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TECHNICAL NOTE

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Automated in situ detection (AISD) of biomolecules

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Abstract In order to facilitate in situ detection of biomolecules in large sample series the processing of whole-mount specimens has been automated. A freely programmable liquid handling system is described by which embryos or similar biological materials are processed. Possible applications include in situ hybridization (ISH), immunocytochemistry (ICC) or reporter gene assays. Process times required for the preparation of whole-mount in situ hybridizations in *Drosophila*, *Xenopus*, *Gallus* and in hydroids were – in part – significantly reduced as compared with manual processing. Application of automated in situ detection (AISD) in random screening is demonstrated in hydroids. Potential further applications are discussed.

Key words *Hydractinia echinata* · Whole-mount in situ hybridization · Expression analysis · Random screen · Differential display

Introduction

In situ hybridization (ISH) techniques are usually applied to characterize the function of genes assumed or already recognized to be important in development. ISH analysis is informative because spatial expression domains become obvious and temporal aspects of gene activity are directly monitored in the course of developmental processes. In respect of spatial resolution, ISH is superior as compared to northern analysis. For visualization of gene expression in a few single cells in the embryo it is the method of choice.

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So-called developmental genes have been isolated in the course of saturation mutagenesis screens, e.g. in *Drosophila melanogaster* (Nüsslein-Volhard and Wieschaus 1980), in *Caenorhabditis elegans* (e.g. Hirsh and Vanderslice 1976) in *Danio rerio* (Mullins and Nüsslein-Volhard 1993; Mullins et al. 1994; Driever et al. 1994; Solnica-Krezel et al. 1994) and in *Arabidopsis* (Jürgens et al. 1991; Mayer et al. 1991), or by screening for homologues. Most of these known control genes have restricted spatiotemporal expression patterns. Vice versa, it should be possible to search for novel developmental genes by using randomly selected probes and screening for conspicuous expression patterns (random screens). This approach would be most interesting in organisms which are not easily accessible for mutational screens and genetic analysis. Random screens have been congenially developed and started in several laboratories working in the field of developmental biology.

Three major problems are encountered when planning random screens on a large or even saturating scale:

1. The method of probe generation and labelling has to be efficient, involving as few steps as possible and it should make allowances to avoid redundancy.

2. Whole-mount ISH has to be carried out in numerous samples.

3. The processed specimens have to be inspected and expression patterns have to be documented as accessible data.

One of us (M.G.) proposed to automatize whole-mount ISH and thus initiated the project. Here we report development and application of a device that allows us to process specimens like embryos or blocks of tissue (biopsies) through serial incubation steps. In respect to the practicability of large scale random in situ screens we thus address the second problem in particular. The aim of this study is to explore AISD in several systems and its application for random screens. Considering the possibility of conducting very large series of ISH preparations under facilitated conditions novel applications of ISH in gene expression analysis are discussed.

Material and methods

Hybridization protocols

Hybridization procedures were performed as described elsewhere (see Table 1) with slight modifications. Briefly, digoxygenin-labelled probes were used. RNA-hybrids were detected by alkaline phosphatase-conjugated antibodies. Antibody incubations which are usually overnight steps at 4°C were reduced to 6 h incubations at room temperature (*Gallus* and *Xenopus*). The incubations with 100 mM NaCl, 100 mM TRIS-Cl pH 9.5, 50 mM $MgCl_2$, 1% Tween 20 and 2 mM levamisole (NTMT) in the chick protocol including an overnight step were replaced by four washes of 20 min each. In *Xenopus*, incubation with 80 g/l NaCl, 2 g/l KCl, 30 g/l TRIS-Cl pH 8.0, 0.1% Triton X 100 (TBSX) was done five times for 1 h each instead of four times for 1 h and one overnight incubation. The incubation step following the high stringency washes was prolonged to 20 min. The program lists are available on request.

