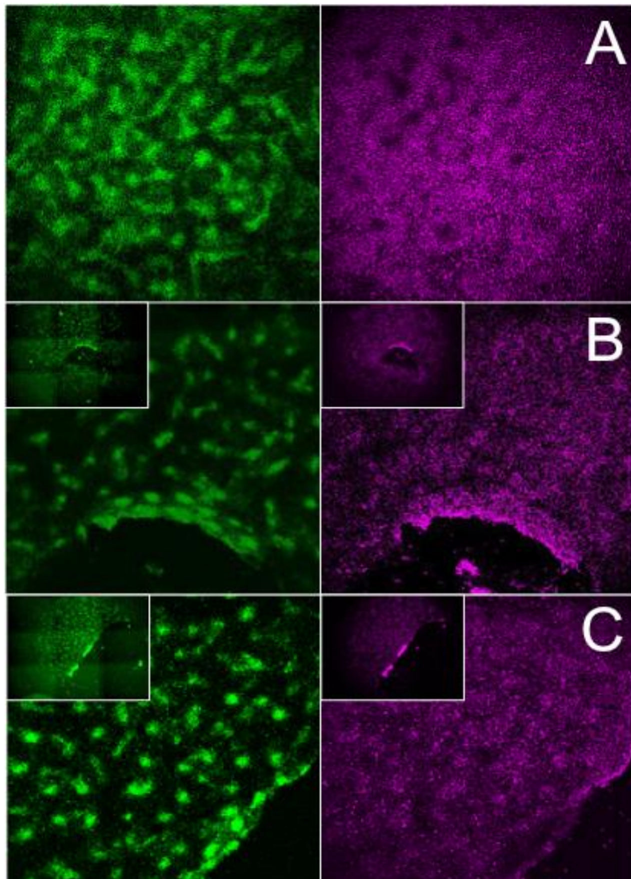


Figure 3: CLSM images and 3x3 Zeiss mosaics (inserts in B and C). Green is EGFP-Sox9, and magenta is DAPI. A) Control, showing the normal Turing pattern in culture. B) 0.5g compressed sample with capillary piston. The stimulus is surrounded by a control pattern, which allows for comparison C) 2.5g compressed sample with capillary piston. Due to cell adhesion, the compressed cells were removed with the pistons upon imaging, and the compressed pattern could not be viewed in this system.

Even though the two devices had very different physical impact on the culture, they sometimes showed the same results (fig 5). Some of the cultures had significant upregulation of Sox9 on the border between uncompressed and compressed areas. In some cultures, there was also a down-regulation in the nearby surrounding uncompressed region (fig 5A-B). The glass devices allowed for visualization of the under- lying compressed region in these border phenomena (fig 5C). This revealed that the compressed region still expressed a Turing pattern, and that the uncompressed region seized to express the pattern. This would make sense since the upregulation of Sox9 would lead to the induction of the diffusing Sox9 inhibitors Wnt and BMP, and ultimately inhibit Sox9 in surrounding tissue. Since the compression upregulates Sox9, this might counteract the effects of the inhibitor in compressed regions, but the effects are still visible in uncompressed parts of the culture since there is no mechanical compression to induce Sox9, and the inhibitors dominate these regions.

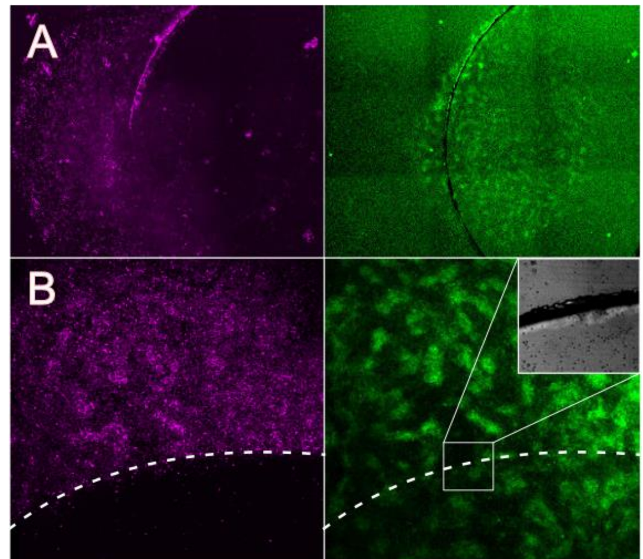


Figure 4: A) Mosaic of homogenous 0.5g pressure on glass cover slides. The DAPI-staining clearly shows the disruptive forces of the unstable system. B) Confocal images of another well showing the boarder (white dots) between compressed (bottom) and uncompressed (top). The brightfield insert shows cells distributed both under and outside the glass.