Heat treatment was performed manually. After initial fixation according to the respective protocol, the specimens were rinsed once with the respective RNase-free buffer (phosphate buffered saline) and transferred to 1.5-ml reaction tubes together with 1 ml fresh buffer. Heat denaturation was performed by immersing the tubes for 5 min in a 95°C water bath. Then the samples were snap-cooled on ice. It is important to resuspend the specimens just before and during heat incubation (twice) and once again during subsequent cooling. After heat treatment, automated processing was started with the prehybridization step. Alternatively, the specimens can be stored in buffer at 4°C for a couple of weeks without loss of quality.

Probe generation and hybrid detection

Probes were prepared as hydrolized riboprobes. For *Drosophila* a random-labelled cDNA-probe was used. For use in the in situ processor the concentration of $MgCl_2$ in the alkaline phosphatase buffer was reduced from 50 to 5 mmol/l. This buffer is stable for at least 3 days and does not form precipitates. In order to detect labelled mRNA hybrids, the specimens were transferred into multiwell plates and subjected to chromogenic substrate reaction under visual control. The reaction mix was composed of alkaline phosphatase buffer with normal (50 mmol/l) $MgCl_2$ concentration and 175 µg/ml 5-bromo-4-chloro-3-indolylphosphate (BCIP) and 225 µg/ml nitrobluetetrazolium (NBT).

Results and discussion

Description of the in situ processor

An automated in situ processor was developed. The device is capable of carrying out the liquid handling steps in whole-mount in situ detection. In principle, protocols for ISH, immunocytochemistry (ICC) and enzyme-based assays of reporter gene activity can be applied. In the current version of the instrument, up to 55 samples are processed in parallel with the possibility of using individual probes for each sample. The instrument consists of a pipetting robot equipped with a rack with a thermostat for sample containers and racks for probes, reagents and wash solutions. The specimens are contained in small flow-through vessels equipped with filter frits as supports (Fig. 1). The outlet of this sample container is connected to a tube which extends into a water bath. The hydrostatic pressure thus generated keeps liquids in the tubes even during long reaction times. The vessels are closed with a sealing cap bearing a small opening in its centre. Reagents are introduced or removed with a specially designed double needle made from concentric tubes. The inner tube is used to deliver reagents without creating pressure on the sample container (Fig. 1A). To remove reagents, the needle is lowered to the sealing position and nitrogen is delivered between the outer and inner tube (Fig. 1B). Sealing is accomplished by matching the diameter of the outer pipette with the aperture of the sealing cap. As the hydrostatic counterpressure is only about 15 mm water, a low pressure is sufficient to empty the sample container. The specimens are immersed in reagent of the subsequent incubation step within a few seconds. The instrument is controlled by software that allows us to set up the procedure along manual protocols in an easy and straightforward manner. At the same time, a large number of parameters are accessible for fine-tuning of a procedure. Protocols can be defined using a personal

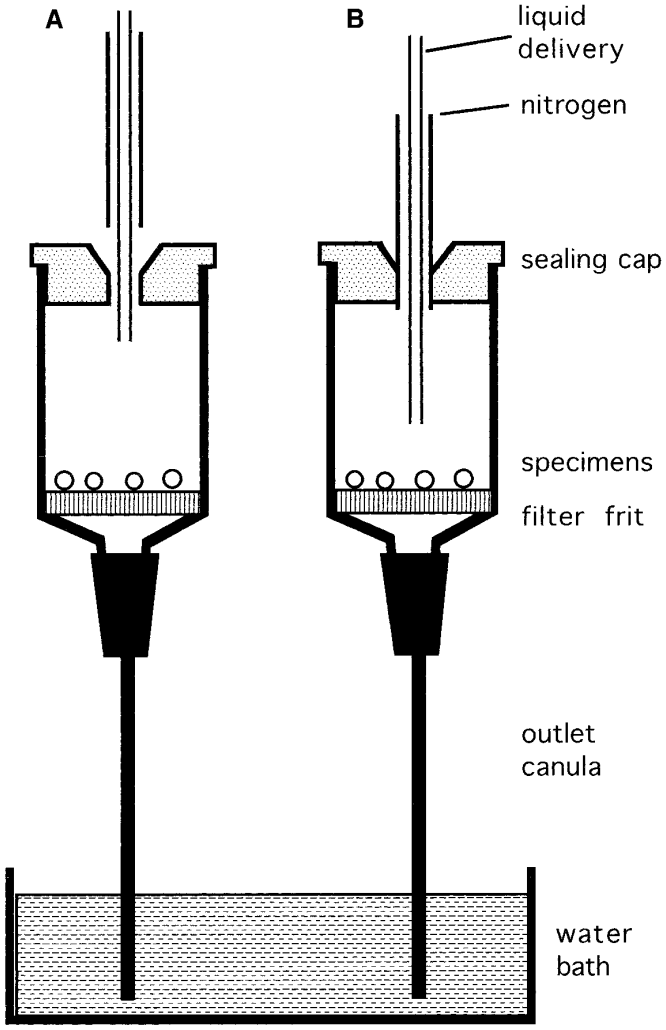

Fig. 1A, B Flow-through sample container of the in situ processor. **A** Liquid delivery. **B** Pipette in sealed position for liquid displacement. For further description see text

computer and are run from a controller unit equipped with a floppy disk drive. In principle, it is possible to start the protocol with the first fixation step and recover specimens from the instrument ready for inspection. The instrument described here is available on the market (Abimed-Analysentechnik, D-40764 Langenfeld, Germany).

AISD in various species – ISH

ISH was performed in all species by using dig-labelled probes and according to protocols modified from the original method by Tautz and Pfeifle (1989).

Embryos of *Xenopus laevis* were processed according to a protocol of Harland (1991) and hybridized to a probe of the *Xenopus* homologue of *brachyury* (*Xbra*; Smith et al. 1991). Automated processing was started by loading fixed embryos in methanol to the in situ processor. The *Xbra*-typic hybridization pattern was obtained in background-free preparations (Fig. 2A).

Developmental stages of hydroids were extensively studied by automated in situ detection (see also below). In Fig. 2B hybridization of a neuropeptide precursor probe (common precursor for Pol-RFamide II, He-RNamide, He-RYamide neuropeptides of *Hydractinia echinata*; Gajewski et al., in press) in nerve cells of *Hydractinia echinata* planula larvae is shown.

Chick embryos, Hamburger and Hamilton-stage 4, were fixed and heat treated instead of undergoing proteinase-K treatment (see below). The specimen depicted in Fig. 2C was stored in buffer at 4°C for 1 month and then hybridized to a chick *goosecoid* (1)-probe (Izpisúa Belmonte et al. 1993) according to a protocol of Wilkinson (1992).

For *Drosophila melanogaster* a shortened version of the protocol originally developed by Tautz and Pfeifle (1989) was used with an important modification. We substituted the proteinase K/post-fixation step by a heat treatment which very effectively denatures proteins and makes the mRNA accessible to the probe. In addition, this treatment avoided the problem of the specimens becoming sticky due to proteinase-K treatment and adhering to each other in the subsequent post-fixation steps. In all systems analysed so far (chicken, fly and hydroids), heat denaturation resulted in much better structure conservation as compared to proteinase-K treatment. The accessibility of mRNA as judged from the intensity of the hybridization signals was equal or even better after heat denaturation. In addition, protocols are significantly shortened. Proteinase-K treatment and refixation, including buffer washes, requires approximately 3 h of processing time. The duration of heat treatment and the subsequent snap-cooling step is 15 min in total. Heat-treated *Drosophila* embryos of different stages were loaded to the processor and hybridized to a *bagpipe* (Azpiazu and Frasch 1993) random-primed cDNA probe. Hybridization signals obtained after AISD were not distinguishable from those obtained after manual processing for all described specimens and probes.