Discussion

The compression systems used in these experiments was prototype constructs, and therefore the results cannot be seen as concrete evidence for an interaction between mesenchymal condensations and the Turing mechanism. The pistons and cover glasses were not completely fixed, and perturbations lead to several uncertainties as to what were Sox9 induced mesenchymal condensations, and what were cell movements from moving the de- vices and pistons. An important consideration for future work with testing mechanochemical effects on patterning through a cell culture system like this, is therefore to reduce vibrations, and maybe even do imaging, compression and culturing at the same place. Another lesson to learn from these experiments is the importance of only compressing a fraction of the cell culture. As seen from the results, a smaller area of compression allows for an internal control in each well. This is very important since Turing patterns are very hard to compare across cultures, and every environment is different. This leads to the third consideration when compression mesenchymal cell cultures: The importance of using transparent pistons. Using the glass cover slips, we were able to observe both compressed and uncompressed cells. This allowed for comparative studies, and the interesting border phenomena could be observed. By working with these three principles: Small area of compression, stability and transparency, one will obtain the best results when experimenting with the digit Turing mechanism.

As mentioned in the results, some wells where characteristic in that they had an upregulation of Sox9 in the border between compressed and uncompressed cells. Sometimes this border was followed by a Sox9 inhibited region. These results suggest that compression could play a role in regulating the morphology of bone patterning. A possible explanation for these observations

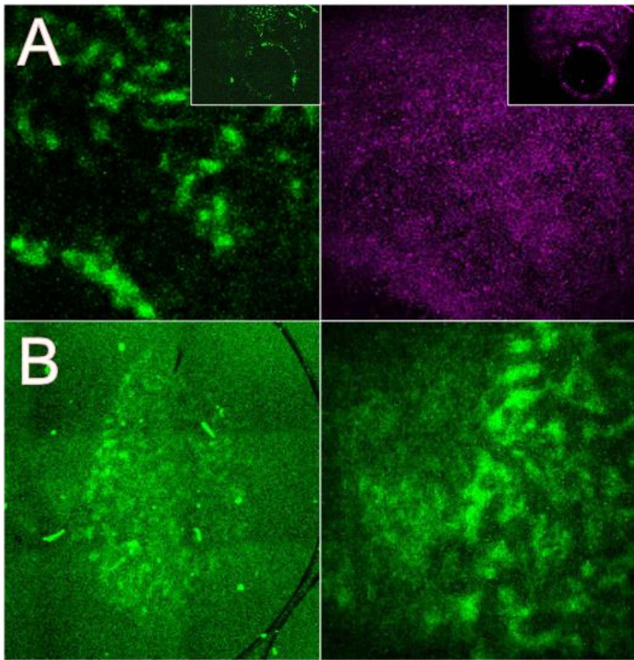
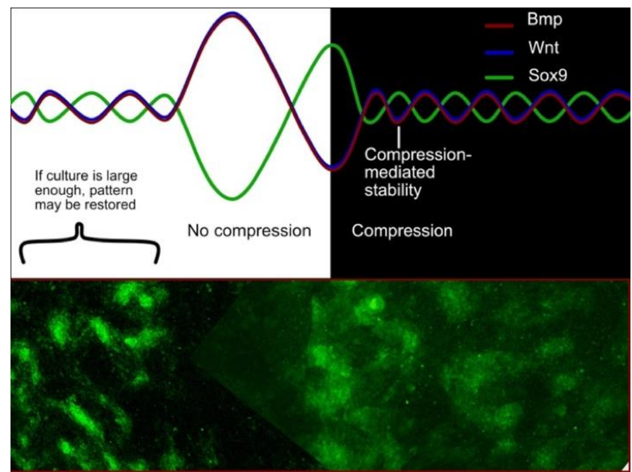


Figure 5: A) Confocal images showing the boarder region between the piston and the culture. GFP (left) and Dapi (right) is clearly upregulated next to the piston, but an area with no expression separates the Turing Pattern from this upregulated region. Inserts shows Zeiss mosaics showing that this behavior exist in the entire region facing the culture. B) Zeiss mosaic GFP (left) and CLSM (right)

could be that since compression is somewhat uniform under the piston, Sox9 is evenly upregulated under the glass. This allows for the formation of a periodic Turing pattern. However, in the border region, mechanically induced Sox9 diffuse outside the glass and creates an upregulated border region. Sox9-induced inhibitors diffuse into the surrounding uncompressed cells, and create a region of Sox9 cells. This wave of inhibition would stop due to the Turing mechanism upregulating local Sox9 at a distance to the compression depending on how much force was applied to the cells. This diffusion of inhibitors would not influence the compressed pattern, since Sox9 is produced through mechanochemical induction, and therefore the overall pattern stays the same under compressed conditions (fig 6). This could mean that the Turing mechanism in the digits helped guide early patterning of Sox9, Bmp and Wnt. Hereafter, Sox9-induced condensations in digit areas would up regulate Sox9 further in these areas, but in interdigital mesenchyme, the Sox9 expression would be downregulated tremendously. It is possible that this mechanism could be factor that helped stabilizing the initial pattern defined by the Turing mechanism discovered by Raspopovic et al.

Conclusions

These experiments serve, as a small explorative examination of how mechanochemical interactions could be relevant in patterning processes of vertebrate development. The study has many associated uncertainties and should be thought as more of as a new perspective, than a definitive proof. In this study, we show new methods for testing the global expression of genes and put forth data suggesting the possibility of interplay between



mesenchymal condensations and patterning of the digits. Hopefully these results will give rise to new ideas and approaches to the rather tricky topic of testing the effects of mechanical forces in developmental patterning processes.