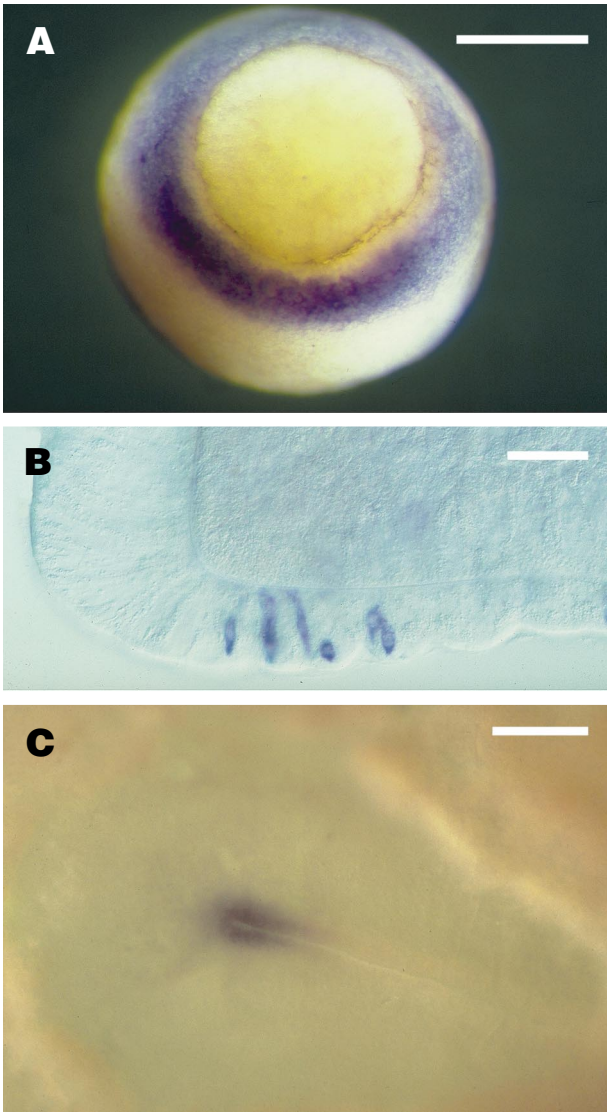

Fig. 2A–C AISD of gene expression in various species. **A** Hybridization of *Xbra* probe to early gastrula stage of *Xenopus laevis*. **B** Planula larva of *Hydractinia echinata* hybridized with a probe to the common precursor of the neuropeptides Pol-RFamide II, He-RYamide and He-RNamide. Only one half of the anterior larva is shown. **C** Stage 4 embryo of *Gallus domesticus* hybridized to chick *gsc*1 (*Scale bars* **A**, **C** 0.5 mm; **B** 50 μm)

By AISD, ISH experiments become less laborious because manual work is restricted to loading and deloading the processor. In addition, however, a substantial gain in performance is achieved since process times decrease remarkably (Table 1). It is obvious that the gain increases as the protocols increase in complexity and length. Time is saved because liquids are exchanged quicker by the robot than by hand. In addition, at no time is there need to suspend the process as a concession to human physiology. Thus overnight incubation steps have been reduced down to actually required duration. Process times even shorter than those listed in Table 1 may be possible – this is a subject of future systematic method development.

Table 1 Process times in automated and manual in situ hybridization

Process time [a]			Protocol according to
	Automated processing	Manual processing	
Xenopus laevis	36 h	4–5 d	Harland 1991, modified
Gallus domesticus	36 h	3–4 d	Wilkinson 1992, modified
Drosophila melanogaster	19 h	1 d	Tautz and Pfeifle 1989, modified
Hydractinia echinata	28 h	2–3 d	Gajewski et al. 1996, modified

[a] Values indicate process duration from starting the hybridization protocol with fixed specimens until the samples are ready for substrate reaction.

All results reported in this study were obtained by processing the specimens in 150-µl exchange volumes. Consumption of liquid media is thus also reduced as compared to manual processing. The overall gain in performance due to automated processing allows us to develop approaches in which the number of samples to be processed is no longer the limiting parameter.

Hybridization of random probes in developmental stages of the colonial hydroid *H. echinata*

In order to test the feasibility of AISD for random screens, 39 probes were prepared from randomly selected cDNAs and 17 probes were generated from randomly primed PCR fragments. Out of these 56 probes 11 showed interesting spatial hybridization patterns (Table 2). Expression is observed either specifically in one cell type or a special cell precursor (indicated as cell type), in one but not other life stages (stage-specific), in only one germlayer or restricted to a typical axial position of the animals.

The probe derived from clone 10* (Table 2) is specific for small differentiating endodermal cells, obviously not gland cells. The cells remarkably express the targeted mRNA exclusively in a narrow zone in the head of the polyp. The zone is band-like in freshly metamorphosed young polyps, the primary polyps (Fig. 3A), located at the level of tentacle insertion and is band-like in adult polyps, too, but slightly shifted upwards in position and forming a zone between apex and tentacle whirl (Fig. 3B). In sexual polyps, cells expressing the mRNA are concentrated at the extreme apex. Sequence information so far available indicates a regular pattern of cysteine residues. Nevertheless, the identity of the gene remains to be determined.

The adult polyp in Fig. 3C (clone 1.11 in Table 2) bears hybridizing cells in the ectoderm of the lower body region exclusively. From BrdU S-phase labelling in *Hydractinia* and other hydroid species it is known that dividing interstitial cells are located precisely in this region (Plickert and Kroiher 1988; G. Plickert, unpublished results). Most interestingly, the probe labels endodermal cells in the middle part of the planula larva (Fig. 3D). It is in this region where some residual proliferative activity is observed after termination of embryogenesis (Plickert et al. 1988; Kroiher et al. 1990). Exactly in this region of the planula, the interstitial cells (I-cells) of the animal occur. This cell population comprises the multipotent stem cells and progenitors of cell types such as nerve cells, nematocytes and gland cells. I-cells pass the mesogloea during metamorphosis and invade the ectoderm of the developing primary polyp (van de Vyver 1964). In the regions of the primary polyp known to be populated by invading I-cells, single cells in the ectoderm hybridize to the probe (Fig. 3E). It is remarkable that the tips of the stolons do not contain hybridizing cells. Since stolon tips have been shown to be devoid of S-phase cells (Plickert et al. 1988) we expect, therefore, that further analysis of this clone will confirm specificity for dividing interstitial cells.

Another position-specific expression pattern is shown in Fig. 3F. Exclusively endodermal cells in the hypostome are targeted by the probe (clone E7 in Table 2). As observed in the pattern of clone 10* (Fig. 3A), the cells are located between the apical mouth and the tentacles. In contrast, the E7-hybridizing cells occur in a broader region.

Three out of the 11 clones and thus a prominent fraction were associated with wound responses. As an exam-

Table 2 Hybridization patterns of randomly selected probes in developmental stages of *Hydractinia echinata*

Clone identity	Specificity of expression	Sequence similarity
17*	Position, germlayer, stage, cell type	dsRNA-binding protein [a]
10*	Position, germlayer, cell type	Cysteine-spacing
8*	Position, cell type	Splicing factor
7*	Position, germlayer, stage, cell type	60S acidic ribosomal protein [a]
3*	Position, germlayer wound response	Trypsinogen [a]
2*	Position, germlayer	Sodium-dependent nucleoside transporter [a]
mp	Position, germlayer, stage, cell type	Metalloprotease [a]
E7	Position, germlayer, wound response	not determined
1.11	Position, cell type	not determined
C7	germlayer, cell type	Cathepsin D [a]
1.15	Position, germlayer, wound response	not determined