Notes and references

[a]: *Nano-Science Center, University of Copenhagen, Universitetsparken 5, 2100 København Ø, Denmark.; E-mail: henrikpinholt@hotmail.com*

[b]: *Systems Biology Program, Centre for Genomic Regulation (CRG), and Universitat Pompeu Fabra (UPF), Dr. Aiguader 88, 08003 Barcelona, Spain.*

1. AJ. Koch, H. Meinhardt, *Rev Mod Phys*, 1994, **66**, 1481-507;
2. AM. Turing, *Phil Roy Biol*, 1952, **237(641)**, 37-72;
3. S. Kondo and T. Miura, *Science,* 2010, **329(5999),** 1616-20
4. E. Wright, MR. Hargrave, J. Christiansen, L. Cooper, J. Kun and T. Evans et al., *Nat Genet,* 1995, **9(1)**, 15-20;
5. E. Wright, MR. Hargrave, J. Christiansen, L. Cooper, J. Kun, T. Evans et al., *Nat Genet,* 1995, **9(1)**, 15-20
6. MM. Kaltcheva, MJ. Anderson, BD. Harfe and M. Lewandoski, *Dev Biol,* 2016, **411(2)**, 266-76

Figure 6: A possible theory on how mechanical forces interacts could interact with the Turing Pattern in the digits.

7. MM. Kaltcheva, MJ. Anderson, BD. Harfe and M. Lewandoski, *Dev Biol,* 2016, **411(2)**, 266-76
8. J. Raspopovic, L. Marcon, L. Russo and J. Sharpe, *Science,* 2014, **345(6196)**, 566-70
9. J. Guiu-Souto, J. Carballido-Landeira,V. Pérez-Villar and AP. Muñuzuri, *Phys Rev, E.* 2010, **82(6)**, 066209
10. R. Sheth, L. Marcon, MF. Bastida, M. Junco, L. Quintana, R. Dahn et al., *Science,* 2012, **338(6113)**, 1476-80
11. AD. Economou, A. Ohazama, T. Porntaveetus, PT. Sharpe, S. Kondo, MA. Basson et al., *Nat Gene,* 2012, **44(3)**, 348-51
12. P. Müller, KW. Rogers, BM. Jordan, JS. Lee, D. Robson, S. Ramanathan et al., *Science,* 2012, **336(6082)**, 721-4
13. S. Sick, S. Reinker, J. Timmer and T. Schlake, *Science,* 2006, **314(5804)**, 1447-50
14. J. Lefèvre, J-F. Mangin, *Com Biol* 2010, **22;6(4)**, e1000749;
15. RO. Prum and S. Williamson, *Proc Roy Biol,* 2002, **22;269(1493)**, 781-92
16. K. Unsicker and K. Krieglstein, *Wiley-VCH,* 2006, 523-578
17. SF. Gilbert and MJF. Baressi, *Sinauer Associates,* 2016, **11**, 810
18. NJ. Steinmetz, EA. Aisenbrey, KK. Westbrook, HJ. Qi and SJ. Bryant, *Acta Biomater,* 2015, **15(21)**, 142-53
19. AJ. Steward, DJ. Kelly, *J Anat,* 2014, **227(6)**, 717-31
20. P. Strzyz, *Nat Rev Genet,* 2016, **17(9)**, 505

21 KM. Warren, MM. Islam, PR. LeDuc and R. Steward, *App Mat Int,* 2016, **8(34)**, 21869-82.

22 T. Mammoto and De. Ingber, *Development,* 2010, **137(9)**, 1417-20

23 J. Foolen, T. Yamashita and P. Kollmannsberger, *J Phys D,* 2016, **49(5)**, 053001

24 P. McMillen and Sa. Holley, *Gen Dev,* 2015, **32**, 106-11

25 LV Beloussov, *Eur phys J E,* 2013, **36(11)**, 132

26 JH. Henderson and DR. Carter, *Bone,* 2002, **31(6)**, 645-53

27 I. Takahashi, GH. Nuckolls, K. Takahashi, O. Tanaka, I. Semba, R. Dashner et al., *J Cell Sci,* 1998, **30(111)**, 2067-76

28 J. Howard, SW. Grill and JS Bois, *Nat Rev Mol,* 2011, **12(6)**, 400-6

29 NW. Goehring and SW. Grill, *Trends in Cell Biol,* 2013, **23(2)**, 72-80

30 M. Mercker, F. Brinkmann, A. Marciniak-Czochra and T, Richter, *Biol Dir,* 2016, **11(22)**, 72-80

31 LG. Morelli, K. Uriu, S. Ares and AC. Oates, *Comp Appr Dev Pat,* 2012, **336(6078)**, 187-91

32 Ma. Wyczalkowski, Z. Chen, BA. Filas, VD. Varner and LA. Taber, *Emb Tod Rev,* 2012, **96(2)**, 132-52

33 L. Truskinovsky, G. Vitale and TH. Smit, *Engi Sci,* 2014, **83**, 124-37

34 P. Thévanaz and M. Unser, *Mic Res Tech,* 2007, **70(2)**, 135-46