[a] Denotes very high homology, identification reliable

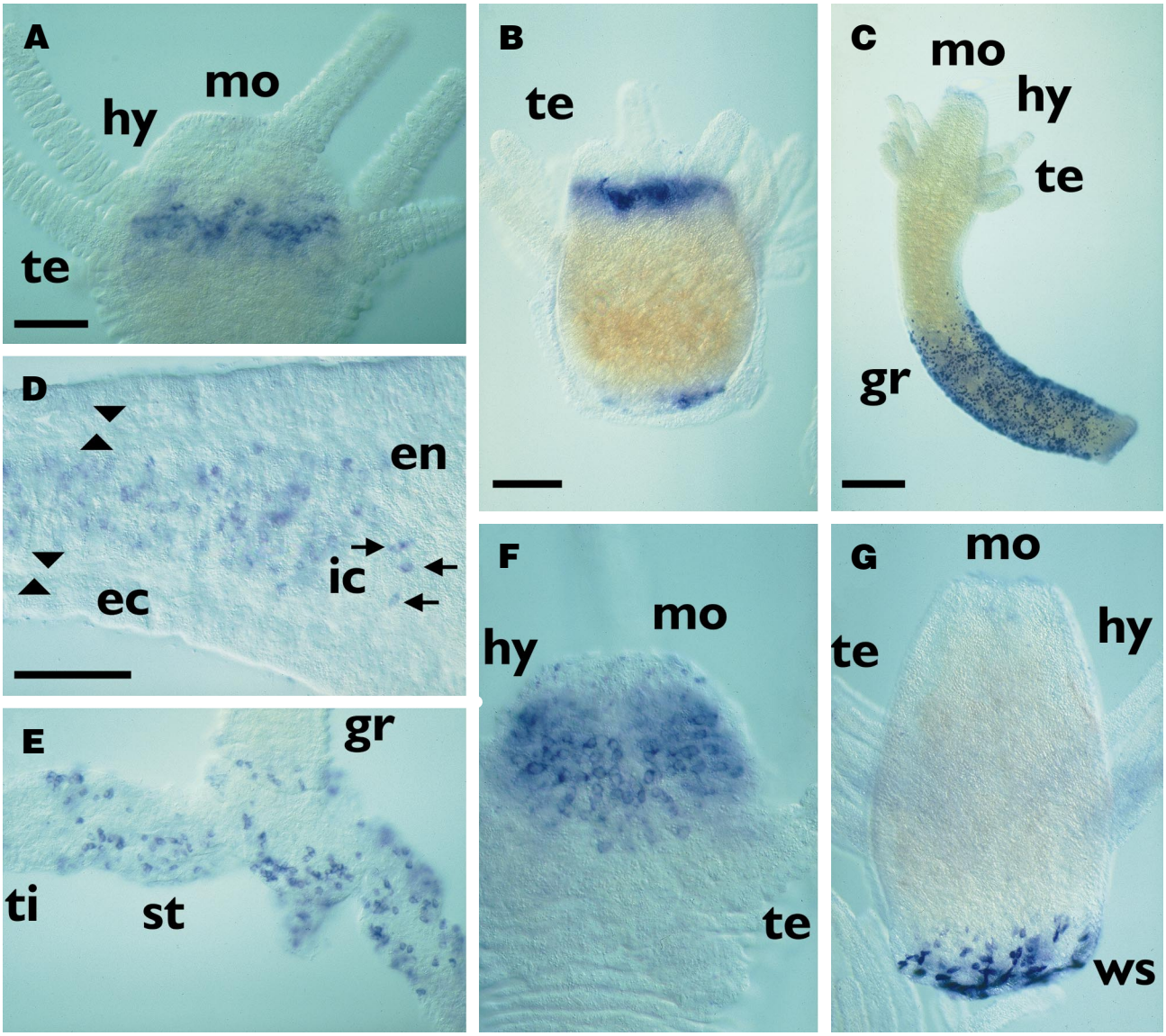

Fig. 3A–G Hybridization patterns of randomly selected probes in developmental stages of *Hydractinia echinata*. **A** Probe 10*, primary polyp. **B** Probe 10*, adult polyp. **C** Probe 1.11, adult polyp. **D** Probe 1.11, planula larva. Note that cells containing label occur in the endoderm exclusively. *Arrowheads* indicate the mesogloea which separates ecto- from endoderm. *Arrows* indicate three cells displaying the typical shape of I-cells. **E** Probe 1.11, basal part of primary polyp including the stolons. **F** Probe E7, primary polyp. **G** Probe 1.15, adult polyp. For further description of the expression pattern see text (*ec* ectoderm, *en* endoderm, *gr* gastric region, *hy* hypostome, *ic* I-cell, *mo* mouth, *st* stolon, *te* tentacle, *ti* stolon tip, *ws* wound surface; *scale bar* **A**, **E**, **F**, 50 µm; **B**, **G**, 100 µm; **C**, 350 µm; **D**, 50 µm)

ple, hybridization of a probe (clone 1.15 in Table 2) in gland cells near the wound surface (the animal was removed from the colony 1 h before fixation) is shown (Fig. 3G). None of the three probes has an exclusive specificity for wound responses. The probe displaying the expression pattern documented in Fig. 3G, for instance, also hybridizes to endodermal cells in specific regions of the primary polyp. Vice versa, the probe derived from clone E7 and documented in Fig. 3E is also wound-specific as it hybridizes to gland cells in the vicinity of tissue lesions. Since hydroids are sedentary animals, any material for RNA-isolation has to be removed from the colony. Removal by cutting necessarily involves tissue damage and thus induction of any wound-response-related transcription. Accordingly, resulting cDNA libraries are enriched with wound-response-related messages. It is plausible that a relatively large fraction of expression patterns observed indicate wound response.

Conclusion

AISD allows us to substantially reduce manual work in many kinds of analytical incubation experiments. In addition to ISH, this includes substrate reactions for enzyme-based reporter gene assays and also ICC. For instance, we applied the method to screen 17 different domain-specific antibodies for cross-reaction in developmental stages of

hydroids (paper in preparation). The principal problem of automated in situ detection, i.e. to automatize efficient liquid exchange without loosing the sample, has been solved. We found that any incubation protocol tested so far could be easily converted into a corresponding program for the instrument described here. In addition to the species shown in this paper this includes in situ hybridization in embryos of zebrafish, Medaka fish and in mouse embryos (data not shown). Results obtained from repeated runs with the same samples and probes were highly reproducible. Once a type of material and the respective protocol is successfully tested on the device, processing work is absolutely reliable. Only technical modifications are nedded to increase the capacity of the instrument. Random in situ screens can be planned on a larger scale than is possible with manual sample processing. Moreover, the extended requirements of complex staining protocols, e.g. multiple colour in situ detection (e.g. Hauptmann and Gerster 1996) or combined ISH/ICC-procedures, would nicely be met by the wide range of possibilities the AISD-processor provides.

Beyond this we propose and explore the following applications of AISD. Much experimental work in developmental biology is based on the comparison of treated and untreated groups. We propose to systematically assay the effects of such treatments by displaying expression patterns. Activities of known genes may be displayed. Shifts in spatial expression domains or ectopic expression could be quickly correlated with the treatment. Alternatively, treatment activation of unknown genes is detected by a conspicuous expression pattern. For this kind of application the above mentioned detection of wound responses – if conducted systematically – would provide a good example. All approaches based on conventional random-primed differential display techniques can be easily extended by directly labelling the PCR product and displaying the corresponding expression pattern by AISD. Moreover, systematic screening for compounds that alter a specific gene expression pattern in a desirable fashion would be feasible. On the other hand, compounds that have to be assayed for teratogenic or organtoxic side effects could also be subjected to AISD analysis by using a set of known indicator genes. Any effects of treatment at the early level of gene activity would become obvious. We would like to summarise these applications under the term "*D*ifferential *D*isplay of *Ex*pression *P*atterns (DDExP)".

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References

Azpiazu N, Frasch M (1993) *tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. Genes Dev 7: 1325–1340

Driever W, Stemple D, Schier A, Solnica-Krezel L (1994) Zebrafish: genetic tools for studying vertebrate developement. Trends Genet 10: 152–159

Gajewski M, Leitz T, Schloßherr J, Plickert G (1996) LWamides from Cnidaria constitute a novel family of neuropeptides with morphogenetic activity. Roux's Arch Dev Biol 205: 232–242

Gajewski M, Schmutzler C, Plickert G (1997) Structures of neuropeptide precursors in cnidaria. In: Vaudry H (ed) Trends in comparative endocrinology and neurobiology. Ann New York Acad Sci (in press

Harland RM (1991) In situ hybridization: an improved whole-mount method for *Xenopus* embryos. Methods Cell Biol 36: 685–695

Hauptmann G, Gerster T (1996) Multicolor whole-mount in situ hybridization to *Drosophila* embryos. Dev Genes Evol 206: 292–295

Hirsh D, Vanderslice R (1976) Temperature-sensitive developmental mutants of *Caenorhabditis elegans*. Dev Biol 49: 220–235

Izpisúa Belmonte JC, De Robertis EM, Story KG, Stern CD (1993) The homeobox gene *goosecoid* and the origin of organizer cells in the early chick blastoderm. Cell 74: 645–659

Jürgens G, Mayer U, Torres Ruiz RA, Berleth T, Misera S (1991) Genetic analysis of pattern formation in the *Arabidopsis* embryo. Development Suppl 1: 27–38

Kroiher M, Plickert G, Müller WA (1990) Pattern of cell proliferation in embryogenesis and planula development of *Hydractinia echinata* predicts the postmetamorphic body pattern. Roux's Arch Dev Biol 199: 156–163

Mayer U, Torres Ruiz RA, Berleth T, Misera S, Jürgens G (1991) Mutations affecting body organization in the *Arabidopsis* embryo. Nature 353: 402–407

Mullins MC, Nüsslein-Volhard C (1993) Mutational approaches to studying embryonic pattern formation in the zebrafish. Curr Opin Genet Dev 3: 648–654

Mullins MC, Hammerschmidt M, Hafter P, Nüsslein-Volhard C (1994) Large scale mutagenesis in zebrafish: in search of genes controlling development in a vertebrate. Curr Biol 4: 189–202

Nüsslein-Volhard C, Wieschaus E (1980) Mutations affecting segment number and polarity in *Drosophila*. Nature 287: 795–801

Plickert G, Kroiher M (1988) Proliferation kinetics can be studied in whole mounts and macerates by means of BrdU/anti-BrdU technique. Development 103: 791–794

Plickert G, Kroiher M, Munck A (1988) Cell proliferation and differentiation during embryonic development and metamorphosis of *Hydractinia echinata.* Development 103: 795–803

Smith JC, Price BMJ, Green JBA, Weigel B, Herrmann BG (1991) Expression of a *Xenopus* homolog of brachyury (T) is an immediate-early response to mesoderm induction. Cell 67: 79–87

Solnica-Krezel L, Schier AF, Driever W (1994) Efficent recovery of ENU-induced mutations from the zebrafish germline. Genetics 136: 1401–1420

Tautz D, Pfeifle C (1989) A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. Chromosoma 98: 81–85

Vyver G van de (1964) Étude histologique du développement d'*Hydractinia echinata* (in Flemish). Cah Biol Mar 5: 295–310

Wilkinson DG (ed) (1992) In situ hybridization: a practical approach. IRL Press, Oxford